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Contributions of Immunoaffinity Chromatography to Deep Proteome Profiling of Human Biofluids

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Abstract

Human biofluids, especially blood plasma or serum, hold great potential as the sources of candidate biomarkers for various diseases; however, the enormous dynamic range of protein concentrations in biofluids represents a significant analytical challenge for detecting promising low-abundance proteins. Over the last decade, various immunoaffinity chromatographic methods have been developed and routinely applied for separating low-abundance proteins from the high- and moderate-abundance proteins, thus enabling much more effective detection of low-abundance proteins. Herein, we review the advances of immunoaffinity separation methods and their contributions to the proteomic applications in human biofluids. The limitations and future perspectives of immunoaffinity separation methods are also discussed.

Keywords

Biofluid; immunoaffinity chromatography; plasma/serum; immunodepletion; immunoenrichment; biomarker discovery; proteomics; affinity proteomics

1. Introduction

Human biofluids are biological fluids that are excreted or secreted from inside the bodies of living people, including but not limited to blood, urine, cerebrospinal fluid (CSF), saliva, tear, and synovial fluid (See Figure 1). Human biofluids, especially blood plasma/serum and urine, are considered the most promising sources for the discovery of novel biomarkers for disease diagnosis and prognosis based on the notion that these biofluids contain disease-associated proteins secreted or leaked from pathological tissues across the body [1–3]. Comparing to other types of specimens such as tissues, biofluids are often easily obtainable through noninvasive procedures, making it particularly attractive for large-scale clinical and/or longitudinal studies. For these reasons, there has been tremendous interest in profiling the biofluid proteomes for the development of biomarkers for various diseases over the last decade [4–10].

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Blood plasma or serum has been the most popular choice for biomarker development and verification/validation. One of the unique challenges in proteome profiling of plasma/serum lies in its extremely large dynamic range of protein concentrations (up to 10–12 orders of magnitude [4]). The 12 most abundant proteins (*e.g.*, albumin, transferrin, immunoglobulins, *etc*) account for ~95% of total protein mass in plasma or serum, which leaves thousands of other moderate- and low-abundance proteins (MAPs and LAPs) in only 5% of total protein mass (See Figure 1). The "masking" effect caused by these high-abundance proteins (HAPs) greatly hampers the detection of LAPs such as cytokines and other clinically important proteins that often present at the sub ng/ml level. Besides blood, urine is also an ideal source for biomarker discovery and has been utilized in more and more proteomics studies [9, 11, 12]. Urine is formed in the kidney by ultrafiltration from the blood, which can be easily accessed and collected in large amounts via non-invasive approaches [13]. Comparing to blood, the composition of urine is less dominated by HAPs, which provides a relatively easier access to LAPs (See Figure 1). Other biofluids, such as cerebrospinal fluid, saliva, tear, and synovial fluid, have also been analyzed for potential biomarkers; however in much more limited number of studies comparing to blood and urine [14–16].

Immunoaffinity chromatography (IAC) approaches have become the most commonly utilized strategies for digging deeper into the biofluid proteomes by both global and targeted proteomics [17–19]. IAC represents a specific type of affinity chromatography where the stationary phase is composed of immobilized antibodies or other affinity reagents on solid support matrix. The underlying principle of IAC is based on the selective non-covalent interaction between antibodies (or affinity reagents) and their specific binding targets or antigens. The purpose of IAC separations is to enrich LAPs of interest by either removing HAPs from the complex samples through immunoaffinity depletion (immunodepletion) or directing capturing low-abundance targets of interest through immunoaffinity enrichment (immunoenrichment). Significant advances in IAC methods for both the depletion and enrichment have been made and these methods have been broadly utilized in proteomics applications for many types of biofluids related to various human diseases [18, 20].

2. Overview of Immunoaffinity Chromatography

The most common schemes for applying IAC either by immunodepletion or immunoenrichment are illustrated in Figure 2. In these schemes, chromatographic matrices (column) or other resins with immobilized antibodies are used to specifically capture target proteins/peptides, and the resulting flow-through fraction (immunodepletion) or bound fraction (immunoenrichment) is collected for further analyses [21, 22]. The immunodepletion strategy is designed to remove the HAPs and enrich LAPs on a global scale [23]. In immunodepletion, complex samples as plasma/serum are first loaded onto a depletion column, and only specific HAPs are selectively captured by the antibodies immobilized on column, while other LAPs flow through directly and are collected (See Figure 2A). To maximize the detection of LAPs, simultaneous removal of multiple HAPs is desired. Therefore, multiple antibodies are often mixed and immobilized onto column in order to remove multiple HAPs. Furthermore, immunodepletion columns targeting different proteins/peptides can also be used in tandem to enable the removal of a relatively large number of HAPs [21]. On the other hand, the immunoenrichment (or immunoaffinity

purification) strategy is more targeted towards specific analytes (*e.g.*, low-abundance proteins, peptides, or PTMs) to enhance the detection of these specific target low abundance analytes of interest, which is ideally suited for coupling with targeted proteomics measurements [24, 25]. In immunoenrichment, only target low-abundance analytes are recognized and bound on column, while all other proteins/peptides are washed away, and the targeted proteins/peptides are further eluted from column for analysis (See Figure 2B).

