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Review

Affinity prefractionation for MS-based plasma proteomics

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The plasma proteome has proven to be one of the most challenging proteomes to profile using currently available proteomics technologies. A plethora of methodologies have been used to profile human plasma in order to discover potential biomarkers for disease and for therapy optimization. Affinity-based prefractionation coupled to MS has been shown to be one of the most successful ways to dig deeper into the plasma proteome. Depletion of high abundant plasma proteins is becoming an initial method of choice in any plasma profiling project. However, several other affinity-based enrichment methods have been published in recent years. Here we review both protein and peptide affinity prefractionation methods coupled with MS-based proteomics. Analysis of the proportion of cellular and extracellular annotated proteins of publicly available MS plasma proteomics data is performed to estimate the analytical depth of various prefractionation methods.

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1 Introduction

When working in the field of clinical proteomics plasma is the sample material that will inevitably cross your path. Plasma is one of the few biological materials that can be sampled at any time, from any patient, with the hope of finding the answer to any clinical proteomics question (what disease does this patient have, will he or she respond to treatment, what is the prognosis, *etc*.).

The main difference between plasma and other samples used in proteomics is that the majority of studies focusing on plasma are not interested in plasma as a body compartment *per se*, but in leakage products potentially present in plasma. This introduces an inherent challenge to the analysis as the proteins of interest will, typically, be present only temporarily and in small amounts. In addition, the high dynamic range of protein concentrations present in plasma, spanning over at least 10 orders of magnitude [1], makes plasma challenging from an analytical point of view, as no analytical method can span a dynamic range this broad.

An additional issue that has to be considered in MS-based proteomics is the added complexity induced by digestion. This challenge is not unique to plasma but is potentially more problematic in plasma as it accentuates the already high dynamic range of concentrations, due to large high abundant proteins such as IgG generating a large number of peptides.

The most common approach to facilitate the proteomic analysis of plasma is to reduce the high range of concentrations by fractionation. “Divide and conquer” has been the motto in plasma proteomics and a number of successful studies have been able to show the positive effect of extensive fractionation on number of identified proteins and analytical depth [2–4]. During the few last years there has been a shift toward performing discovery proteomics in tissue or cell lines, and then target validation in plasma using, for example, multiple reaction monitoring (MRM). The inherent analytical challenges of plasma are unfortunately still a main issue and therefore fractionation has been shown to be beneficial even prior to targeted MS analysis [5–7].

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Abbreviations: IMAC, immobilized metal affinity chromatography; **M-LAC,** multilectin affinity chromatography; **MRM,** multiple reaction monitoring

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The fact that the majority of plasma proteomics studies are biomarker orientated discovery studies which do not explore the biological function of plasma means that analysis of subproteomes within plasma may be sufficient. The aim is then not to cover the entire plasma proteome, but to enrich for potentially interesting tissue leakage proteins.

Affinity prefractionation in plasma proteomics holds great potential, not only by reducing the complexity of samples and enriching for biomarkers, but also by increasing throughput. Affinity prefractionation often generates fewer fractions compared with other prefractionation techniques. Throughput is an important aspect in particular when dealing with clinical material with large biological variation and hence a need for large sample series.

As described above there are three main analytical challenges in MS-based plasma proteomics; proteins of interest are present in low concentration, there is a high dynamic range of concentrations and additional complexity is induced by digestion. Affinity prefractionation can basically target any of these analytical challenges and depending on the aim and analytical method of the study prefractionation methods have different pros and cons.

In this section, we review affinity prefractionation methods used both for targeted and nontargeted MS-based plasma proteomics. The distribution of cellular localization (annotated among identified proteins from different affinity methods) is evaluated to get an insight to the analytical depth in relation to number of proteins identified.

2 Protein-based prefractionation

Protein-based prefractionation mainly facilitates the analysis of plasma either by reducing the dynamic range of concentration and/or by enriching for potentially interesting sub groups of proteins. Performing prefractionation on the protein level enables additional downstream fractionation both on the protein and on the peptide level.

