Recent advances in proteolysis and peptide/protein separation by chromatographic strategies

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This review gives a broad glance on the progress of recent advances on proteolysis and peptide/protein separation by chromatographic strategies in the past ten years, covering the main research in these areas especially in China. The reviewed research focused on enzymatic micro-reactors and peptide separation in bottom-up approaches, and protein and peptide separation in top-down approaches. The new enzymatic micro-reactor is able to accelerate proteolytic reaction rate from conventionally a couple of hours to a few seconds, and the multiple dimensional chromatographic-separation with various models or arrays could sufficiently separate the proteomic mixture. These advances have significantly promoted the research of protein/peptide separation and identification in proteomics.

proteolysis, enzymatic digestion, chromatographic separation, peptide enrichment, mass spectrometry

1 Introduction

Proteomics has provided an analytical tool for elucidating cellular and biological systems at the molecular level. Compared to genome analysis of a certain species, the proteomic analysis is challenging because of its relatively large population, wide dynamic range, varieties of complexes, and continuous change with time and space. Two approaches have been exploited for proteome analysis. One is the top-down strategy, which involves the separation of proteins by 2D gel electrophoresis, followed by the digestion of specific fractions using a proteolytic enzyme and the identification of proteins by tandem mass spectrometry (MS/MS). The other is the bottom-up strategy, which is performed by the initial digestion of all proteins in a sample, followed by the separation of peptides and the identification of proteins by MS/MS.

No matter by which means, it could be seen that the digestion of proteins is indispensable before they could be identified. The typical protocol for protein digestion is accomplished by enzymatic hydrolysis. However, the conventional digestion performed in solution suffers such drawbacks as enzyme autodigestion, low efficiency, extended incubation time and manual operation. To overcome these shortcomings, more attention has been paid to the immobilized enzymatic reactors [1]. In recent years, numerous enzyme immobilization methods have been developed. Proteolytic enzymes could be covalently bonded, trapped, or physically adsorbed onto different carriers, which in turn significantly affect properties of the immobilized enzyme.

With the demands of proteome analysis, high resolution and high throughput peptide/protein based separation techniques have been developed. The 2D-PAGE has limitations in protein analysis for high (>150 kDa) and low (<10 kDa) molecular weight proteins as well as proteins with extreme pI values. Another limitation of 2D-PAGE is that it cannot be on-line coupled with MS to achieve a rapid multi-
dimensional protein separation and identification [2]. As a result, multi-dimensional liquid phase based separation methods using different electrophoretic and/or chromatographic techniques have been developed as complementary tools. The feasibility of multi-modular combinations of HPLC, isoelectric focusing (IEF), chromatofocusing (CF), capillary electrophoresis (CE) as well as combinations of different HPLC modes provides numerous options for the separation of protein complexes and peptides.

2 Enzymatic micro-reactors in bottom-up approaches

2.1 A capillary micro-enzymatic reactor immobilized with nanoparticles

Previous enzyme immobilization was carried out on the inner surface of a fused silica capillary [3, 4]. An immobilized metal-ion chelating capillary microreactor was innovatively developed by Zou et al. [5], which showed advantages, such as ease in regeneration, good reproducibility and less consumption of samples. Enzyme immobilization on a portion of a silica capillary was achieved by photo-initiated polymerization by Bossi et al. [6]. With the rapid development of nanotechnology, the immobilization of enzymes was performed on various nanomaterials, such as nanoparticles, nanofibers, mesoporous materials and single enzyme nanoparticles [7], offering large surface areas, pore sizes tailored to protein molecule dimensions, functionalized surfaces, multiple sites for interaction or attachment and reduced mass transfer limitation.

Yang et al. conducted much work concerning the immobilized enzyme reactors with nanomaterials. They reported protein digestion inside the nanoreactor channels of mesoporous silica of SBA-15, and evaluated the performance by peptide mass mapping [8]. After 10-min incubation, the mass spectrum of the digests released from the mesoporous silica-based nanoreactors revealed the presence of eight peptides covering 58% of the myoglobin sequence with an intense signal (signal/noise ratio >70). In comparison, the conventional overnight in-solution digestion of proteins under otherwise identical conditions generated three peptides (27% sequence coverage). A microchip reactor packed with metal-ion chelated magnetic silica microspheres (200 nm in diameter) was newly developed by the same group [9]. In this investigation, magnetic microspheres were first modified with tetrathylorthosilicate (TEOS), and then reacted with GLYMO-IDA, formed by the reaction of glycidoxypropyltrimethoxysilane (GLYMO) and iminodiacetic acid (IDA). The copper ion and trypsin were subsequently introduced onto the surface. Finally, the prepared microspheres were packed into the microchannel by applying a strong magnetic field to make an on-chip enzymatic microreactor. Taking an RPLC fraction of protein extracted from the rat liver as the sample, the digestion could be completed within 5 min, and 23 unique peptides, corresponding to 7 proteins, were identified, demonstrating the potential application in proteome analysis.