The utility of IAC is highly dependent on the quality of affinity reagents, typically antibodies [19]. A "good" antibody for IAC should meet two requirements: (a) a high intrinsic affinity toward target protein, and (b) reversible interactions between antibody and antigen that can be easily de-stabilized. Two main types of antibodies are commonly used in IAC, namely polyclonal and monoclonal antibodies [17, 19]. Polyclonal antibodies are produced as a heterogeneous population of antibodies from multiple clones of B-cells, which can recognize and bind a variety of epitopes on a single antigen with diverse affinity [26]. Sera of immunized animals are the main sources of polyclonal antibodies. Due to their easy accessibility and relative low cost, polyclonal antibodies are widely used for developing IAC methods. However, there are a number of limitations in using polyclonal antibodies. Firstly, sera are usually available in limited supply and could vary from animal to animal. Therefore, it is difficult to obtain consistent quality of antibodies from multiple batches or lots. To obtain high quality antigen-specific antibodies, polyclonal antibodies must be purified against the target antigen via affinity chromatography [19]. However, it is often impractical to acquire a large amount of purified antigen proteins for this purpose. Alternatively, anti-peptide antibodies have been developed to selectively capture the proteolytic peptides of target proteins for their quantification in human biofluids, which is exemplified in the SISCAPA approach (Stable Isotope Standards and Capture by Anti-Peptide Antibodies) [24, 27]. In such case, synthetic peptides conjugated with a carrier protein are used to immunize host animals. Antibodies targeting unique epitopes (or peptide-specific) are produced to specifically recognize the proteolytic peptides. Compared to proteins, synthetic peptides are commercially available in highly purified forms and in large quantities, facilitating the production of a peptide-specific antibody population for IAC analysis. Another limitation of using polyclonal antibodies is that the polyclonal antibodies may consist of a number of antibodies targeted at different epitopes of the same antigen, which makes it difficult to find a favorable eluting condition to simultaneously destabilize multiple types of antigen-antibody interactions that are different in nature [28]. Therefore, potential lack of quantitative elution and low recovery of target protein should be taken into consideration in polyclonal antibody-based IAC applications. For IAC applications in human biofluids for proteomic profiling, polyclonal antibody-based IAC methods have been mainly applied for the selective depletion of HAPs and MAPs where high specificity of capture is preferred over quantitative elution (Table 1).

In contrast, monoclonal antibodies are typically produced by fusing myeloma cells with the spleen cells from a mouse that has been immunized with the desired antigen to produce hybridomas. Very high affinity monoclonal antibodies can be identified by screening large numbers of hydridoma supernatants since many therapeutic monoclonal antibodies have reported affinities of ~10^{-9} M or better [29]. Automated screening of monoclonal antibodies using MS-based platform has also been recently reported [30]. The monoclonal antibodies

constitute a homogeneous population that has monovalent affinity since they bind to the same epitope on an antigen [26]. Compared to polyclonal antibodies, monoclonal antibodies have the advantages of consistent specificity and reproducibility. Since all antibodies bind to the same epitope, it is relatively easy to find a gentle elution condition for IAC [19]. Therefore, monoclonal antibodies are preferred for immunoaffinity enrichment applications. Besides antibodies, other affinity reagents such as immobilized metal ions [31], metal oxides [32], lectins [33], and aptamers [34] have also been applied for affinity chromatography-based applications.

In a typical IAC approach, antibodies are immobilized on a solid stationary phase to facilitate the selective and strong binding of antibodies to their target antigens, as well as the subsequent washing (if desired) and elution processes. Conventional matrices for immobilization of antibodies include natural carbohydrate-based materials (*e.g.*, agarose, dextrose and cellulose), synthetic organic polymers and inorganic materials, such as silica and zirconia [17]. Cross-linked agarose is the most popular matrix for IAC, due to its excellent biocompatibility and extraordinary chemical stability in a broad pH range and most solvents. However, agarose beads and other soft gel matrices are not compatible to a highly pressurized system, such as HPLC. In contrast, silica, polystyrene and other highly cross-linked materials have good mechanical strength and stability, thus can be used in a HPLC-based IAC system, namely HPIAC [17]. Various commercial products and kits have been developed over the years for immunoaffinity separation of proteins in human biofluids (Table 1). Immunoaffinity columns are commonly produced with immobilized mammalian IgG or avian immunoglobulin yolk (IgY) antibodies. These two kinds of antibodies can be produced in a relatively high quantity from serum or eggs of immunized animals [19]. IgY antibodies have several advantages over IgG including higher antibody yield and less cross-reactivity toward non-targeted human proteins [18, 35].

3. Immunoaffinity Chromatography Approaches

In the following sections, we will discuss different IAC approaches with some details focusing on immunodepletion and immunoenrichment.

3.1 Immunoaffinity depletion

Immunodepletion of HAPs has become a relatively routine sample preparation strategy for biofluid proteome profiling, in which multiple antibodies are mixed in an optimized ratio and immobilized on solid matrices for removing multiple HAPs simultaneously [18, 36]. While immunodepletion kits in spin column formats, such as Vivapure anti-HAS kit (Sartorius) for removing albumin, Qproteome (Qiagen) for removing albumin and IgG, have been available for several decades now, Pieper *et al.* were the first to report on the concept of multi-component immunoaffinity subtraction chromatography in an LC column format for reproducible removal of up to 10 plasma HAPs to enhance plasma proteome profiling in 2003 [37]. Two commercial LC column products, Multi-affinity removal system (MARS) Hu-6 by Agilent [36] and ProteomeLab™ IgY12 by Beckman Coulter [35], were shortly made available for removal of 6 and 12 HAPs in blood plasma/serum, respectively; these two immunodepletion columns were further improved into the MARS Hu-14 kit (Agilent)

and the Seppro® IgY14 system (Sigma Aldrich), respectively, for removal of top 14 HAPs. Similarly ProteoPrep® 20 (Sigma Aldrich) was developed for removing 20 HAPs in plasma. The detailed list of current commercially available immunoaffinity depletion systems were provided in Table 1. Compared to spin columns, the LC column-based products utilizing automated LC systems provide a number of advantages in effective removal of targeted proteins, such as minimal carryover, good reproducibility, and minimal nonspecific binding [38]. Besides these single-stage depletion systems, an IgY-based SuperMix depletion column has been developed to enable the removal of ~50 MAPs by applying it with IgY12 or IgY14 column in tandem to further enrich LAPs prior to follow-up analysis [21, 39]. In our experience, a typical LC depletion column will offer reproducible depletion for 100–200 biological samples with a shelf life for several years, which provides a great potential for large-scale biomarker discovery and verification studies.

