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Strategies for Characterization of Low-Abundant Intact or Truncated Low-Molecular-Weight Proteins From Human Plasma

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Abstract
Low-molecular-weight region (LMW, MW ≤ 30 kDa) of human serum/plasma proteins, including small intact proteins, truncated fragments of larger proteins, along with some other small components, has been associated with the ongoing physiological and pathological events, and thereby represent a treasure trove of diagnostic molecules. Great progress in the mining of novel biomarkers from this diagnostic treasure trove for disease diagnosis and health monitoring has been achieved based on serum samples from healthy individuals and patients and powerful new approaches in biochemistry and systems biology. However, cumulative evidence indicates that many potential LMW protein biomarkers might still have escaped from detection due to their low abundance, the dynamic complexity of serum/plasma, and the limited efficiency of characterization.
approaches. Here, we provide an overview of the current state of knowledge with respect to strategies for the characterization of low-abundant LMW proteins (small intact or truncated proteins) from human serum/plasma, involving prefractionation or enrichment methods to reduce dynamic range and mass spectrometry-based characterization of low-abundant LMW proteins.

1. INTRODUCTION

1.1 LMW Proteins in Human Serum/Plasma Represent a Treasure Trove of Diagnostic Molecules

Human blood collected and accumulated a vast trove of biochemical molecules while going through different organs or tissues, which can ultimately reflect the physiological or pathological status of the body [1,2], and thereby provide valuable information for early diagnosis and prognosis, as well as for the monitoring of therapeutic efficiency. Cumulative evidence suggests that low-molecular-weight (LMW) region of human serum proteome, including small intact proteins, truncated fragments of larger proteins, along with some other small components, has been implicated in various human diseases such as cancer [3], diabetes [4], cardiovascular [5], and infectious diseases [6] and represents a treasure trove of diagnostic molecules. There are several facts that might support this view. To begin with, the LMW proteins are usually made up of physiologically important proteins such as cytokines, growth factors, peptide hormones, as well as proteins/peptides originating from normal cell or tissue leakage as a result of cell death or damage, which are believed to be more important to reflect valuable disease-related information than high-abundant proteins from a diagnostic point of view [7,8]. For instance, the LMW protein family human serum amyloid A (SAA), a hot spot in the field of biomarker research, has been associated in various types of cancers such as ovarian cancer [9], lung cancer [10], pancreatic cancer [11], as well as the other diseases like coronary artery disease, obesity, and severe acute respiratory syndrome [12–14]. Moreover, increasing evidence shows that tumor cells excrete biomolecules (<10 kDa) to the bloodstream that are specific to the tumor type at a rate proportional to the tumor age/size [15,16]. Second, the LMW proteins are readily to enter the blood. In contrast to larger proteins, which enter the bloodstream intact only if they are actively secreted, or if the vascular wall becomes permeable owing to disease, the LMW proteins are small enough to enter the blood passively and produce diagnostic traces [2]. In fact, many LMW proteins and peptides that potentially contain disease-specific information were found to be fragments derived from
larger parent molecules that are normally too large to passively diffuse through the endothelium into the circulation. Third, proteinase-mediated ex vivo enzymatic cleavage as part of the coagulation and complement activation pathways also leads to the produce of the LMW protein biomarkers. This is really true as circulating blood already contains an abundance of protein fragments apparently derived from cells and tissues that are produced in vivo. For instance, Villanueva and coworkers showed that diagnostic protein fragments can be generated ex vivo by circulating enzymes derived from the diseased tissue microenvironment acting on exogenously derived peptides produced by serum collection methodology [17].

1.2 Characterization of LMW Proteins From the Human Serum/Plasma Is Analytically Challenging

However, identification of LMW proteins from human serum/plasma is analytically challenging due to several aspects. First, the dynamic complexity of serum/plasma, spanning more than 10 orders of magnitude in terms of concentration, greatly hinders the characterization of LMW proteins. As we known, many high-abundant proteins exist at concentrations billion-fold higher than those of the low-abundant LMW proteins thereby masking the potential LMW protein biomarkers and hindering their detection. For instance, in the human plasma proteome, 22 abundant proteins, including albumin, immunoglobulin, fibrinogen, alpha 1-antitrypsin, alpha 2-macroglobulin, are responsible for 99% of the bulk mass of the total protein content in human plasma [7,18]. Some proteins like albumin can even reach concentrations of 35–50 mg/mL in serum [8]. Second, compounded by the relatively low concentration of LMW biomarkers present in the circulation (levels of some LMW proteins in serum were estimated to be as low as picomole or even femtomole), accurate and consistent comprehensive measurement is difficult. Moreover, cumulative evidence shows that many LMW proteins in serum consisted of truncated soluble forms resulting from an in vivo or ex vivo cleavage, which further lowers the concentration of these proteins and decreases their detectability. Indeed, degradation of target biomarkers occurs during transportation and storage of blood, leading to serious false-positive and false-negative results. Third, filtration of molecules through the glomerular capillary wall (glomerular filtration) is highly dependent on molecule size and is referred to as the filtration-size threshold [19], which means the size of a protein generally determines how fast it would be cleared from the blood by kidney filtration and uptake by the liver. For instance, insulin achieves 100% renal filtration with a blood half-life of only 9 min [20]. Therefore, LMW molecules generated in vivo would be cleared
rather quickly, thereby reducing the concentration of the molecule to potentially undetectable levels. Last but not least, the effective way for a small proteins to avoid to be rapidly cleared as mentioned earlier and stay in circulation is to hitch a ride with a carrier protein. This is really true as many studies have showed that LMW biomarkers are associated non-covalently with high-molecular-weight (HMW) protein [21]. Therefore, removing HMW proteins from the sample may further dilute the samples from LMW biomarkers.