2.1 High abundant protein depletion

High abundant protein depletion is by far the most common prefractionation technique in plasma proteomics and there are numerous depletion systems available on the market, most of them based on antibody affinity. At present depletion systems removing one [8, 9], two [10–12], three [13], six [10, 11, 14–16], seven [17], 12 [16, 18, 19], 14 [20], 20 [21], and 58 [22] high abundant proteins from plasma have been described in the literature.

There are several reasons why depletion systems are so widely used in plasma proteomics. As the ten most abundant proteins in plasma represent approximately 90% of the total protein mass in human plasma and the top 22 represents approximately 99% [23], removal of one or several of these proteins shifts the analysis toward other, potentially more interesting, less abundant proteins. High abundant protein depletion thereby specifically targets the high dynamic range of concentrations found in plasma. The low number of fractions generated (flow through and eluate), where usually only one fraction (flow through) is used in the downstream analysis, makes combination with a wide variety of downstream fractionation methods feasible. The possibility to automate the depletion by utilizing an FPLC system further increases the reproducibility and through put.

Several studies have been published comparing different depletion systems using downstream MS analysis [10, 24, 25]. However, extensive comparison using several depletion methods with same sample and downstream analysis is, to our knowledge, lacking.

We have performed a general meta analysis of affinity prefractionation methods including several depletion studies, which will be discussed later in this review.

Depletion strategies have been used upstream to a broad range of MS-based techniques, including both targeted [26–28] and nontargeted approaches [3, 29]. The main critique toward depletion systems has been that more proteins than intended are removed from the sample, which has been shown in several publications [8, 14–16, 18]. The capacity of antibody columns could also be a potential limitation when removing a high number of proteins as a large starting volume of plasma could be needed to detect low abundant proteins and peptides. Further, using many antibodies will most likely increase the costs. Combinations of high abundant protein depletion with other affinity prefractionation methods will be described below.

2.2 ProteoMiner beads technology

ProteoMiner is a novel technology that utilizes a combinatorial library of hexa-peptides coupled to beads, each bead being present in equal numbers [30]. Through interactions with the peptide ligands, proteins are retained by the matrix, but as there is a limited number of each bead only a limited amount of each protein will be retained. The high dynamic range of concentrations is thereby reduced, without removing any specific protein. The potential drawback with this approach is that protein concentration differences between samples could be altered, thereby complicating downstream quantification. The positive effect of ProteoMiner beads on number of identified proteins ($n = 1559$) [31] has been shown, however no quantitative plasma proteomics studies using the ProteoMiner technology in combination with MS have yet been published.

2.3 Carbonylated protein enrichment

Instead of targeting the high dynamic range of concentrations affinity, purification of carbonylated proteins is an attempt to enrich for potentially interesting proteins. ROS are known to play a role in a vast number of diseases including cancer, heart disease, and Alzheimer disease [32, 33, 34]. As a result of exposure to ROS irreversible oxidation of

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amino acid side chains can occur, introducing aldehyde or keton groups [35]. By biotinylation of oxidized proteins with biotin hydrazide and affinity selection using monomeric avidin affinity chromatography, carbonylated protein have been enriched from murine plasma and subsequently analyzed with LC/MS/MS [36]. Although only 146 proteins were identified, the low proportion of resident plasma proteins identified (2%) and the high proportion of tissue derived proteins (92%) indicates the potential of this method in biomarker studies. As this method focus on a specific modification the versatility is, of course, limited and further studies are needed to show that the technique could be applied to human plasma.

3 Peptide-based prefractionation

Peptide-based fractionation rarely directly targets the high dynamic range of concentrations, but more often reduces the complexity induced by digestion or targets subproteomes within the plasma proteome.