3.1.1 Multi-affinity removal system (MARS)—The MARS column from Agilent Technologies was the first commercially available multi-component immunoaffinity depletion system [36, 40]. Initially, this column consisted of 6 polyclonal IgG antibodies for 6 HAPs including albumin, IgG, IgA, transferrin, α-1-antitrypsin and haptoglobin (so called MARS Hu-6) [41]. Antibodies were immobilized onto column through their Fc regions, which ensured easy protein access to the affinity binding sites with reported depletion efficiency higher than 99% for each target protein [40]. MARS Hu-6 was applied to many proteomics applications in biofluids [42–44]. Later on, MARS Hu-7 column was found to deplete fibrinogen plus the original six HAPs [45]. The most recently product of MARS is the Hu-14 column which allows the depletion of 8 more HAPs including fibrinogen, α-acid glycoprotein, α-macroglobulin, IgM, apolipoproteins A-I & A-II, complement C3 and pre-albumin, approximately 95% of the human plasma proteins [46]. The MARS Hu-14 depletion has also been widely used in recent proteomic applications, including plasma [46–53], urine [54], CSF [55–57], and tissue proximal fluids [58].

3.1.2 IgY-based single-stage and dual-stage depletion systems—The IgY12 depletion system based on avian polyclonal IgY antibodies was developed shortly after the MARS Hu-6, which initially targeted 12 HAPs [20, 35, 59]. The IgY12 system was later improved to IgY14 for removing 14 HAPs in human plasma and the product is now commercialized as Seppro® IgY14 from Sigma Aldrich [39, 60–62]. Both IgY14 and MARS Hu-14 are very popular depletion products for proteomics applications since the performance characteristics of MARS Hu-14 and IgY14 are very comparable with both products offering options of multiple loading capacities (customization options available as well) [63]; however, the IgY antibodies appeared to display the least nonspecific binding [64]. Similar to MARS Hu-14, IgY-14 was broadly applied to proteomics studies, including plasma [65–68] and CSF [69, 70].

The concept of a SuperMix column was later developed to be applied in tandem with IgY12 or IgY14 so that the detection of LAPs can be further enhanced by depleting an additional number of MAPs [21, 39, 71]. The SuperMix column was developed by immunizing chickens with the protein mixture from IgY14-depleted human blood plasma as mixed antigens, and the purified antibodies were further immobilized and packed into the

SuperMix column to target a relatively large number of MAPs [21]. Figure 3 shows an example of the configuration of tandem IgY14 and Supermix depletion and overlays of representative LC chromatograms of three replicates of a reference plasma sample [39]. In our original characterization of the system, we observed that the SuperMix depleted at least 50 MAPs from plasma [21], providing significant enhancement for the detection of LAPs (*e.g.*, a 100-fold enrichment of LAPs comparing to the 20-fold enrichment by the IgY12 alone).

All the depletion columns (MARS Hu-14, IgY-14, or SuperMix) can be easily implemented and automated using conventional HPLC with the protocol of using the column typically consisting of loading the sample, collecting the flow-through proteins (LAPs), washing, eluting bound proteins, and column regeneration. In our experience, a typical LC depletion column will offer reproducible depletion for 100–200 biological samples with a shelf life for several years, which provides a great potential for large-scale biomarker discovery and verification studies. More recently, a microscale chromatographic depletion system using IgY-14 resin was reported to facilitate more effective depletion of small amounts of human CSF or serum samples, where only ~6 μL serum or 600 μL CSF were used [72].

3.1.3 Combinatorial peptide ligand library (CPLL)—The concept of combinatorial peptide ligand library approach, first commercialized by Bio-Rad Laboratories as ProteoMiner kit, is that the library consists of millions of hexapeptides capable of interacting with most proteins in any given proteome. In principle, each unique hexapeptide binds to a unique protein sequence. Because the bead capacity limits binding capacity, high-abundance proteins quickly saturate their ligands and excess proteins are washed out during the procedure. On the contrary, LAPs are captured on their specific ligands, thereby leading to reduced dynamic range of the biofluid proteome or so called “equalizing of protein concentrations” [73–76]. Several studies have explored the applications of ProteomeMiner in proteome profiling [77–80]. The ProteoMiner kit and depletion columns are conceptually different and complementary. The depletion columns achieve early complete removal of highly abundant proteins, but will suffer non-specific loss of other LAPs. On the other hand, ProteoMiner only offer partial removal of HAPs and reduce the dynamic range given the “equalizing” principle. In a recent study by Gil-Dones *et al.* [76], it was clearly demonstrated that ProteoMiner was not as effective for enhancing the detection of LAPs as depletion columns. For better depletion of HAPs and enrichment of LAPs, more details such as sample overloading degree, the way of peptide ligand grafted on bead, and chemical modification of peptide ligand library, should be optimized [75].