Liu and Yang et al. reported a microreactor prepared by entrapping trypsin in a microchip coated with gold nanoparticle network, and applied it for the efficient on-line proteolysis of low-level proteins and complex extracts of the mouse macrophages [10]. The nano-structured coating was assembled via a layer-by-layer electrostatic binding of poly(diallyldimethylammonium chloride) and gold nanoparticles (AuNPs), as shown in Figure 1. The maximum proteolytic rate of the immobilized trypsin was 400 mM min$^{-1}$ (mg of enzyme)$^{-1}$, and trace proteins down to fmol were digested. By such an enzymatic reactor, proteins isolated from the mouse macrophages were digested, and 497 proteins were identified by 2D-HPLC-ESI-MS/MS. The same group has explored macroporous materials as novel catalytic systems for efficient

![Figure 1](a) The workflow schematic diagram of the identification of the protein mixture from mouse macrophages by coupling the immobilized enzyme microchip reactor combined with SCX-RPLC/MS/MS; (b) the assembly process of PDDA/AuNPs multilayers and trypsin immobilization on the PET microchannel (from Ref. [10] with permission).
and controllable enzyme micro-reactors [11]. Without increasing the enzyme or protein concentrations, this simple digestion approach exhibits high proteolysis efficiency and selectivity due to the in situ rapid adsorption of both enzymes and proteins from bulk solutions into the macropores of the catalysts, where the target substrates and enzymes are significantly concentrated and confined in the nanopores to realize a rapid digestion. This nanoporous reaction system has been applied to the analysis of a complex biological sample, where 293 proteins were identified, while 100 proteins were obtained by the standard overnight in-solution digestion. Liu et al. have described the kinetics of proteolytic micro-reactions in nanoporous materials [12], and speculated that the effect of a confined space and the ingress and diffusion of proteins into porous cavities could accelerate or limit the first proteolytic step requiring the encounter between the substrates and enzymes.

2.2 Organic carriers for enzyme immobilization

As a significant material, organic polymer monoliths emerged about 15 years ago, and were at first used as the stationary phase for HPLC. Due to the large variety, organic polymer monoliths have been widely used in proteome analysis [13, 14]. As such materials could offer high permeability, good biocompatibility, and rapid mass transfer, they have been employed as the carriers for enzyme immobilization.

Foret et al. developed an immobilized trypsin reactor based on poly(GMA-EDMA) monolith via a one-step reaction. Cytochrome-c was digested in less than 30 s at 25 °C, yielding a coverage sequence of 80%, comparable to 3 h digestion performed in solution at 37 °C [15]. Zhang et al. also studied poly(GMA-EDMA) based enzymatic microreactors [16, 17]. They developed a simple on-line protein digestion, separation, and identification system by coupling a monolithic enzymatic microreactor with nano-HPLC-ESI-MS/MS. The performance of such a monolithic microreactor was demonstrated through the digestion of cytochrome-c with a residence time of 7 s, yielding a coverage sequence of 54.8%. They found that the addition of a small amount of ACN was more favorable for protein digestion compared to that performed in pure aqueous solution.

Ye et al. coupled a monolithic poly(GMA-EDMA) microreactor with CE via a fluid joint, and the detection of peptides was enhanced by post-column derivatization and laser-induced fluorescence detection [18]. More than 20 peaks of the digest of α-lactalbumin were resolved, and the overall analysis, including on-line digestion and separation, lasted about 16 min. Zou et al. coupled a poly(GMA-EDMA) monolith-based nanoliter scale microreactor with RPLC-MS/MS for proteome studies [19]. 590 ng total cell lysate of Saccharomyces cerevisiae was digested by the microreactor with an incubation time of 1 min, and 1578 unique peptides, corresponding to 541 proteins, were identified. Compared with the conventional free trypsin digestion in solution for 16 h, the number of identified proteins was decreased by 13.3%.