3.1 Cystein containing peptide enrichment

Cystein enrichment takes advantage of the reactive cysteinyl residue and a thiol-affinity resin is used to enrich for cysteinyl containing peptides [37]. Whiteaker *et al.* [11] found no positive effect on the number of identified proteins from plasma when using cysteinyl enrichment (135 identified proteins compared with 147 in crude plasma) and suggested that this could be due to the fact that approximately 95% of cysteinyl peptides in human plasma are contributed by two highly abundant proteins: albumin and transferrin. This is supported by the high number of proteins ($n = 1977$) identified using cystein enrichment in combination with high abundant protein depletion [29], where 12 high abundant proteins (among them albumin and transferrin) where depleted prior to digestion and cystein enrichment. This also illustrates the benefit of combining complementary methods for reducing the different factors contributing to the complexity of the plasma proteome. Further the "cystein depleted" fraction yielded almost as many identified proteins ($n = 1914$) and proteins present in subng/mL concentration was identified from both cystein enriched and cystein depleted fractions.

4 Combined protein/peptide prefractionation

4.1 Glyco-affinity enrichment

Affinity purification of glycosylated proteins is a large field in plasma affinity fractionation. Approximately 50% of all proteins are thought to be glycosylated [38] and glycoproteins secreted into the bloodstream are a major part of the plasma proteome [39]. The oligosaccharide units of glycoproteins are linked either to Aspargine side chains by *N*-glycosidic bonds (*N*-glycoproteins) or to serin and threonin side chains by *O*-glycosidic bonds (*O*-glycoproteins). Alteration of protein glycosylation is known to play a role in several pathological states including cancer [40] and immune diseases [41], making it an interesting subproteome to study within the plasma proteome. This review will only discuss glyco-affinity as a prefractionation method for proteomics analysis and will not include analysis of glycans. For a recent review on glycomics, see Gesslbauer *et al.* [42].

A vast number of strategies have been employed to enrich for glycoproteins from plasma, with the majority of them being based on *lectin affinity* or *hydrazide chemistry*, recently some *other carbohydrate affinity* approaches have also been described.

As the endpoint of the enrichment often is a set of glycosylated peptides these strategies both reduce the complexity of plasma by looking at a potentially interesting subproteome within the plasma proteome (glycosylated proteins) and by reducing the complexity induced by digestion by only looking at a fraction of the tryptic peptides generated (*i.e.*, the glycosylated peptides from the glycoproteins).

4.1.1 Lectin affinity enrichment

Lectins are a group of proteins with affinity for carbohydrates. Lectin affinity ranges from specific sugars to groups of carbohydrate chains, thereby making it possible to enrich for either one specific type or several types of glycoproteins or peptides. Both single lectin [43] and serial lectin [38] affinity purification methods have been described as well as multilectin affinity chromatography (M-LAC) [44]. M-LAC has successfully been used in combination with high abundant protein depletion [45] where proteins in the low ng/L concentration range (HER-2, 5–20 ng/mL) were identified [46]. Recently a "reversed" M-LAC approach have been described where high abundant protein depletion and M-LAC has been used in combination to deplete both high and "mid-abundant" proteins from plasma, taking advantage of the fact that many mid-abundant plasma proteins are glycosylated showing that 56% of the identified proteins emerged in the nonglycosylated fraction [47].

4.1.2 Hydrazide chemistry enrichment

In hydrazide-based glycoprotein enrichment, *N*-glycosylated proteins are selectively enriched. Initially, all glycoproteins are covalently coupled to a hydrazide resin, nonglycosylated proteins are then washed off and nonglycosylated peptides are subsequently removed by tryptic digestion. *N*-Glycosylated peptides are then selectively eluated through cleavage with PNGase, an enzyme that specifically cleaves *N*-glycosylation sites [48].

Proteins present in sub-ng/mL in plasma have been identified after hydrazide-based glycoprotein enrichment

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using both targeted [49] and nontargeted [50] MS approaches. Combining hydrazide-based glycoprotein enrichment with upstream high abundant protein depletion and downstream two dimensional chromatographic separation has been shown to improve the analytical depth, identifying several proteins in the pg/mL to ng/mL concentration range [51].

The potential of analyzing the nonglycosylated peptides of the hydrazide enriched glycoproteins was highlighted in a recent article showing more than two times as many identified proteins (nonglyco $n = 1486$, glyco $n = 662$) from the nonglycosylated peptides as opposed to the glycosylated peptides [29].