3.2 Immunoaffinity enrichment

Immunoaffinity enrichment, also called immunoaffinity purification, has been broadly applied as traditional biochemical approaches for enrichment of specific protein targets for decades. In particular, immunoaffinity enrichment approaches have found broad applications in targeted proteomics. For example, the coupling of antibody-based enrichment and targeted mass spectrometry has become a routine approach for developing so called mass spectrometric immunoassays (MSIA) [81, 82]. Many types of affinity reagents have been developed to enable different types of proteomic applications. The affinity reagents can be

used to capture specific target proteins by antibodies, or specific classes of proteins such as glycosylated proteins by using lectins [33] or hydrazide resins [83]. The affinity reagents can also be applied to target specific peptides by anti-peptide antibody [24], specific types of PTMs including phosphorylation by immobilized metal ions [31], and metal oxides [32], and other PTMs [84] by antibodies, and specific type of peptides such as cysteine-containing peptides using thiol-affinity resins [85, 86]. Herein, we discuss several major types of immunoaffinity reagents for enrichment other than full antibodies.

3.2.1 Protein ligands—Some natural or recombinant proteins have specific recognition and binding capacity of certain target proteins. For example, bacterial Protein A and Protein G are ideal ligands for immunoglobins by binding to the Fc regions of IgG [87]. A type of recombinant protein ligand, known as Affibody, has also been developed by mimicking or complementing the structures and binding activities of an antibody [88, 89]. Lectins are glycoproteins that have higher affinity for sugar moieties in glycoproteins [90]. The multi-lectin affinity chromatography (M-LAC) has been developed as an effective approach for enriching and analyzing glycoproteins from human biofluids [33, 91]. For instance, M-LAC has been coupled with depletion and other separations to achieve a deep coverage of the plasma proteome by LC-MS/MS [91, 92].

3.2.1 Single chain/domain antibodies—Single chain/domain antibody is another type of protein ligand that consists of a fragment of antibody from a single chain or a single domain [93, 94]. Single chain antibody (Single chain Fv, scFv) was the minimal form of functional antibodies, which comprised of the VH and VL segments in a single polypeptide chain joined by a short flexible linker, and each V-region has three complementarity determining regions (CDRs) in direct contact with antigen; while single domain antibody was an antibody fragment which consisted of a single monomeric variable antibody domain [95]. Single chain/domain antibodies demonstrated several advantages over the traditional whole antibody, including the ease of cloning in bacteria, minimally immunogenic, and better tissue penetration due to the smaller size and better stability [96]. Especially, the advance in phage display technology further facilitates the production of single chain antibodies for proteome research and other applications [97, 98].

Phage display is a technology that enables the detection of interaction between the displayed protein and other test molecules, including protein, peptide and DNA, which could be used for selection of proteins, peptides, or antibodies with affinity and specificity to a molecule or protein of interest from phage libraries [99]. The construction of a phage display library (could be a protein library, peptide library, antibody library) was accomplished by inserting DNA fragments into phage or phagemid genomes which proteins were further expressed on the phage coat. Specifically, the technique was named antibody phage display (APD) when an antibody was displayed, which allowed *in vitro* selection of antibodies of virtually any specificity, greatly facilitating recombinant production of antibody reagents to create antibody libraries [100]. The use of single-chain antibody library or antibody microarray to screen human serum to discover novel cancer biomarkers has been reported [101, 102].

3.2.3 Anti-peptide or anti-PTM antibodies—The development of immunoaffinity enrichment approaches to targeted specific peptides or specific types of PTMs is another

emerging area in proteomics. The first demonstration of using anti-peptide antibodies was reported as the “Stable Isotope Standards and Capture by Anti-Peptide Antibodies” (SISCAPA) approach [24]. SISCAPA has been advanced to become an important technology for enabling the detection of LAPs in biofluids by targeted MS without the need of extensive LC fractionation [25, 103]. SISCAPA also combines the power of peptide level immunoaffinity enrichment with the analytical capability of selected or multiple reaction monitoring (SRM/MRM, which enables sensitive quantitation of corresponding protein biomarkers and targets [103, 104]. Although not extensively applied to biofluids yet, immunoaffinity enrichment using antibodies against specific types of PTMs has also been developed and extensively applied in proteomics studies, including protein phosphorylation [105, 106], ubiquitination [107, 108], acetylation [109, 110], and lately methylation [111].

4. Proteomic applications of IAC in biofluids

Figure 4 summarizes a general workflow for applying IAC methods in proteomics profiling of human biofluids samples. For any biomarker discovery or verification studies, the experimental design will be the key for its chance of success. As recommended in a recent publication, clearly defined clinical question, selection of subjects, sufficient demographic data, and appropriate statistical approaches including adequate sample size are all important factors in the experimental design [10]. Depletion of HAPs is often required for plasma/serum samples, which can be performed by HPLC using IgY/MARS/ Proteoprep columns. After removal of HAPs, the remaining MAPs and LAPs account for about 5–10% of the original protein mass (~20-fold enrichment of LAPs). If further removal of MAPs is required (typically in plasma/serum), the SuperMix column can be used, which results in a flow-through of ~1% of original protein mass corresponding to ~100-fold enrichment of LAPs. In contrast, urine is less dominated by HAPs. However, pre-concentration methods, such as ultrafiltration, protein precipitation, dialysis, lyophilization, and ultracentrifugation, need to be applied to concentrate urine proteins prior to down streaming processing for proteome analysis [13, 112]. Among these methods, ultrafiltration has been reported to be the best method for concentration and cleanup of protein components from urine [112]. Besides depletion, enrichment strategies such as M-LAC for glycoproteins [91] and SISCAPA [25] can also be applied to enrich LAPs either independently or in combination with the depletion strategies. The enriched samples are then analyzed either by shotgun LC-MS/MS proteomics for discovery studies or by LC-SRM based targeted proteomics for verification studies. In the following sections, we review some examples of applications using different biofluids.