The organic membrane is another type of carriers that could be used for the immobilization of enzymes. Papers involving organic membrane-based enzymatic reactors have been published. Jiang et al. prepared a microreactor by immobilizing trypsin on the columns packed with cellulose membrane modified by glycidyl methacrylate, and the apparent Michaelis-Menten kinetics constant (Km) and Vmax values were 0.12 mM and 0.079 mM min⁻¹ (mg of enzyme)⁻¹, respectively [20]. Tyan et al. exploited microchips based reactors by binding enzymes to the self-assembled alkanethiols monolayers of gold surfaces with N-ethyl-N-(3-dimethyl aminopropyl) carbodiimide hydrochloride and N-hydroxysuccinimide as coupling agents [21]. The erythrocyte protein sample was used to evaluate the performance of the enzymatic reactor. After digested by the immobilized trypsin on the functionalized SAMs surface, and analyzed by 2D nano-HPLC-ESI-MS/MS system, 272 proteins were identified. Liu developed a novel microchip trypsin reactor on the basis of a layer-by-layer approach [22]. Natural polysaccharides, positively charged chitosan and negatively charged hyaluronic acid were assembled onto the surface of a poly(ethylene terephthalate) microfluidic chip to form a microstructured and biocompatible network for trypsin adsorption. Using such a reactor, the maximum proteolytic velocity was found to be 600 mM min⁻¹ (mg of enzyme)⁻¹, thousands of times faster than that in solution, and several standard proteins were identified at the concentration of 0.5 ng μL⁻¹ with the digestion time less than 5 s.

2.3 Sol-gel encapsulation for immobilization

Sol-gel encapsulation has been used for a decade as a powerful biomolecule encapsulation technique. Formation of a sol-gel begins with the partial or complete hydrolysis of a suitable precursor to form an aqueous solution. After hydrolysis, the sol is used to allow continued evolution of the sol particles. During this time, further hydrolysis and condensation reactions occur, resulting in a wide range of linear, branched and colloidal polysilicates. The hydrolyzed precursor is mixed with a buffered aqueous solution containing the biomolecules which could be entrapped during the polymerization reaction and gelation [23]. The encapsulation process occurs under mild conditions, and the enzymes retain their native structures and bioactivity for a prolonged period. Liu et al. have developed various sol-gel techniques to modify polymer microfluidic chips for trypsin immobilization, including silica, alumina, and titania sol-gel [24, 25].

2.4 Proteomic application of microfluidic enzymatic reactors

Most immobilized enzymatic micro-reactor applications
aim at protein identification by peptide mapping. Peptide mapping is typically performed using enzymatic cleavage of the protein, and the resulting degraded products are subsequently identified using electrospray ionization-mass spectrometry (ESI MS) or matrix-assisted laser desorption/ionization-mass spectrometry (MALDI MS). Ekstrom et al. [26] described a microchip enzymatic reactor integrated with a sample pretreatment robot and a microdispenser for transfer of digested proteins directly to a MALDI target plate for automated MS analysis. Such a microchip enzymatic reactor allowed online enzymatic digestion of protein samples within 1–3 min, approximately 200–1000 times faster than digestion in solution. Immobilization of the protease has the advantage of avoiding autolytic interferences from the proteolytic enzyme in the mass spectra. By using porous silicon as a carrier for the immobilized enzyme in the microchip reactor, a large surface area and thus a high catalytic turnover are obtained. This integrated system provided a throughput of 100 samples in 3.5 h.

Figure 2 illustrates the process of enzyme immobilization and subsequent protein identification in a multilayer assembled microchip [27]. Such an assembled membrane provides a biocompatible network with high enzyme-loading capacity. The maximum rate of reaction of the adsorbed trypsin in a microchannel was significantly accelerated. The efficient on-chip proteolysis was obtained within a few seconds and the results of protein identification are comparable with those from in-solution digestion. Both a high surface-to-volume ratio and microstructured confinement within the microchannel induce order-of-magnitude enhancement of catalytic efficiency. Wu et al. [28] explored an on-line digestion protocol to couple the microreactor integrated with an SST electrode with a replaceable silica-capillary-nanospray tip attached to ESI MS. The experimental data shows that the microreactors are suitable for high-throughput analysis of proteins. Numerous micro enzyme-reactors have been developed for biosensors because they are superior in reducing the reaction time and use minimal amount of reagents [29, 30].

