Focusing Review

Analytical Platforms for Mass Spectrometry-Based Proteomics

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Abstract
Mass spectrometry-based proteomics platforms have been widely used as ‘proteome sequencers’ to characterize the proteomes of a wide range of organisms. The work-flow generally involves multiple steps of sample preparation, peptide purification/concentration/pre-fractionation, and nanoLC/MS/MS measurement. This review focuses on our contributions to the technical development of current proteomics platforms, and includes a consideration of the limitations of these systems, together with the prospects for developing superior new-generation proteome sequencers.

Keywords: Proteomics; Mass spectrometry; NanoLC; Sample pretreatment

1. Introduction
Since the first introduction of the word “proteome” in 1994 by Mark Wilkins as an alternative to the term “the protein complement of the genome”, the field of proteomics has expanded enormously (Fig. 1), especially following the completion of the human genome project in 2001. The development of nano-scale liquid chromatography coupled with tandem mass spectrometry (nanoLC/MS/MS) has played a crucial role in this development. In other words, technological advances have underpinned proteomics in much the same way that next-generation sequencers (NGS) have advanced genomics. The technical challenges in proteomics arise mainly from the fact that proteins are composed of 20 amino acids as well as their post-translationally modified forms, whereas there are only 4 nucleic acid bases in DNA/RNA. In addition, protein amplification is difficult, whereas DNA can be easily amplified. Furthermore, both the dynamic range of concentration and the chemical complexity arising from the variety of proteoforms in proteome samples are very large. Therefore, in order to analyze the entire proteome, we require very high resolution in both LC and MS, in addition to efficient methods for sample pre-fractionation or enrichment of particular target proteins or peptides.

Attempts to determine the amino acid sequences of peptides using tandem MS began at least 30 years ago. For example, Hunt et al. used capillary LC coupled with fast atom bombardment ionization triple quadrupole tandem MS in the early 90's and succeeded in identifying MHC class 1 peptide [1]. Fig. 2 shows the current workflow for shotgun proteomics [2], in which proteins are firstly fragmented into peptides by a sequence-specific protease such as trypsin. Then, the digested peptides are separated by nanoLC, ionized by electrospray ionization, and analyzed by tandem MS to obtain partial amino acid sequences. These are searched against the complete sequence information in the protein amino acid sequence database to identify peptides uniquely. Simultaneously, the MS signals at either the MS1 or MS2 level are used for peptide quantitation.

This paper reviews analytical platforms using proteomic nanoLC/MS/MS and related technologies, focusing on our contributions over the past 20 years. The limitations of current systems are considered, as well as the prospects for developing new-generation proteome sequencers.

2. Protein extraction and digestion using phase-transfer surfactants
Proteins are localized within cellular components such as the cytoplasm, cell membrane, nucleus and mitochondria, or secreted into biofluids such as blood, urine, and cerebrospinal fluid (CSF). These proteins have a variety of physicochemical properties such as hydrophobicity and
isolectric points, and in order to comprehensively analyze them, it is necessary to extract them efficiently and reproducibly from the samples of interest without any bias. So far, various approaches have been developed to extract proteins, using organic solvents such as acetonitrile and methanol [3], surfactants such as octyl glucoside and sodium dodecyl sulfate (SDS) [4,5], and chaotropic reagents such as urea and guanidine hydrochloric acid [4,6] as protein solubilizers and cell lysis reagents in combination with ultrasonicators or homogenizers. In general, the protein solubilization ability of surfactants is greater than that of organic solvents and chaotropic agents. However, surfactants with high solubilization ability often inhibit the activity of proteases such as trypsin and Lys-C. Furthermore, it is difficult to completely remove the surfactant from the sample solution, and residual surfactant adversely affects the MS performance. In other words, solubilizers that can solubilize highly hydrophobic proteins, that do not inhibit the protease activity, and that can be removed before nanoLC/MS/MS are preferred for shotgun proteomics. We searched for a solubilizing agent that satisfies these three criteria, and selected sodium deoxycholate (SDC) out of 27 candidates [7]. Although the solubilization ability of SDC for membrane proteins is equivalent to that of SDS, we surprisingly found that 1% SDC promotes the activity of trypsin and Lys-C. Furthermore, since the hydrophobicity of

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**Fig. 1.** Number of published papers found using the search terms ‘proteome’ and ‘proteomics’ in PubMed.