4.1 Plasma/Serum

Plasma/serum is one of the most well studied and characterized biofluids for biomarker discovery and validation. Significant effort and progress has been made in extending and characterizing the plasma/serum proteome using various IAC approaches. The initial application of multi-component depletion by Pieper *et al.* demonstrated significant enhancement of the detection of plasma LAPs by 2-DE following the removal of major proteins [37]. Liu *et al.* demonstrated that enhanced detection of LAPs in plasma could be achieved with high reproducibility and specificity by employing immunodepletion strategy

[38]. The comparison between different immunodepletion columns has also been carried out [113, 114]. Polaskova *et al.* compared six different immunodepletion columns by using 2-DE, in which Seppro IgY system demonstrated the best results [114]. To further enhance the detection and coverage of LAPs, Qian *et al.* reported the application of tandem IgY12 and SuperMix depletion to human plasma by successfully removing up to top 60 abundant proteins in plasma, demonstrating nearly doubled plasma proteome coverage compared to single stage depletion and the effective detection of many LAPs with reported normal concentrations of approximately 100 pg/ml to 100 ng/ml [21]. Moreover, the IAC methods are very flexible to be coupled with downstream fractionation, enrichment, or detection techniques such as 2-DE or 1D or 2D-LC-MS/MS for digging deeper into the plasma proteome. For instance, a number of other techniques, including Cysteine-peptide enrichment [115], N-linked glycopeptide enrichment [116], multi-lectin affinity chromatography [91], off-gel electrophoresis [117], isoelectric focusing [91], have been coupled with immunoaffinity depletion for more extensive plasma proteome profiling.

Besides global profiling, the immunodepletion strategy has been extensively applied as a useful tool in targeted quantification studies of low-abundance plasma proteins in plasma/serum [67, 118–124]. For example, Liu *et al.* developed a prostate specific antigen assay in serum by combining immunoaffinity depletion and SRM detection, which showed very good correlation with conventional immunoassays result from other independent clinical serum samples [118]. Keshishian *et al.* demonstrated multiplexed quantitative assays for LAPs with limits of quantification in 1–10 ng/mL range in plasma by coupling depletion with limited fractionation and targeted mass spectrometry [59]. Furthermore, Shi *et al.* developed a so-called “high-pressure, high-resolution separations coupled with intelligent selection and multiplexing” (PRISM) method for ultrasensitive detection of LAPs in plasma, which demonstrated accurate and reproducible quantification of proteins at concentrations at the 50–100 pg/mL levels in plasma by integrating by coupling IgY14 with PRISM and SRM [67]. In a recently large scale targeted quantification study of cancer-associated proteins in human plasma, Hüttenhain *et al.* demonstrated reproducible quantification of 182 proteins in MARS Hu-14 depleted plasma, spanning five orders of magnitude in abundance and reaching below a concentration of 10 ng/mL [121].

While immunodepletion is highly effective for enhancing proteome analysis of blood plasma/serum, concerns still exist for its applications in biomarker related studies, especially in the aspect of non-specific loss of LAPs due to the non-characteristic protein binding to HAPs or to antibodies. For example, Keshishian *et al.* demonstrated that troponin T was largely lost during IgY14 immunodepletion [120]. Yadav *et al.* systematically analyzed the bound HAPs fraction and showed that 101 proteins could be detected with high confidence, and suggested both bound and depleted fractions should be analyzed from immunodepletion for biomarker discovery using plasma/serum [125].

As an important alternative to immunodepletion, immunoaffinity enrichment has also been broadly applied to plasma/serum related biomarker studies. The utility of antibodies for developing high selective mass spectrometric, targeted immunoassays for clinical important proteins in human plasma/serum has been well demonstrated [81, 82, 126]. The development of peptide-level immunoenrichment as exemplified by SISCAPA has also

clearly demonstrated the value for quantification of potential low-abundance biomarkers [24]. Following the initial work by Anderson *et al.,* Whiteaker *et al.* developed an automated and multiplexed approach for quantification of potential protein biomarkers by combining multiplexed SISCAPA and MRM analysis [103]. SISCAPA was also applied to develop multiplexed assays for a known biomarker of cardiac injury (troponin I), and an emerging cardiovascular marker interleukin 33, which could be detected at 1–10 ng/mL in plasma [127]. SISCAPA has been demonstrated for multiplexing up to 50-plex and able to extend the limits of detection to sub-ng/ml range of protein concentration [103, 104]. Ramirez *et al.* recently also demonstrated the utility of single chain antibody array for detecting low-abundance cancer biomarkers in human serum [101]. By combining a single chain antibody library and protein microarray for screening ovarian cancer serum for novel potential biomarkers, they identified 19 promising scFvs and verified 6 top candidates using full-length antibodies [101].