4.1.3 Other carbohydrate affinity enrichments

To selectively enrich for sialylated peptidies, Larsen *et al.* [52] described a novel approach from plasma using high abundant protein depletion in combination with titanium dioxide chromatography showing high selectivity for sialylated peptides. In another publication, Zhang *et al.* [53] showed selective enrichment of nonenzymatically glycated D-glucose peptides by combination of high abundant protein depletion and boronate affinity chromatography. Using this approach they could show a slightly higher degree of nonenzymatically glycosylated proteins among patients with diabetes 2 patients and patients with impaired glucose tolerance as opposed to healthy controls.

As many of the high abundant proteins are glycosylated one risk with glycol-affinity enrichment is that the high dynamic range of concentrations is not significantly reduced as many plasma proteins are glycosylated. One way to circumvent this challenge is to analyze both the glycosylated and the nonglycosylated protein fractions [29].

4.2 Metal affinity-based fractionation

Despite being quite frequently used in proteomics [54], only a limited number of studies have applied immobilized metal affinity chromatography (IMAC) to plasma or serum samples. IMAC cupper has been used as a selective surface on the SELDI MS platform enriching for cupper binding proteins [55], as well as in combination with MALDI TOFMS, using magnetic beads enriching for cupper binding peptides [56]. Cupper has affinity to the imidazole ring of the histidine side chain and can therefore be used to enrich for histidine containing proteins or peptides.

Iron and gallium both have affinity for phosphopeptides [54] and have been used together with MALDI TOFMS to enrich for both peptides and proteins from plasma and serum [57, 58].

As IMAC primarily has been used prior to MALDI TOFMS or by SELDI TOFMS, where only the number of peaks and not the identities of the proteins are evaluated, comparison with other methods is difficult.

5 Prefractionation specifically for targeted MS

5.1 Protein immunoaffinity coupled to MRM analysis

Antibody-based validation assays such as ELISA assay have long been the golden standard in protein chemistry. However, for many potential biomarkers there are no ELISA kits available and development of ELISA kits is time consuming and relatively expensive. Unspecific binding is always a risk and multiplexing comprises an additional challenge. Nicol *et al.* [5] recently showed that they could couple four different antibodies to hydrazide beads, enrich the targeted proteins from plasma, digest, and subsequently measure their concentrations using MRM. Using this approach they obtained linearity over two to three orders of magnitude and showed CVs $\leq$11%, illustrating how targeted prefractionation in combination with targeted MS can be used to quantify proteins of interest present in plasma at low ng/mL concentrations. This targeted MS approach reduces the potential problem with nonspecific binding to the antibody, since MRM-methods offer high specificity due to selection both on the peptide and on the fragment level. However, using the above described approach one is still dependent on the availability of antibodies to the proteins of interest and one has to be certain that the quantitative measurement is not compromised by the immunocapture.

5.2 Peptide immunoaffinity coupled to MRM analysis

Antipeptide antibodies coupled to magnetic beads have been used in combination with high abundant protein depletion to facilitate quantitative analysis of low abundant proteins in plasma using MRM. Absolute quantifications of peptides present in ng/mL have also been achieved in a similar way using the SISCAPA methodology; stable isotope standards and capture by antipeptide antibodies [6, 59, 60]. As antipeptide antibodies are rarely commercially available, this method is quite labor-intense and greatly limits the throughput if used as a part of a validation workflow. However, using this approach linearity over three orders of magnitude, accuracy of 20% and CVs below 10% has been achieved, illustrating its potential in targeted proteomics [59].

A possible option to the time consuming development of specific antibodies, is to perform prefractionation based on other chemical characteristics of peptides using, for example, affinity to specific amino acids (*e.g.*, cysteine, histidine). Another attractive option would be to employ narrow range peptide IEF [61–63] as prefractionation prior MRM and design the enrichment based on the p*I* of the peptides.

6 Discussion and concluding remarks

Plasma proteomics is in general very tightly linked with the discovery and establishment of biomarkers. The clinical need for plasma markers has never been bigger, not only for diag-

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nosis, but also for selection of the most effective therapies as well as exclusion of ineffective or toxic treatments. Therapy options increase continuously, however markers for personalized therapy are lacking behind. In last 10 years, plasma proteomics has been loaded with expectations to generate biomarkers to solve many of these clinical bottle necks. It can clearly be seen that methods with novel affinity prefractionation techniques have to proceed hand-in-hand with development of mass spectrometers to achieve better sensitivity and a broad dynamic range.