**Fig. 2.** Current work-flow of shotgun proteomics.
SDC increases under acidic conditions, we developed a method to remove SDC from the sample solution by liquid-liquid partitioning between ethyl acetate-acidic aqueous solutions prior to LC/MS/MS. Because SDC is transferred from the aqueous phase to the organic phase, we named it a phase-transfer surfactant (PTS). In addition to SDC, we found that sodium lauroylsarcosinate (SLS) also works as a PTS, and the mixture of SDC and SLS was found to be effective for unbiased protein extraction [8]. The PTS method has been applied to a wide range of samples, including cultured cells [7], tissues [9], microorganisms [8], and the oral metaproteome [10]. Furthermore, the PTS method can extract proteins efficiently from formalin-fixed paraffin-embedded tissue sections, and indeed, also removes paraffin from sample solutions during liquid-liquid partitioning [11]. Besides the PTS method, RapiGest, a commercial acid-cleavable surfactant, has been used as a surfactant removable after digestion [12]. Although the protein-solubilizing ability of RapiGest is higher than that of SDC, the number of identified peptides was lower than that in the case of the PTS method [7]. This is probably because the trypsin activity in the presence of RapiGest is lower than that with PTS, and some hydrophobic peptides are co-precipitated with the cleavage products of RapiGest. Another widely used method for protein extraction/digestion is a filter-aided sample preparation (FASP) method using SDS and an ultrafiltration filter [13]. We compared the PTS and FASP methods using EphH4 cells and found that the PTS method is better, especially when the sample amount is small [14]. The PTS method is also applicable for ultrasensitive phosphoproteomics, as will be described later.

3. Development of StageTip miniaturized SPE columns for peptide purification and separation

In shotgun proteomics, it is necessary to fractionate, desalt, purify and concentrate approximately 10 pg to 10 μg of peptide mixtures prior to nanoLC/MS/MS analysis. In the early 2000’s, however, there was no commercial solid-phase extraction (SPE) device that could recover several fmol (pg) of peptides quantitatively and reproducibly. For example, Stewart et al. reported that the recovery of 50 fmol of albumin-digested peptides from a commercial SPE product was only 10% [15].

The Empore SPE disk from 3M Co. with 0.5 mm thickness contains chromatographic particles fixed in a Teflon mesh, and has been used as a preconcentration cartridge for capillary electrophoresis [16] and as an online trap column for LC to quantify drugs in serum samples [17]. We considered that if we could prepare large numbers (hundreds) of small disk SPEs from one disk sheet for the analysis of trace peptide samples, we could expect higher reproducibility and recovery. Thus, we started to prepare a prototype micro SPE column using the tapered structure of the pipette tip. Because of the softness of the Empore disk, we directly inserted the disk into the pipette tip and pushed it in as far as it would go with a syringe plunger. This was named a stop-and-go-extraction tip (StageTip), because peptides can be stopped or released by changing the combination of multiple disks or manipulating the elution solvents. The maximum loading capacity of a 0.9 mm diameter disk with >80% recovery for albumin-digested peptides was 25 μg for C18-StageTip and 100 μg SCX-StageTip. As regards the minimum sample amounts for C18-StageTip and SCX-StageTip, 5 fmol and 20 fmol afforded >80% recovery with a disk of 0.4 mm diameter [18,19]. Depending on the application, different sizes of disks as well as multiple disk formats can be prepared, and the largest was a disk with a diameter of 2 mm fixed on a 1 mL pipette tip. It is also possible to process 96 samples at once by combining a 96-well adapter and a centrifuge. In addition, an example of connecting C18-StageTip online to MS and applying it to peptide separation by isocratic elution has been reported [20]. This format has been modified and is now a commercial product [21].