The IAC approaches have been routinely coupled with both global discovery and targeted verification for disease-biomarker related studies [114, 128], and the applications include pancreatic cancer [124], ovarian cancer [129, 130], breast cancer [131, 132], renal cancer [133], gastric cancer [134], prostate cancer [135, 136], osteoarthritis [137–139], cardiovascular disease [120, 140], *etc.*

4.2 Urine

Urine perhaps represents one of the most non-invasive, easily accessible biofluids available for diagnostic or prognostic tests. A number of studies have successfully demonstrated that urinary proteins and metabolites are promising biomarkers for diseases, such as kidney disease, prostate cancer, breast cancer, diabetes, atherosclerosis and osteoarthritis [141, 142]. Compared to plasma/serum, the urine proteome has a more limited dynamic range of protein concentrations and is less dominated by HAPs. Nevertheless, a number of studies have applied immunoaffinity-based separation to extend the proteome coverage and search for potential biomarker candidates in urine. Adachi *et al.* combined the albumin depletion and LC-MS/MS, which successfully identified more than 1500 proteins in normal urine [143]. Furthermore, by utilizing immunodepletion coupled to HPLC-Chip-MS/MS detection, He *et al.* identified 1641 urinary proteins with high confidence and provided a panel comprising 18 urinary proteins to assess the human health status from urine samples of 100 male and 100 female healthy donors [144]. Martin-Lorenzo *et al.* evaluated two immunodepletion approaches including ProteoPrep Immunoaffinity Albumin and IgG Depletion Kit (Sigma) and MARS Hu-14, and they demonstrated that using OasisR HLB cartridge purification in combination with albumin depletion by ProteoPrep kit as the best option for urine proteome profiling from patients with proteinuric renal disease [145]. Magagnotti *et al.* compared three depletion strategies including IgY, MARS and a home-made depletion column for improving coverage of the human urine proteome, and showed IgY depletion followed by ethanol precipitation was the most efficient method for exploring urine proteome [146]. The immunoaffinity based separation has also been applied to various biomarker discovery and verification studies in urine, such as bladder cancer [54], osteoarthritis [147], diabetic nephropathy [148, 149], and renal transplant [150].

4.3 Other Biofluids

4.3.1 Cerebrospinal fluid (CSF)—CSF is a clear, colorless body fluid found in the brain and spine, and plays critical roles in many physiological processes in the central nervous system. CSF contains abundant small metabolites, peptides, and proteins. Changes in their concentrations directly reflect the internal milieu of the brain. Therefore, CSF is considered as a good candidate for the discovery of new biomarkers to improve early diagnosis of neurological diseases, such as Alzheimer's disease [151, 152]. CSF has low protein content (*e.g.*, ~0.35 mg/mL) but a wide dynamic range spanning at least 9 orders of magnitude. Due to the low total protein mass available from a typical CSF sample, Cunningham *et al.* applied a modified IgY14-based spin column to analyze CSF obtained from mice, which led to the identification of 289 proteins [153]. Furthermore, Hyung *et al.* designed a microscale depletion system using IgY14 resin, which could be used to analyze small volume of CSF and plasma with low flow rate [72]. By combining immunoaffinity separation with high sensitivity and resolution LC-MS/MS detection, Schutzer *et al.* first established a comprehensive proteome map for normal human cerebrospinal fluid, which consisted of 2630 proteins [154]; using the same analytical strategy they further compared the CSF proteomes of Neurologic Post Treatment Lyme disease (nPTLS) patient, Chronic Fatigue Syndrome (CFS) patient and normal healthy control, and reported on a number of CSF proteins that could be useful for distinguishing nPTLS and CFS [155]. Recently, by combining immunodepletion of 14 HAPs, high-pH reverse-phase separation and high resolution MS/MS detection, Zhang *et al.* constructed a most up-to-date proteome map for CSF, which consisted of 3256 non-redundant proteins [156]. Immunoaffinity separation has also been applied in CSF for biomarker studies of several other neurological diseases, such as Parkinson's disease [157], Huntington's disease [158].

4.3.2 Synovial fluid (SF)—Synovial fluid (SF) is a serum filtrate located among the joint, where it also receives protein contributions from the surrounding tissues, articular cartilage, synovial membrane and bone.

In order to identify potential biomarkers for rheumatoid arthritis and osteoarthritis, Mateos *et al.* applied immunoaffinity depletion followed by LC-MS/MS detection, which lead to the identification of 136 differential proteins [159]. More recently, by using immunoaffinity depletion combined with extensive fractionation and high resolution MS/MS detection, Balakrishnan *et al.* presented an in-depth analysis of the synovial fluid proteome from patients with osteoarthritis, which successfully detected 677 proteins [160].

5. Conclusion and perspectives

Significant advances have been made over the last decade in the IAC approaches in the aspects of both immunoaffinity depletion and enrichment. Given the challenge of the enormous dynamic range of protein concentrations in human biofluids, IAC has made tremendous contributions to enable more effective detection of LAPs in these biofluids and identify potential biomarkers for various disease conditions by both global proteome profiling and targeted quantification. The development of targeted MS immunoassays based

on either anti-protein antibodies or anti-peptide antibodies has become much more automated and higher throughput [81, 103].

While IAC has become an important tool for proteomic analyses of biofluids, there are several caveats that need to be taken into consideration in the experimental design. First, there is a significant concern of non-specific or specific loss of LAPs during immunodepletion. This is especially the case for the SuperMix system since it contains a large number of different antibodies and could deplete or partially deplete many more proteins. Therefore, the SuperMix system was proposed as a fractionation approach rather than a depletion approach. Second, for immunoaffinity enrichment, the quality (specificity, binding affinity) of antibody is still critical. It is still costly to make a good antibody in terms of the time and cost for enriching specific targets. Third, another valid concern for IAC lies in the appropriate loading amount. Determining the optimal loading amount is essential for high efficient binding of target proteins which enables effective depletion as well as high enrichment efficiency. Finally, most IAC approaches are still quite labor intensive and only offer moderate throughput for sample processing. Despite of these concerns, we anticipate the field of immunoaffinity chromatography will continue to advance in the areas of novel affinity reagents, automation for high throughput IAC separations and assay developments, and integration of other advanced devices (*e.g.*, microfluidics-based devices [161]), and mass spectrometry platforms to make even more significant contributions in both global proteomics analyses of human biofluids and large-scale high throughput targeted quantification of biofluid samples for biomarker verification and development.