### 2. STRATEGIES FOR THE PREFRACTIONATION OR ENRICHMENT OF SMALL INTACT OR TRUNCATED PROTEINS FROM THE HUMAN PLASMA

As mentioned earlier, characterization of low-abundant and LMW proteins is a very difficult task. In order to simplify the biomarker discovery from this LMW region, a wide range of technologies is utilized, which are mainly fall into two strategies (Fig. 1), prefractionation to reduce the

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**Fig. 1** Strategies for prefractionation or enrichment of low-abundant LMW proteins from human serum/plasma.
dynamic complexity and enrichment of LMW proteins to enhance the detection sensitivity.

2.1 Prefractionation Methods to Reduce Dynamic Complexity

By modifying the sample protein distribution, prefractionation strategies allow proteins initially present at low concentrations to be accessible for analysis. Hence, many different fractionation/separation techniques, considering protein’s biochemical and biophysical properties such as molecular weight, isoelectric point, and hydrophobicity, as well as the efficiency and reproducibility, have been developed and applied in a multidimensional fashion to enhance detection of low-abundance proteins in human serum/plasma [22,23]. Due to the great “masking” effect created by high-abundance proteins on the LMW proteins detection, the depletion of high-abundance proteins has become one of the most commonly applied strategies to reduce dynamic complexity of serum/plasma. Indeed, Tu and coworkers reveal that removal of the top 7 or top 14 high-abundance proteins has been shown to result in a 25% increase in the number of identified proteins [24]. So far, there are several different approaches commonly used to remove high-abundance proteins from human serum/plasma, including organic solvent precipitation, centrifugal ultrafiltration, or affinity-based techniques.

2.1.1 Organic Solvent Precipitation

The simplest approach to deplete high-abundant proteins from serum would be to precipitate the proteins with organic solvent (methanol or acetonitrile) or ammonium sulfate (or other inorganic salt). Most of the HMW proteins (MW > 20kDa) would be precipitated, and only lower-molecular-weight ones would remain in the supernatant. So far, acetonitrile precipitation, which has been shown to effectively precipitate large, abundant proteins out of serum, is believe to be a superior method to remove serum albumin and immunoglobulins from serum [25]. This may due to the facts that ACN not only denatures these large proteins allowing for their removal but causes the small, low-abundance proteins and peptides normally bound to these carrier proteins to dissociate, thus making them available for detection [8]. Indeed, our experience suggests that mass spectrometry (MS) analysis of serum LMW proteins was greatly improved with enhanced signal intensity and higher resolution (Fig. 2), which facilitated tracing of the targeted proteins [26]. However, caution is required, as the risk of coprecipitating the low-abundant proteins would be great.
2.1.2 Centrifugal Ultrafiltration

Centrifugal ultrafiltration, which utilizes both centrifugation force and a semipermeable membrane to retain HMW proteins, while LMW proteins pass through the membrane (filtrate) of nominal molecular weight cutoff, is another common strategy for the depletion of high-abundant proteins from human serum/plasma. However, it is critical to disrupt the peptide/protein–protein interactions prior to centrifugal ultrafiltration to released LMW components bound to larger species, as many physiologically important low-abundant proteins are often found to be bound to carrier proteins in plasma [18]. Moreover, it is important to recognize that retention of a protein by centrifugal ultrafiltration membrane is determined by a variety of factors, including molecular weight, molecular shape, electrical charge, sample concentration, buffer composition, and operating conditions. Therefore, multiple studies showed that HMW proteins will still appear in the filtrate when use different commercially available filter membranes. One of the reasons was supposed to be that the shape of some HMW proteins changes after denature, thereby leading their size change and making them pass through the membrane. Therefore, multidimensional separation, such as the combination of centrifugal ultrafiltration and reversed-phase

Fig. 2 Electrophoretic comparison (A) and MALDI-TOF-MS profiles of low-molecular-weight proteins (B) of serum sample before (1) and after (2) ACN precipitation. Loading sample size of electrophoresis is 0.3 μL serum (lane 1), 7 μL supernatant derived from ACN precipitation of 5 μL serum (lane 2), respectively.

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solid-phase extraction, was recommend to improve the enrichment of LMW proteins and peptides [27].

2.1.3 Affinity-Based Techniques
The organic solvent precipitation and centrifugal ultrafiltration approaches mentioned earlier are quite nonselective because it generally removes the HMW proteins. To remove high-abundant proteins from serum more specifically, many techniques based on affinity matrices such as antibodies, antibody-mimicking peptides, or other kinds of natural compounds are often employed. For instance, the most popular method to remove HSA is using Cibacron Blue-based affinity chromatography [28,29]. For depletion of immunoglobulin, the second most abundant serum/plasma protein, the use of immobilized protein A or G media, which specifically binds to the Fc region of IgG, is the technique of choice [30]. However, the depletion of HSA by dye-based resins is not entirely straightforward due to the enormous load of HSA species in serum and plasma and nonspecific binding of some other serum proteins as well [31]. Moreover, Cibacron Blue dye does not only show affinity for albumin but also for NAD, FAD, and ATP-binding sites of proteins, which often results in the unwanted removal of proteins of interest [32–34]. In addition, the protein A for antibody capture also shows widely varying degrees of affinity for the antibody isotypes and subtypes, which results in incomplete removal of some subtypes of IgG. Therefore, the use of specific ligands for the capture of HSA and IgG, such as antibodies, was employed. Steel and coworkers demonstrated to develop an immune-affinity resin using monoclonal antibodies against HSA to effectively removal of both full-length HSA and many of the HSA fragments present in serum, which shows markedly better performance than dye-based resins in terms of both the efficiency and specificity of albumin removal [31]. Furthermore, when a protein G affinity resin is used together with the immunoaffinity resin, IgG and HSA can be simultaneously removed from the samples.