It is useful to employ StageTip not only for desalting, but also for pre-fractionation prior to nanoLC/MS/MS. For this purpose, StageTip has the unique feature that stacking different disks can provide different functionalities [22]. When reversed-phase separation under acidic conditions is used for nanoLC/MS/MS, the pre-fractionation separation should be as orthogonal as possible. Thus, SCX and SAX are often used. Reversed-phase LC at high pH is also used because the separation efficiency is generally higher than that of ion-exchange chromatography, although the separation orthogonality is not so high. When ion exchange disks are used, the sample solution needs to be desalted beforehand, for example, using C18-SCX-C18 type. For SCX, the resin type can be also used between two C18 disks, such as polysulfoethyl A (PolyLC) [19]. After desalting the sample solution with C18 at the top, SCX fractionation is done by salt concentration, and then desalting is done at the second C18 for subsequent nanoLC/MS/MS analysis. Furthermore, we examined two-dimensional fractionation using C18-SCX-StageTip. Since fractionation by organic solvent concentration in the first-dimension C18 disk did not affect the retention on the second-dimension SCX disk, it was possible to perform fractionation by salt concentration immediately after elution into SCX. The eluted fractions were desalted and concentrated with C18-StageTip, and two-dimensional fractionation could be performed. It was also possible to use SAX instead of SCX. Since the retention of tryptic peptides to SAX is performed under alkaline conditions, the reverse-phase disk does not contain C18 silica beads but SDB (poly(styrene-divinylbenzene) copolymer) beads.
When the simplified fractionation method using StageTip was applied to E. coli proteome samples, we obtained about 1.8 times more peptides, compared with the case where no fractionation was performed [23]. Recently Adachi et al. reported that peptide elution from SCX-StageTip with strongly acidic solutions resulted in a significant improvement in the number of identified peptides, not only for unmodified peptides but also for phosphorylated peptides [24]. Although high-resolution separation cannot be expected in StageTip-based separation, this approach is very simple and no special equipment is required. In addition, StageTip is disposable, so there is no need to consider carry-over or contamination of previously analyzed samples, which is advantageous for a robust analytical platform.

4. Phosphopeptide enrichment analysis

Post-translational modifications (PTM) of proteins increase the functionality and dynamic character of the proteome. Protein phosphorylation is one of the most ubiquitous PTMs, and plays important roles in modulating intracellular signal transduction. Therefore, information about the modified sites, as well as the modified proteins, is important for understanding cellular functions. In order to comprehensively measure protein phosphorylation by means of shotgun proteomics, it is essential to enrich phosphopeptides after protein digestion, because the abundance of phosphorylated peptides is generally lower than that of unmodified peptides. It is known that the phosphate group has high chemo-affinity for metal ions or metal oxides. Based on this property, immobilized metal ion affinity chromatography (IMAC) and metal oxide chromatography (MOC) have been used to enrich phosphorylated molecules. However, these approaches cannot be applied to phosphoproteomics since metal ions and metal oxides have also affinity for acidic unmodified peptides. Since the amounts of acidic unmodified peptides are much larger than those of phosphopeptides in cells, much higher selectivity for phosphopeptides is required to enrich phosphopeptides for phosphoproteomics. We have developed several approaches to improve selectivity using StageTip-based IMAC or MOC systems.

4.1. IMAC-C18-StageTip

StageTips for phosphopeptide enrichment were prepared with IMAC resin packed on top of a C18 disk, where the C18 was used to remove phosphate ions employed for phosphopeptide elution from the IMAC resin. In order to improve the enrichment selectivity, several approaches have been developed, such as methyl esterification of carboxyl groups [25] or combined prefractionation [26]. We simply optimized the pH conditions during the enrichment steps [27]. In combination with database searching using a customized de-phosphorylated protein database, we successfully identified hundreds of phosphorylated sites using IMAC StageTips in a 96-well format [28].