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Highlights

- The enormous dynamic range of protein concentrations in human biofluids represents a significant analytical challenge for discovering low-abundance protein biomarkers.
- Immunoaffinity chromatography has become an essential method for enabling the detection of low-abundance proteins in human biofluids.
- The advances of immunoaffinity chromatographic methods including immunodepletion and immunoenrichment are reviewed.
- Proteomics applications of immunoaffinity approaches to human biofluids are highlighted.

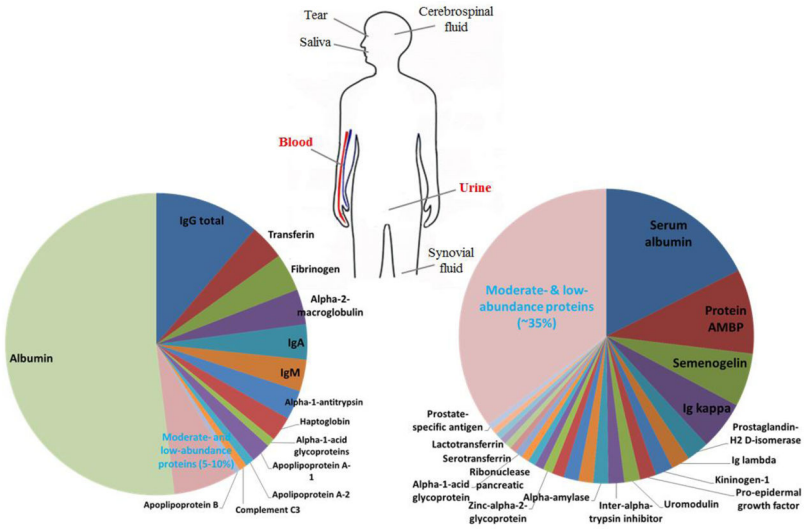

Figure 1.

Biofluids in human. Main sources and origins of human biofluids are depicted; the compositions of protein mass for both plasma/serum (data from in-house protein identification result and protein abundances were based on spectral count) and urine (data from Li *et al.* [162]) are shown in pie charts on the left and right hand side, respectively.

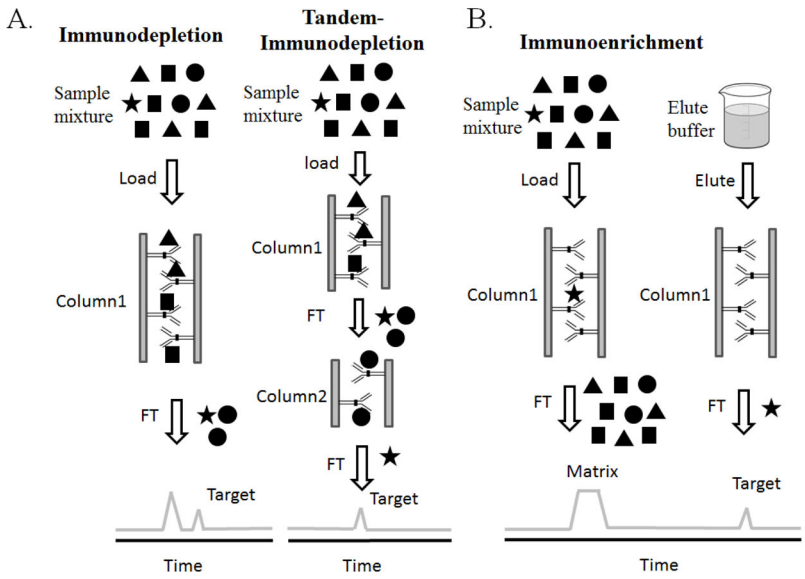

Figure 2.

The main workflows of immunodepletion (A) and immunoenrichment (B). For immunodepletion, the Star and Circles represents LAPs and MAPs, respectively. For Immunoenrichment, the Star represent the target molecules and other symbols are sample matrix.