Affinity prefractionation is used to "zoom in" on potentially interesting targets in plasma and reduce the sample complexity. Depending on the fractionation method affinity prefractionation usually targets one or several of the following analytical challenges in plasma; high dynamic range of concentrations, biomarkers present in low concentrations, and increased complexity induced by digestion.

Comparing different fractionation methods is a great challenge in proteomics. First, downstream analytical MS setup will greatly affect the outcome. Second, as the aim of the experiment varies a lot the endpoint is hard to evaluate. Number of identified proteins, tissue origin of the proteins, types of proteins identified, or specificity of enrichment, are all aspects that could be important to evaluate. In targeted MS, this is of course easier as the target proteins are already specified. The challenge then lies more in reducing the sample complexity without loosing any quantitative information.

As many of the plasma biomarkers used today (such as prostate specific antigen, CA-125, and troponin-t) are tissue leakage proteins the big question is how to reach to that level of analytical depth in discovery projects. As listed above plasma proteomics researchers have tackled the problem both with various affinity depletion and enrichment, as well as brute force using extensive LC/MS/MS analysis.

To evaluate the analytical depth of different affinity prefractionation methods, a plot of distribution of cellular localization (as annotated by Ingenuity Pathway Analysis, application version 5.5.1, content version 1002, Ingenuity Systems) was made here both for datasets from different fractionation approaches as well as the proteins identified in the human plasma proteome project (HPPP) (Figs. 1 and 2). This comparison should not be seen as a head-to-head comparison as it is complicated by the wide variety of protein identifiers used in proteomics studies, the various criteria used for identification, the different MS techniques used, and a reliance on synonyms used for many gene names. However, it does provide a general trend on the relation between the number of identified proteins and types of proteins identified from the different affinity fractionation approaches.

One general observation when looking at the plots is that the fraction of cellular proteins seems to be inversely correlated with fraction of extracellular proteins (*i.e.*, the larger the portion of extracellular proteins, the smaller portion of cellular proteins). This is true particularly for the intracellular

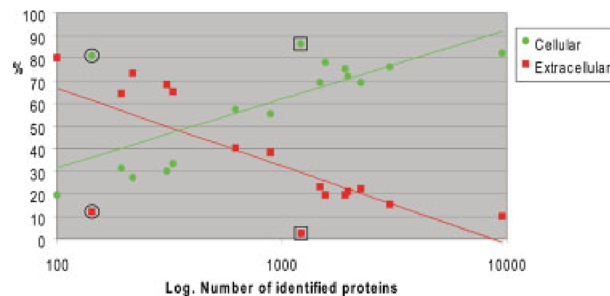

Figure 1. Correlation between cellular and extracellular proteins and number of identified proteins, based on the 16 data sets described in Fig. 2. Each data set has been divided into two subclasses: cellular proteins (green) and extracellular proteins (red). Percent of total number of proteins in each data set belonging to the cellular or extracellular subclass (*Y*) is plotted against total number of proteins in the data set (*X*). Circled dots shows protein distribution from carbonyl enrichment [36] and squared dots shows proteins distribution from depletion of 58 proteins [22].

proteins. Further, the proportion of cellular proteins detected also seems to be correlated with the total number of proteins identified in the study, where a high number of identified proteins is reflected by a large proportion of cellular proteins. One method that stands out in this regard is the affinity enrichment of carbonylated proteins (indicated by circles in Fig. 1 and arrow 1 in Fig. 2), identifying over 40% cytoplasmic proteins and as few as 10% extracellular proteins, despite the rather low number of identified proteins ($n = 143$). This suggests a good zoom-in effect, and shows the potential of this method when looking for targets of oxidative stress. Extensive depletion (58 proteins) of high and mid-abundant proteins, as performed in ref. [22] clearly affects the fraction of extracellular proteins, well over the effect accounted for by the removal of the 58 proteins. The result of the depletion is an increase in both cytoplasmic and nucleic proteins as compared with the general trend (squared in Fig. 1 and arrows 2 and 3 in Fig. 2).