However, it still leaves behind many high-abundance proteins, though the single-antibody columns that are specific to IgG and HSA can capture a good percentage of their target protein. Thus, the selective simultaneous removal of more than a single high-abundance protein became more desirable. During the last decades, there has been a gradual development of several multiple affinity removal columns for the simultaneous depletion of even more high-abundant proteins. The multiple affinity removal system (MARS) (Agilent Technologies, Santa Clara, California), which
combines the specificity of antibody–antigen recognition and the efficiency of standard liquid chromatography, was one of the first MARS on the market. The affinity column removes HSA, IgG, transferrin, alpha 1-antitrypsin, haptoglobin, and immunoglobulin by using six affinity-purified polyclonal antibodies that bonded to polymeric microbeads via their Fc region.

A number of other commercial products, based upon specific antibodies to the high-abundance proteins or using ion-exchange mechanism, have also been designed to remove high-abundant proteins from serum and plasma [35]. These products are claimed to be ranging from the depletion of 4 proteins all the way up to more than 20 proteins, such as Seppro IgY14 and ProteoPrep 20 (Sigma Aldrich), as well as ProteoSpin (Norgen Biotek Corp.) However, cautions are required, as recent evidence has shown that increasing the number of depleted proteins from 12 to 20 had only little beneficial effect and could in fact even increase the removal of peptides and proteins of interest which are associated with the abundant proteins [24,36]. In addition, the next layer of abundant protein, moderately abundant proteins, after separating the highly abundant proteins, becomes an obstacle to access the low-abundant proteins. To tackle this challenge, Fang and coworkers further developed the IgY-microbead system by immunizing chickens with a flow-through fraction of IgY12 column and constructing the column with affinity-purified IgY antibodies against the flow-through proteins of IgY12 column, which enabled specific capturing of 207 moderately abundant proteins and thereby enables deeper and more effective access into the population of LAPs [37].

2.2 Enrichment of Low-Abundance LMW Proteins

Even though there are several methods available as mentioned earlier to fractionate or remove proteins in the sample to decrease the dynamic range. The LMW proteins in human serum/plasma still present a complex mixture. It remains a big challenge to identify the target protein from serum/plasma without enrichment or isolation. As a subsequence, an efficient enrichment or isolation procedure is needed for further reduction of sample’s complexity, thereby for the purification and identification of targeted proteins.

2.2.1 Affinity Enrichment of Low-Abundance LMW Proteins

In contrast to depletion techniques, specific enrichment processes allow for fast and high specific detection at picomolar or even femtomolar quantities of target protein.
One of such enrichment processes is magnetic beads coated with ligands of interest, which enable identifying low-abundant LMW protein faster, more efficiently, and at lower cost than other methods. This may primarily due to the fact that magnetic particles comprise a convenient solid support for a variety of assays and procedures based on affinity purification. For instance, the high density of the beads allows rapid capture by magnetic devices, while the visibility of the beads ensures reliable collection of all target protein bound in the immunoprecipitation/pull-down application, which allows concentration of low-abundant target protein samples from milliliter down to microliter scale. Anderson and coworkers reported a SISCAPA (stable isotope standards and capture by antipeptide antibodies) method for specific antibody-based capture of individual tryptic peptides from a digest of whole human plasma based on a simplified magnetic bead protocol [38]. With this protocol, selected tryptic peptides of α1-antichymotrypsin and lipopolysaccharide-binding protein were enriched relative to a high-abundance serum albumin peptide by 1800- and 18,000-fold, respectively, as measured by multiple reaction monitoring (MRM). In contrast to the magnetic bead, which is of excellent properties for small-scale experiments, the experimental features of antibody microarrays have the advantages of performing the simultaneous enrichment of several hundreds of proteins in parallel [39]. Desrosiers and coworkers demonstrated a proteomic analysis of human plasma proteins using antibody arrays coated with 350 antibodies directed against signaling proteins after removal depletion of high-abundance proteins [40]. The results showed that antibody microarrays allowing the identification of 61 lower-abundance plasma proteins using 350 antibodies directed against signaling proteins, and authors suggest that this strategy is a valuable approach for detecting potential plasma biomarkers. An alternative and innovative strategy is based on a combinatorial solid-phase library, also called ProteoMiner (ProteoMiner technology, BioRad) [41]. This combinatorial solid-phase library contains a large and highly diverse library of hexapeptides bound to a chromatographic support, allowing each unique hexapeptide to bind to a unique protein sequence. When the sample is added, the limiting binding capacity of the beads enables high-abundance proteins to rapidly saturate their ligands, and excess protein is washed out.