4.2. MOC-C2-StageTip

In the case of metal oxides, the selectivity for phosphopeptides can be improved by the addition of aromatic carboxylic acid [29], octanesulfonic acid [30], acidic amino acid [31], or other additives. We carefully examined the effect of additives using MOC-C2-StageTip in which a C2 or C8 disk was used as a frit and metal oxide resins such as titania or zirconia were packed on top of the frit. As a result, we found that aliphatic hydroxy acids such as lactic acid dramatically improved the phosphopeptide selectivity, and could be easily removed prior to LC/MS/MS [32]. This is because the affinity between hydroxy acid and MOC is greater than that between carboxylate and MOC, and weaker than that between phosphate group and MOC, so the acidic peptides cannot be trapped in the presence of hydroxy acid. We named this approach HAMMOC (Hydroxy Acid Modified Metal Oxide Chromatography). HAMMOC can identify thousands of phosphorylated peptides directly from human cell lysates without pre-fractionation for the first time. We applied HAMMOC to Arabidopsis phosphoproteome analysis and discovered that tyrosine phosphorylation exists in plants to much the same extent as in mammals [33].

Titania has different crystal forms, such as anatase and rutile. The anatase form with a controlled amount of coordinated water gave the best performance in the presence of lactic acid [34], while the rutile form calcined at 800 °C was found to have sufficient phosphorylated peptide selectivity even in the absence of hydroxy acid [35]. Moreover, when we used piperidine or pyrrolidine at different concentrations as the eluent, different elution selectivity was observed [36]. We recently further improved the coverage of the phosphoproteome by selective elution depending on the number of phosphoryl groups of peptides from a single titania-HAMMOC using hydrophilic interaction chromatography (HILIC). We successfully identified 11,300 nonredundant phosphopeptides from triplicate analyses of 100 μg of HeLa cell lysates using this approach [37]. For high-sensitivity phosphoproteome analysis, the PTS protocol is also effective. We applied the PTS-HAMMOC method to 1 μg of HeLa cells [38]. The phosphopeptides were directly injected onto a miniaturized LC column using a nitrogen-pressure-driven cell. As a result, we identified 1011 unique phosphorylated sites based on 995 unique phosphopeptides from a single analysis of 104 HeLa cells. This was the most sensitive phosphoproteomics system at that time. A similar method was also used in a high-throughput phosphoproteomics platform named EasyPhos [39]. Since the PTS-HAMMOC
method provides high recovery and high reproducibility, it was also employed for targeted analysis of phosphopeptides using LC/MS/MS with selective reaction monitoring mode [40].

5. Analytical columns for nanoLC/MS/MS systems

Typical nanoLC columns are prepared using C18 silica particles with sub 2-10 μm diameter packed into fused silica capillaries with 12-100 μm inner diameter, where sintered silica particles or silicate-polymerized ceramics have been prepared as frits [41-45]. On the other hand, in ESI-MS, an electrospray needle with a tapered end can be used as an analytical column to pack the particles without a frit. This approach is attractive for LC/MS because the post-column dead volume is minimized. In the 2000’s, it was believed that the opening size of the column must be smaller than or equal to the average diameter of the packing materials to retain them [46-48]. However, the column is easily blocked during the packing process [49], because the particle size is almost equal to the size of the outlet, and a single particle can completely block the outlet. To overcome this problem, we developed a “stone-arch” column, where the opening size is two- to five-fold larger than the average particle size, and particles at the end of the column arch over the opening; these self-assembled particles function as a frit [50]. This ‘stone-bridge’ column has been widely used in many laboratories, either in the form of self-made or commercial products. Monolithic columns are another type of fritless column. Both silica-based and organic polymer-based materials have been used [51,52].