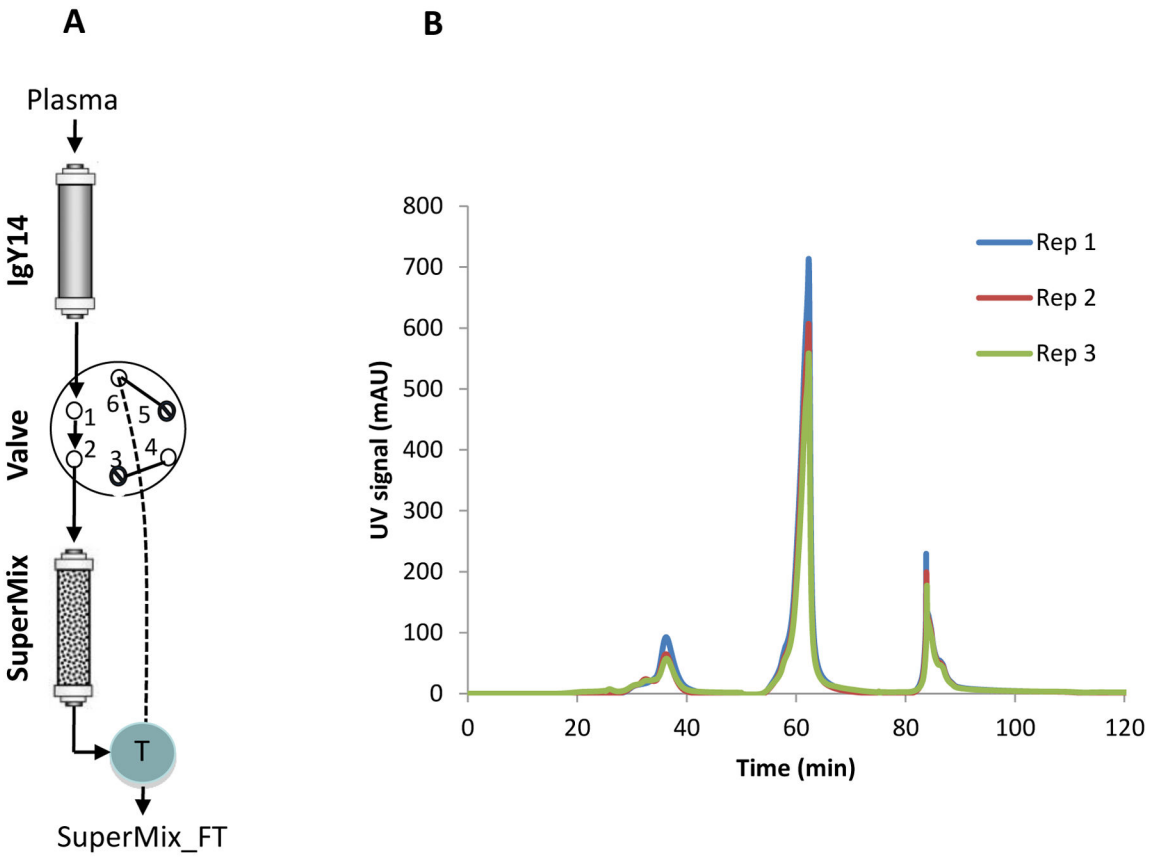

Figure 3.

Immunodepletion using the tandem IgY14-SuperMix configuration. (A) IgY14 and SuperMix columns are connected through a 6-port valve. By switching the valve positions, the SuperMix flow-through (SuperMix_FT), IgY14 bound, and SuperMix bound fractions can be collected consecutively. (B) Representative LC chromatograms of 3 replicates of the tandem IgY14-SuperMix separations during the initial, middle and late stage of the column life of a reference plasma sample.

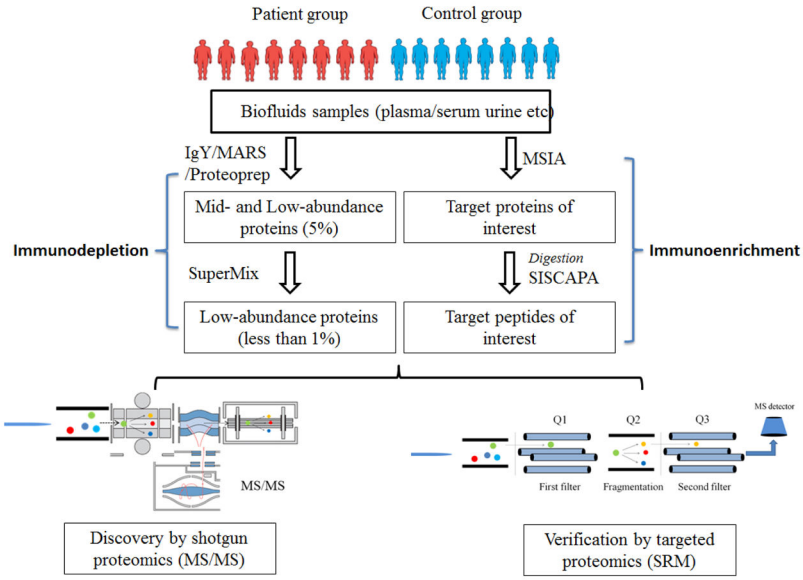

Figure 4.

General workflow of applying immunoenrichment and immunodepletion for a biomarker related study. Biofluid samples (*e.g.*, plasma/serum or urine) are subjected to immunodepletion or immunoenrichment to reduce sample complexity. Immunodepletion is used to remove high abundant species, such as HAPs and MAPs, prior to MS-based proteomic studies. Target proteins or peptides can be selectively enriched through immunoenrichment by using MSIA or SISCAPA approaches, which are especially useful for targeted proteomics.

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Table 1

HAPs in plasma targeted by the commonly commercially available immunoaffinity depletion columns.

	Vivapure	Qproteome	ProteoPrep	Mars Hu-6	Mars Hu-14	Seppro IgY-14	ProteoPrep 20
Albumin	√	√	√	√	√	√	√
IgG		√	√	√	√	√	√
Transferrin				√	√	√	√
Fibrinogen					√	√	√
IgA				√	√	√	√
a-2-Macroglobulin					√	√	√
IgM					√	√	√
a-1-Antitrypsin				√	√	√	√
Complement C3					√	√	√
Haptoglobin				√	√	√	√
Apolipoprotein A-I					√	√	√
Apolipoprotein A-II					√	√	√
Apolipoprotein B						√	√
a-1-Acid Glycoprotein					√	√	√
Transthyretin					√		
Ceruloplasmin							√
Complement C4							√
Complement C1q							√
IgD							√
Prealbumin							√
Plasminogen							√
Number of depleted HAPs	1	2	2	6	14	14	20
Vendors	Sartorius	QIAGEN	Sigma Aldrich	Agilent	Agilent	Sigma Aldrich	Sigma Aldrich