However, cautions are required, as one potential pitfall remains with regarding to the elution of target molecules for specific enrichment. If the antibodies have very high selectivity, then in the case of low-abundance proteins, only a very small amount of proteins will be eluted from the antibody, which are easily lost through the following procedures.
2.2.2 Enrichment of Low-Abundance LMW Proteins Based on Nanotechnology

Advances in microfabrication offer great opportunities to provide “nanoharvesting” agents designed specifically to capture and amplify classes of LMW proteins. In fact, in the past few years, a variety of types of nanoparticles have been applied to capture and separate proteins from complex mixtures [42–46].

Among these nanomaterials, golden nanoparticles (GNPs) are found to be readily available, relatively stable, have low toxicity, and excellent compatibility with biomolecules [47]. Self-assembly monolayer technology further increases GNP solubility and improves interfacial interactions. By coating with selection of functional motifs, GNPs can be functionalized to harvest LMW proteins [48]. Recently, Khoury and coworkers [49] demonstrated a poly(acrylic acid)-coated gold NPs created by the layer-by-layer method to be used to selectively harvest positively charged LMW biomarkers (<10 kDa) such as SDFR and FITC-labeled peptide in one step. In addition, specialized affinity bait-containing hydrogel particles, which simultaneously conduct molecular sieve chromatography and affinity chromatography in one step, have shown great promise for LMW proteins harvesting. Cumulative evidence shows that hydrogel particles can conduct enrichment and encapsulation of selected classes of proteins and peptides from complex mixtures of biomolecules such as native human serum and urine, purify them from endogenous high-abundance proteins such as albumin, and protect them from degradation during subsequent sample handling [45,50,51].

Carbon nanotubes are another type of important nanomaterial useful for enrichment of low-abundant LMW proteins, due to their extraordinary electronic and mechanical properties. CNTs offer a number of advantages for enrichment of proteins that may result in more efficient enrichment than nanoparticles. CNTs have a large inner volume which allows molecules to be encapsulated, and this volume is more easily accessible because the end caps can be easily removed. In addition, they have distinct inner and outer surfaces for functionalization to be biocompatible and to be capable of recognizing proteins [52]. Broadly, CNTs may be classed either as single-walled nanotubes (SWNTs) or multiwalled nanotubes (MWNTs). MWNTs are composed of several coaxial layers of SWNTs, which allow them interact strongly with organic molecules, and thereby might offer interesting opportunities for the development of new, unique adsorbent material for the capture of LMW proteins. Li and coworkers described a novel peptidome
analysis approach using multiwalled carbon nanotubes as an alternative adsorbent to capture endogenous peptides from human plasma [53]. By coupling with liquid chromatography–mass spectrometry, this method allowed detection of 2521 peptide features (m/z 300–1800 range) in about 50 μL of plasma.

Due to its well-defined pore network and surface reactivity, the use of mesoporous silica surfaces as a size-exclusion method for harvesting the peptides and small proteins has also been widely reported [42,54]. In addition, the chemical and structural modification of porous silicon enables optimization of the ionization of the porous silicon surface, which allows it to be sensitive and compatible with silicon-based microfluidics and microchip technologies. Based on this, nanoporous surfaces have been used to capture LMW peptides from human plasma. For instance, Fan and coworkers have described a peptide extraction approach based on nanoporous silica thin films to successfully detect low concentrations of hepcidin from human body fluids collected from 119 healthy volunteers and 19 inflammation patients [55]. Recently, Tang and coworkers reported an approach for the adsorption and enrichment of low-abundance protein in mammalian cell extract by using nanostructured surface-imprinted polymers with sufficiently thin shells and high surface-to-volume ratios [56].

3. CHARACTERIZATION OF LMW PROTEINS BASED ON MS

By virtue of its high sensitivity, high resolution, high accuracy, and high throughput, MS has become the primary tool for identification and quantification of proteins from serum/plasma. For characterization of LMW proteins from plasma, there are two fundamentally different MS-based strategies: discovery-based identification and targeted quantification.

3.1 Discover-Based Identification of LMW Proteins

With a discovery-based strategy, the goal is usually to discover proteins that are associated with the ongoing physiological and pathological events, and thereby a further elucidation of LMW protein identities or clinical validation. MS-based strategies that most frequently used in the identification of proteins always involve a combination of gel- or LC-based separation techniques and MS, such as SDS-PAGE or 2-DE coupled with MALDI/ESI-MS(/MS), and LC-MALDI/ESI-MS(/MS). However, prior efforts
in the search for serum and plasma protein biomarkers utilized gel-based separation technologies showed that gel cannot readily separate and distinguish molecules of less than 10 kDa in size [57]. In contrast, high-performance liquid chromatography (HPLC) separation, coupling with MS, is highly automated and has the capacity to separate low-abundance proteins for subsequent detachment and thus widely used to separate the proteins in serum/plasma before or after high-abundant protein depletion. In a typical ESI-MS/MS-based experiment, especially LC-MS/MS approach, protein is digested into peptides, and then peptide ions are selected in the mass spectrometer for fragmentation to generate tandem mass spectra for sequencing, which involving automatic and sophisticated bioinformatics tools. In laser desorption/ionization techniques (SELDI or MALDI) based experiment, many reports of biomarker were limited to a general description of protein peaks, due to the inherent low resolution and mass accuracy of SELDI-TOF-MS or limited capability of tandem MS/MS for protein analysis by MALDI-TOF-MS. Therefore, an additional efficient isolation procedure is needed for a further elucidation of LMW protein identities. Our group has developed an approach combining ZipTip-C18 pipette tip desalting, acetonitrile (ACN) precipitation, reverse-phase C18 HPLC purification, and MALDI-TOF-MS analysis (Fig. 3), which we believed is suitable to profiling, purification, and identification of low-abundance, LMW targeted proteins in human serum. Using this workflow, we purified and identified 10 SAA isoforms with or without truncations from sera of advanced-stage cancer patients [26].