In shotgun proteomics with a data-dependent acquisition mode, MS data are acquired by means of an MS survey scan (MS1 scan) followed by MS/MS scans (MS2 scan) to measure the m/z values of the peptide precursor ion in MS1 and its fragment ions in MS2. Therefore, any improvements in MS performance, such as detection sensitivity, scan speed, mass accuracy, mass resolution and fragmentation efficiency, lead directly to an increase in the number of identified peptides/proteins. Extensive efforts have been made to improve the MS instrumentation used in proteomics over the past 20 years. As reviewed by Shishkova et al. [53], MS with a higher scan speed can identify more peptides, and the number of identified peptides increases linearly as LC peak capacity in an 80-min gradient increases, although the number becomes saturated when peak capacity is more than 600 and the scan speed is less than 22.5 Hz. This means that LC peak capacity or peak width should be optimized by considering both MS scan speed and sample complexity. If the sample complexity is reduced, the maximum number of identified peptides can be achieved through shorter gradient analysis with a smaller peak width matching the MS scan speed. Based on this strategy, Bekker-Jensen et al. performed high-pH semi-orthogonal reversed-phase separation to generate 46 fractions, followed by 33-min gradient LC/MS/MS with 20 Hz MS2, using an analytical column packed with 1.9 μm C18 particles [54]. More than 580,000 peptides/12,000 proteins were successfully identified.

On the other hand, since LC peak capacity increases gradually as the gradient time increases, another approach to increase the proteome coverage is to use high-resolution LC with an expanded separation window without prefractionation. This approach is called ‘one-shot proteomics’ or ‘single-shot proteomics’. Generally, the use of smaller particles and longer columns increases the separation efficiency. Compared to a prefractionation-based approach, the one-shot approach is expected to have higher recovery/sensitivity because it does not require any additional step prior to LC/MS/MS. However, the one-shot approach with small-particle-packed long columns has the drawback that both smaller particles and longer columns lead to higher column pressure, which must be kept below the pressure limit of the LC system employed. Considering that standard 15 cm columns packed with 3 μm silica particles (75-100 μm inner diameter) generate 12-15 MPa at 1 mm/s linear velocity, the maximum length that can be used in general HPLC systems is 35-50 cm at room temperature. In the field of proteomics, 40-200 cm long columns packed with 1-3 μm C18 particles [55-58] have often been reported. We have used much longer (200 to 1100 cm) C18 monolithic silica columns [59-62], since the column pressure can be manipulated to control the monolith structure without sacrificing column performance. This ‘one-shot’ LC/MS/MS strategy without prefractionation is effective to reduce the number of peptides per MS scan and to increase the number of MS/MS events. Ionization suppression is also reduced due to the reduction of peptide co-elution. As a result, as many as 7,241 proteins were identified in one day (triplicate analyses with an 8-hour gradient time), and 9,510 proteins were identified from 5 types of iPSC cells and 3 types of fibroblasts in 8 days [61].

Previously, we characterized a “dark zone” of analytical difficulty in human proteome analysis by plotting [the number of identified proteins] versus [the number of identified proteins per hour] for 21 human proteomics datasets published in 2009—2014 to identify more than 5000 proteins [2]. Fig. 3 shows the dark zone plot in 2009-2014 together with the data of Bekker-Jensen et al. reported in 2017 [54]. As the overall performance of MS instruments including scan speed was better in 2017, the dark zone is smaller, as expected. But, very interestingly, the one-shot approach with a longer gradient did not benefit from the new MS instrumentation, because in long gradient analysis, the scan speed is not the limiting factor. For the one-shot approach with the longer gradient, a more
Fig. 3. Identification efficiency of proteins. Meta-analysis of 22 publications from 2009-2014 that identified >5,000 human proteins shown by black circles; the inaccessible “dark zone” is shown in grey. Data reported by Bekker-Jensen et al. in 2017 are shown by a red triangle.

Fig. 4. The jPOST environment consisting three functions such as data repository, data re-analysis and database.
sensitive instrument would be beneficial to overcome the peak dilution.

6. Prospects for high-throughput, high-sensitivity and high-coverage analytical platforms for proteomics

As described above, current analytics platforms are still not mature enough to meet the demands of the scientific community, despite continuing advances in MS instrumentation and related technologies. This review has not covered data analysis methodology, but in the future, more advanced data analysis techniques are likely to contribute greatly to achieving high-throughput, high-sensitivity, high-coverage proteomic analysis. To promote this, it will be important to share raw proteomics data within the scientific community through public data repositories such as jPOST (Fig. 4) [63, 64], one of the partner repositories of ProteomeXchange consortium as shown in Fig. 5.

Fig. 5. The ProteomeXchange consortium consisting of 6 public repositories including jPOST.

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