Another feature that stands out is the large fraction of plasma membrane proteins detected by hydrazide chemical enrichment. This is in line with the high proportion of *N*-glycosylated proteins on the plasma membrane, showing good selectivity of this method (Fig. 2, arrow 4).

In plasma prefractionation, surprisingly little is so far done using affinity enrichments toward important intracellular regulation mechanisms such as ubiquitinations, sumosylations, or protein acetylations. However, as shown in Fig. 1, the fraction of cellular proteins also increases markedly with increased number of identified proteins, so the importance of a broad coverage of the plasma proteome should not be underestimated.

There is yet no golden standard method in plasma proteomics and so far plasma proteomics projects have not provided clinically used biomarkers. However, it should be pointed out that all methods evaluated in this comparison, except for the carbonylated protein enrichment and the

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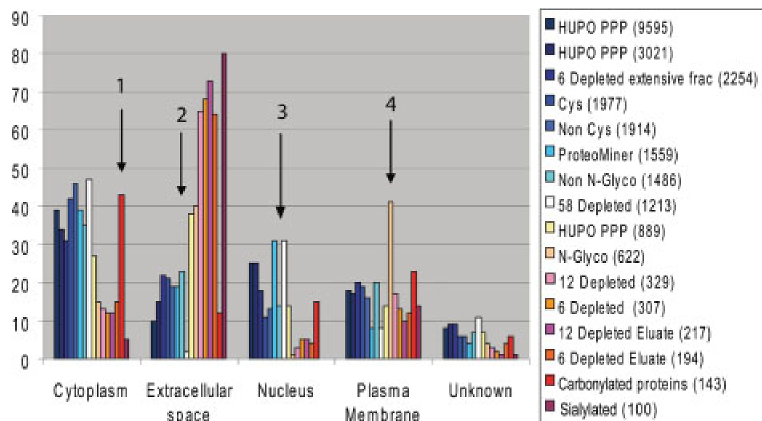

Figure 2. Distribution of protein localization as denoted in Ingenuity Pathway Analysis. Percentage of total number of identified proteins on *Y*-axis. Total number of proteins identified within brackets. Derived from data from (A) HUPO PPP (9595) [64], (B) HUPO PPP (3021) www.peptideatlas.org/hupo/hppp/, (C) 6 depleted extensive frac (2254) [3], (D) Cys (1977) [29], (E) non-Cys (1914) [29], (F) ProteoMiner (1559) [31], (G) non-*N*-glyco (1486) [29], (H) 58 depleted (1213) [22], (I) HUPO PPP (889) www.peptideatlas.org/hupo/hppp/, (J) *N*-glyco (662) [29], (K) 12 depleted (329) [16], (L) 6 depleted (307) [16], (M) 12 depleted eluate (217) [16], (N) 6 depleted eluate (194) [16], (O) oxidized proteins (143) [36], (P) *O*-glyco (100) [52]. Arrows indicates important outliers in the plot: (1) large fraction of cytoplasmic proteins using carbonylated protein enrichment, (2) low fraction of extracellular proteins after 58 proteins depleted, (3) high proportion of nuclear proteins after 58 proteins depleted, (4) large fraction of plasma membrane proteins after *N*-glyco enrichment.

ProteoMiner beads, have used upstream high abundant depletion, thereby showing the versatility of this technique. Although rarely used as the only separation step in plasma proteomics, depletion has become as close to a golden standard as there is in plasma affinity fractionation today. However, the great break through is still ahead of us. Very few projects can reach low abundant proteins in plasma and at same time profile a large number of samples. The future of the field is likely to be combination of powerful prefractionation methods in combination with improved MS, to the extent that proteins present in the ng/mL to pg/mL range can be profiled quantitatively from 40 to 100 plasma samples in discovery phase, followed by rapid shift to directed analysis of selected markers for validation in several hundred samples.

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