3.2 Targeted Quantification of LMW Proteins

In targeted quantification of LMW proteins, several strategies have been applied, which are mainly fall into two categories: selected reaction monitoring (SRM, also termed MRM) and parallel reaction monitoring (PRM).

Due to its high specificity and sensitivity, MRM is presently the most widely used MS-based targeted proteomic approach [58,59]. MRM is typically carried out in triple quadrupole (QQQ) mass spectrometers, in which the first mass analyzer (Q1) is set to only transmit the target ion of the selected peptide, the collision energy is optimized to produce diagnostic charged fragments from this ion in the second mass analyzer (Q2), and the third mass analyzer (Q3) is set to transmit one to three selected diagnostic fragments for monitoring (Fig. 4A). The advantages of MRM compared to other quantitative analytical methods such as Western blotting, ELISA, and
immunohistochemistry include multiplexed detection and the ability to use spiked-in, stable isotope-labeled standards to foster the absolute or precise relative quantification of endogenous analytes. The MRM assay approach has been applied to the measurement of various low-abundant specific peptides in tryptic digests of plasma, such as C-reactive protein [60] and prostate-specific antigen [61].

PRM, first published in 2012, is a targeted proteomics strategy where all product ions of the target peptides are simultaneously monitored at high-resolution and high mass accuracy [62]. The principle of PRM is comparable to SRM/MRM, but it permits the parallel detection of all target product ions in one high-resolution mass analysis, as the third quadrupole of a QQQ mass spectrometer is substituted with a high-resolution and accurate-mass analyzer in PRM case (Fig. 4B). There are several advantages of PRM when compared to MRM. To begin with, the PRM performs a full scan for fragment ions, instead of recording just 3–5 transitions as in MRM, enables high-quality quantitative measurements. Moreover, the background interference could be greatly eliminated with high resolution and accuracy,
thus improve the detection limit and sensitivity in complex background effectively. In addition, requiring minimal upfront efforts for selection of target transitions and optimization of fragmentation energy facilitates assay development. Thus, PRM was believed to be suitable for quantification of multiple proteins in low abundant in complex sample. For instance, Kim and coworkers have developed a PRM assay to selectively measure isotypes of SAA and successfully measured five allelic variants (1α, 1β, 1γ, 2α, 2β) of SAA1 and SAA2 in plasma samples from lung cancer patients [63]. Ronsein et al. have developed a PRM method for quantification of the high-density lipoprotein proteome in plasma samples using 15N-labeled apolipoprotein A-1 as an internal standard [64].

4. FUTURE PERSPECTIVES AND CONCLUDING REMARKS

Despite various advancements in characterization of low-abundant LMW proteins from human serum/plasma, there are still many challenges remain. For instance, even though with significant recent advances, current proteomics technologies still fall short of being able to reliably detect in blood plasma low ng/mL to sub-ng/mL protein concentrations, a level of detection often required for discovering disease-specific biomarkers. Moreover, the depletion of high-abundance proteins could potentially alleviate their “masking” effect; however, the removal or reduction of the high-abundance proteins without affecting the minor proteins represents a challenge. Furthermore, sample preparation introduces preanalytical variations and poses major challenges to analyze the serum proteome. Therefore,
the possibility to develop new technologies or strategies allowing selective identification and quantification of LMW proteins from serum/plasma may accelerate the discovery rate of potential biomarkers and drug targets.

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REFERENCES

[1] S.M. Hanash, S.J. Pitteri, V.M. Faca, Mining the plasma proteome for cancer biomarkers, Nature 452 (2008) 571–579.
[2] L.A. Liotta, M. Ferrari, E. Petricoin, Clinical proteomics: written in blood, Nature 425 (2003) 905.
[3] E.F. Petricoin, C. Belluco, R.P. Araujo, L.A. Liotta, The blood peptidome: a higher dimension of information content for cancer biomarker discovery, Nat. Rev. Cancer 6 (12) (2006) 961–967.
[4] C. Törn, C-peptide and autoimmune markers in diabetes, Clin. Lab. 49 (1–2) (2003) 1–10.
[5] A. Dolci, M. Panteghini, The exciting story of cardiac biomarkers: from retrospective detection to gold diagnostic standard for acute myocardial infarction and more, Clin. Chim. Acta 369 (2006) 179–187.
[6] M.G. Gravett, M.J. Novy, R.G. Rosenfeld, A.P. Reddy, T. Jacob, M. Turner, A. McCormack, J.A. Lapidus, J. Hitti, D.A. Eschenbach, C.T. Roberts Jr., S.R. Nagalla, Diagnosis of intra-amniotic infection by proteomic profiling and identification of novel biomarkers, JAMA 292 (4) (2004) 462–469.
[7] M. De Bock, D. de Seny, M.A. Meuwis, A.C. Servais, T.Q. Minh, et al., Comparison of three methods for fractionation and enrichment of low molecular weight proteins for SELDI-TOF-MS differential analysis, Talanta 82 (1) (2010) 245–254.
[8] S. Hu, J.A. Loo, D.T. Wong, Human body fluid proteome analysis, Proteomics 6 (23) (2006) 6326–6353.
[9] E.F. Petricoin, A.M. Ardekani, B.A. Hitt, P.J. Levine, V.A. Fusaro, S.M. Steinberg, G.B. Mills, C. Simone, D.A. Fishman, E.C. Kohn, L.A. Liotta, Use of proteomic patterns in serum to identify ovarian cancer, Lancet 359 (9306) (2002) 572–577.
[10] W.M. Gao, R. Kuick, R.P. Orchekowski, D.E. Misek, J. Qiu, A.K. Greenberg, W.N. Rom, D.E. Brenner, G.S. Omenn, B.B. Haab, S.M. Hanash, Distinctive serum protein profiles involving abundant proteins in lung cancer patients based upon antibody microarray analysis, BMC Cancer 5 (2005) 110.
[11] K. Yokoi, L.C. Shih, R. Kobayashi, J. Koomen, D. Hawke, D. Li, S.R. Hamilton, J.L. Abbuzzese, K.R. Coombes, I.J. Fidler, Serum amyloid A as a tumor marker in sera of nude mice with orthotopic human pancreatic cancer and in plasma of patients with pancreatic cancer, Int. J. Oncol. 27 (5) (2005) 1361–1369.
[12] B.D. Johnson, K.E. Kip, O.C. Marroquin, P.M. Ridker, S.F. Kelsey, L.J. Shaw, C.J. Pepine, B. Sharaf, C.N. Bairey Merz, G. Sopko, M.B. Olson, S.E. Reis, National Heart, Lung, and Blood Institute, Serum amyloid A as a predictor of coronary artery disease and cardiovascular outcome in women: the National Heart, Lung, and
Blood Institute-Sponsored Women’s Ischemia Syndrome Evaluation (WISE), Circulation 109 (6) (2004) 726–732.

[13] Y. RZ, M.J. Lee, H. Hu, T.I. Pollin, A.S. Ryan, B.J. Nicklas, S. Snitker, R.B. Horenstein, K. Hull, N.H. Goldberg, A.P. Goldberg, A.R. Shuldiner, S.K. Fried, D.W. Gong, Acute-phase serum amyloid A: an inflammatory adipokine and potential link between obesity and its metabolic complications, PLoS Med. 3 (6) (2006) e287.

[14] T.T. Yip, J.W. Chan, W.C. Cho, T.T. Yip, Z. Wang, T.L. Kwan, S.C. Law, D.N. Tsang, J.K. Chan, K.C. Lee, W.W. Cheng, V.W. Ma, C. Yip, C.K. Lim, R.K. Ngan, J.S. Au, A. Chan, W.W. Lim, Ciphergen SARS Proteomics Study Group, Protein chip array profiling analysis in patients with severe acute respiratory syndrome identified serum amyloid a protein as a biomarker potentially useful in monitoring the extent of pneumonia, Clin. Chem. 51 (1) (2005) 47–55.

[15] A.I. Mehta, S. Ross, M.S. Lowenthal, V. Fusaro, D.A. Fishman, E.F. Petricoin, L.A. Liotta, Biomarker amplification by serum carrier protein binding, Dis. Markers 19 (2003) 1–10.

[16] E.W. Deutsch, J.K. Eng, H. Zhang, N.L. King, A.J. Nesvizhskii, B. Lin, H. Lee, E.C. Yi, R. Ossola, R. Aebersold, Human plasma peptide atlas, Proteomics 5 (2005) 3497–3500.

[17] J. Villanueva, D.R. Shaffer, J. Philip, C.A. Chaparro, H. Erdjument-Bromage, A.B. Olshen, M. Fleisher, H. Lilja, E. Brogi, J. Boyd, M. Sanchez-Carbayo, E.C. Holland, C. Cordon-Cardo, H.I. Scher, P. Tempst, Differential exoprotease activities confer tumor-specific serum peptidome patterns, J. Clin. Invest. 116 (1) (2006) 271–284.

[18] R.S. Tirumalai, K.C. Chan, D.A. Prieto, H.J. Issaq, T.P. Conrads, et al., Characterization of the low molecular weight human serum proteome, Mol. Cell. Proteomics 2 (10) (2003) 1096–1103.

[19] W.M. Deen, M.J. Lazzara, B.D. Myers, Structural determinants of glomerular permeability, Am. J. Physiol. Renal Physiol. 281 (2001) F579–96.

[20] L.F. Prescott, J.A. McAuslane, S. Freeston, The concentration-dependent disposition and kinetics of inulin, Eur. J. Clin. Pharmacol. 40 (1991) 619–624.

[21] M.S. Lowenthal, A.I. Mehta, K. Frogale, R.W. Bandle, R.P. Araujo, B.L. Hood, T.D. Veenstra, T.P. Conrads, P. Goldsmith, D. Fishman, E.F. Petricoin 3rd, L.A. Liotta, Analysis of albumin-associated peptides and proteins from ovarian cancer patients, Clin. Chem. 51 (10) (2005) 1933–1945.

[22] H.J. Lee, E.Y. Lee, M.S. Kwon, Y.K. Paik, Biomarker discovery from the plasma proteome using multidimensional fractionation proteomics, Curr. Opin. Chem. Biol. 10 (2006) 42–49.

[23] H. Wang, S.G. Clouthier, V. Galchev, D.E. Misek, U. Duffner, C.K. Min, R. Zhao, J. Tra, G.S. Omenn, J.L. Ferrara, S.M. Hanash, Intact-protein-based high-resolution three-dimensional quantitative analysis system for proteome profiling of biological fluids, Mol. Cell. Proteomics 4 (2005) 618–625.

[24] C. Tu, P.A. Rudnick, M.Y. Martinez, K.L. Cheek, S.E. Stein, R.J. Slebos, D.C. Liebler, Depletion of abundant plasma proteins and limitations of plasma proteomics, J. Proteome Res. 9 (2010) 4982–4991.

[25] K. Merrell, K. Southwick, S.W. Graves, M.S. Esplin, N.E. Lewis, C.D. Thulin, Analysis of low-abundance, low–molecular–weight serum proteins using mass spectrometry, J. Biomol. Tech. 15 (4) (2004) 238–248.

[26] J. Li, Z. Xie, L. Shi, Z. Zhao, J. Hou, X. Chen, Z. Cui, P. Xue, T. Cai, P. Wu, S. Guo, F. Yang, Purification, identification and profiling of serum amyloid A proteins from sera of advanced-stage cancer patients, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 889–890 (2012) 3–9.
[27] X. Lai, F.A. Witzmann, S. Liangpunsakul, Characterization of peptides and low molecular weight proteins in plasma from subjects with hepatocellular carcinoma, A Proteomics 1 (1) (2014) 6.

[28] J. Travis, R. Pannell, Selective removal of albumin from plasma by affinity chromatography, Clin. Chim. Acta 49 (1973) 49–52.

[29] E. Gianazza, P. Arnaud, A general method for fractionation of plasma proteins: dye-ligand affinity chromatography on immobilized Cibacron Blue F3-GA, Biochem. J. 201 (1982) 129–136.

[30] J.N. Adkins, S.M. Varnum, K.J. Auberry, R.J. Moore, N.H. Angell, R.D. Smith, D.L. Springer, J.G. Pounds, Toward a human blood serum proteome: analysis by multidimensional separation coupled with mass spectrometry, Mol. Cell. Proteomics 1 (2002) 947–955.

[31] L.F. Steel, M.G. Trotter, P.B. Nakajima, T.S. Mattu, G. Gonye, T. Block, Efficient and specific removal of albumin from human serum samples, Mol. Cell. Proteomics 2 (4) (2003) 262–270.

[32] N. Zolotarjova, J. Martosella, G. Nicol, J. Bailey, B.E. Boyes, W.C. Barrett, Differences among techniques for high-abundant protein depletion, Proteomics 13 (2005) 3304–3313.

[33] M. Zhou, D.A. Lucas, K.C. Chan, et al., An investigation into the human serum “interactome”, Electrophoresis 25 (9) (2004) 1289–1298.

[34] S.R. Gallant, V. Koppaka, N. Zecherle, Dye ligand chromatography, Methods Mol. Biol. 421 (2008) 61–69.

[35] Y. Gong, X. Li, B. Yang, et al., Different immunoaffinity fractionation strategies to characterize the human plasma proteome, J. Proteome Res. 5 (6) (2006) 1379–1387.

[36] S. Roche, L. Tiers, M. Provansal, et al., Depletion of one, six, twelve or twenty major blood proteins before proteomic analysis: the more the better? J. Proteomics 72 (6) (2009) 945–951.

[37] X. Fang, L. Huang, S. Sikora, D. Hinerfeld, S. Tam, P. Gagné, G.G. Poirier, C. Kusumoto, K. Obata, D.Q. Yang, W. Zhang, Digging deeper and faster into proteome by IgY-immunoaffinity fractionation, J. Biomol. Tech. 18 (1) (2007) 10–11.

[38] N.L. Anderson, A. Jackson, D. Smith, D. Hardie, C. Borchers, T.W. Pearson, SISCAPA peptide enrichment on magnetic beads using an in-line bead trap device, Mol. Cell. Proteomics 8 (5) (2009) 995–1005.

[39] V. Thulasiramam, et al., Reduction of the concentration difference of proteins in biological liquids using a library of combinatorial ligands, Electrophoresis 26 (2005) 3561–3571.

[40] R.R. Desrosiers, E. Beaulieu, M. Buchanan, R. Béliveau, Proteomic analysis of human plasma proteins by two-dimensional gel electrophoresis and by antibody arrays following depletion of high-abundance proteins, Cell Biochem. Biophys. 49 (3) (2007) 182–195.

[41] L. Guerrier, P.G. Righetti, E. Boscetti, Reduction of dynamic protein concentration range of biological extracts for the discovery of low-abundance proteins by means of hexapeptide ligand library, Nat. Protoc. 3 (5) (2008) 883–890.

[42] M. Gaspari, M. Ming-Cheng Cheng, R. Terracciano, X. Liu, A.J. Nijdam, L. Vaccari, E. di Fabrizio, E.F. Petricoin, L.A. Liotta, G. Cuda, S. Venuta, M. Ferrari, Nanoporous surfaces as harvesting agents for mass spectrometric analysis of peptides in human plasma, J. Proteome Res. 5 (2006) 1261–1266.

[43] D. Geho, M.M. Cheng, K. Killian, M. Lowenthal, S. Ross, K. Frogale, J. Nijdam, N. Lahar, D. Johann, P. Herrmann, G. Whiteley, M. Ferrari, E. Petricoin, L. Liotta, Fractionation of serum components using nanoporous substrates, Bioconjug. Chem. 17 (2006) 654–661.
R. Terracciano, M. Gaspari, F. Testa, L. Pasqua, P. Tagliaferri, M.M. Cheng, A.J. Nijdam, E.F. Petricoin, L.A. Liotta, G. Cuda, M. Ferrari, S. Venuta, Derivatized mesoporous silica beads for MALDI-TOF MS profiling of human plasma and urine, Proteomics 6 (2006) 3243–3250.

A. Luchini, D.H. Geho, B. Bishop, D. Tran, C. Xia, R.L. Dufour, C.D. Jones, V. Espina, A. Patanarut, W. Zhou, M.M. Ross, A. Tessitore, E.F. Petricoin 3rd, L.A. Liotta, Smart hydrogel particles: biomarker harvesting: one-step affinity purification, size exclusion, and protection against degradation, Nano Lett. 8 (2008) 350–361.

C. Fredolini, F. Meani, K.A. Reeder, S. Rucker, A. Patanarut, P.J. Botterell, B. Bishop, C. Longo, V. Espina, E.F.I. Petricoin, L.A. Liotta, A. Luchini, Concentration and preservation of very low abundance biomarkers in urine, such as human growth hormone (hGH), by Cibacron Blue F3G-A loaded hydrogel particles, Nano Res. 1 (2008) 502–518.

Z. Zhong, et al., More recent progress in the preparation of Au nanostructures, properties, and applications, Anal. Lett. 36 (2003) 3097–3118.

A. Wang, et al., Gold nanoparticle-assisted protein enrichment and electroelution for biological samples containing low protein concentration—a prelude of gel electrophoresis, J. Proteome Res. 5 (2006) 1488–1492.

L.R. Khoury, R. Goldbart, T. Traitel, G. Enden, J. Kost, Harvesting low molecular weight biomarkers using gold nanoparticles, ACS Nano 9 (6) (2015) 5750–5759.

R. Magni, B.H. Espina, L.A. Liotta, A. Luchini, V. Espina, Hydrogel nanoparticle harvesting of plasma or urine for detecting low abundance proteins, J. Vis. Exp. (90) (2014) e51789.

R. Magni, A. Luchini, Application of hydrogel nanoparticles for the capture, concentration, and preservation of low-abundance biomarkers, Methods Mol. Biol. 1606 (2017) 103–113.

R. Hirlekar, M. Yamagar, H. Garse, M. Vij, V. Kadam, Carbon nanotubes and its applications: a review, Asian J. Pharm. Clin. Res. 9 (4) (2009) 17–27.

X. Li, S. Xu, C. Pan, H. Zhou, X. Jiang, Y. Zhang, M. Ye, H. Zou, Enrichment of peptides from plasma for peptidome analysis using multiwalled carbon nanotubes, J. Sep. Sci. 30 (6) (2007) 930–943.

Y. Hu, A. Bouamrani, E. Tasciotti, L. Li, X. Liu, M. Ferrari, Tailoring of the nanotexture of mesoporous silica films and their functionalized derivatives for selectively harvesting low molecular weight protein, ACS Nano 4 (1) (2010) 439.

J. Fan, S. Niu, A. Dong, J. Shi, H.J. Wu, D.H. Fine, Y. Tian, C. Zhou, X. Liu, T. Sun, G.J. Anderson, M. Ferrari, G. Nie, Y. Hu, Y. Zhao, Nanopore film based enrichment and quantification of low abundance hepcidin from human bodily fluids, Nanomedicine 10 (5) (2014) 879–888.

Y. Tang, Y. Yao, X. Yang, T. Zhu, Y. Huang, H. Chen, Y. Wang, H. Mi, Well-defined nanostructured surface-imprinted polymers for the highly selective enrichment of low-abundance protein in mammalian cell extract, New J. Chem. 40 (2016) 10545–10553.

L.A. Liotta, E.F. Petricoin, Serum peptidome for cancer detection: spinning biologic trash into diagnostic gold, J. Clin. Invest. 116 (1) (2006) 26–30.

V. Lange, P. Picotti, B. Domon, R. Aebersold, Selected reaction monitoring for quantitative proteomics: a tutorial, Mol. Syst. Biol. 4 (2008) 222.

D.C. Liebler, L.J. Zimmerman, Targeted quantitation of proteins by mass spectrometry, Biochemistry 52 (22) (2013) 3797–3806.

E. Kuhn, J. Wu, J. Karl, H. Liao, W. Zolg, B. Guild, Quantification of C-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and 13C-labeled peptide standards, Proteomics 4 (2004) 1175–1186.
[61] D.R. Barnidge, M.K. Goodmanson, G.G. Klee, D.C. Muddiman, Absolute quantification of the model biomarker prostate-specific antigen in serum by LC-MS/MS using protein cleavage and isotope dilution mass spectrometry, J. Proteome Res. 3 (2004) 644–652.

[62] S. Gallien, E. Duriez, C. Crone, M. Kellmann, T. Moehring, B. Domon, Targeted proteomic quantification on quadrupole-Orbitrap mass spectrometer, Mol. Cell. Proteomics 11 (2012) 1709–1723.

[63] Y.J. Kim, S. Gallien, V. El-Khoury, P. Goswami, K. Sertamo, M. Schlesser, G. Berchem, B. Domon, Quantification of SAA1 and SAA2 in lung cancer plasma using the isotype-specific PRM assays, Proteomics 15 (2015) 3116–3125.

[64] G.E. Ronsein, N. Pamir, P.D. von Haller, D.S. Kim, M.N. Oda, G.P. Jarvik, T. Vaisar, J.W. Heinecke, Parallel reaction monitoring (PRM) and selected reaction monitoring (SRM) exhibit comparable linearity, dynamic range and precision for targeted quantitative HDL proteomics, J. Proteomics 113 (2015) 388–399.