3 Peptide separation in bottom-up approaches

3.1 Multidimensional LC separation methods

Bi-phase column was first introduced by Yate III et al. [31] as on-line multidimensional protein identification technology (MudPIT) where the SCX and RPLC materials are sequentially packed into a single micro-capillary column. Zeng et al. [32] reported an integrated column that consisted of one strong cation exchange column and one reversed phase column. These two columns were directly connected to fulfill an on-line SCX-RPLC-MS/MS analysis of mouse liver proteins. Nine pH gradients generated from citric acid-trimethylamine buffer or 10 mM citric acid-ammonium hydroxide buffer were utilized instead of salt gradients which is commonly used in sample elution of SCX column so that SCX fractions could be directly loaded into RPLC column. The exclusion of salt-remove steps in conventional SCX-RPLC on-line or off-line separation approach is the key point to achieve the rapid 2D-LC separation and MS/MS identification. On this basis Zeng et al. developed a SCX and SAX (strong anion exchange chromatography) combined
prefractionation strategy named Yin-Yang multi-dimensional chromatography. It was used to provide an unbiased profiling of protein expression and phosphorylation of mouse liver tissue [33]. Peptides generated from SCX column with elution buffer at pH 2.5 were further separated by SAX column which used a series of pH gradient buffers for gradient elution. Thereafter SAX fractions together with other SCX fractions were analyzed by nano-RPLC-MS/MS. This Yin-Yang MDLC was demonstrated to bear comprehensive separation ability towards peptide complexes with widespread pI values.

Jiang et al. used a SCX trap column instead of commonly used RP trap column for automated on-line nanoflow RPLC-MS/MS separations [34]. SCX trap column was employed to act as RP trap column to gain higher peak capacity. SCX trap column was coupled with nanoflow RPLC-MS/MS to fulfill multidimensional separations by introducing several volatile salt gradient steps to elute peptides bound to SCX trap column gradually. Another application of this SCX trap column system was investigated by Wang et al. in the same research group [35]. As SCX trap column instead of RP trap column was used, good separation performance as well as good proteomic coverage was presented. Peptides, especially basic and hydrophilic peptides with pI > 4.5 and GRAVY < −0.5, were found to be more efficiently analyzed.

Using tandem anion/cation-exchange columns could rapidly generate positively charged, negatively charged and neutral fractions [36], thus proteins or peptides with extensive pI values could be obtained and detected. To some extent, hybrid-phase columns such as SCX-RPLC or RPLC with SCX trap column exhibit higher peak capacity and wider dynamic range than single-dimensional HPLC column. Nevertheless, the incompatibility of the elution conditions of different ion-exchange chromatography (IEX) as well as IEX/RPLC columns limits the use of these “hybrid” 2D-LC systems in comprehensive proteome separation. pH gradients or volatile salt gradients are exclusively allowed in these systems which would limit the separation ability of IEX columns.

3.2 Miscellaneous two-dimensional chromatography techniques

Some other 2D LC modes, size exclusion chromatography (SEC) coupled with RPLC as MDLC separation systems, were reported [37] and used successfully in proteomic analysis of yeast, immunoglobulin fusion proteins, cytochrome b56 complex and complex protein or peptide mixtures. Qian et al. [38] developed an automatic immobilized metal affinity chromatography (IMAC)-capillary RPLC-ESI MS/MS system, by which all procedures needed in phosphopeptide analysis including IMAC enrichment, RPLC separation, and nanospray MS/MS, could be done automatically under the control of the MassLynx program. The platform was applied to the identification of phosphorylation sites of recombinant human telomeric repeat binding factor 1 treated with kinase in vitro, and two phosphorylation sites were defined.

Affinity columns for high-abundance proteins depletion in human plasma were widely investigated. Novotny et al. [39] made a lectin microcolumn to investigate glycoproteins in human serum. The serum sample after six highest abundant protein depletion was further preconcentrated through a lectin column for glycoproteins. The trapped glycoproteins were eluted and analyzed utilizing LC-MS. About 271 glycoproteins were identified.

The latest study of online integration of multiple sample pretreatment steps, involving denaturation, reduction, and digestion with (column switched) microflow RP-HPLC-ESI-MS/MS for high-throughput proteome profiling, has been reported by Zhang et al. [40, 41]. As shown in Figure 3, native proteins were online denatured and reduced within a heater, digested with an immobilized trypsin microreactor, and analyzed by microflow µRPLC-ESI-MS/MS. In comparison to the conventional off-line urea denaturation protocol, even more unique peptides were obtained by online heating in triplicate within a significantly shortened pretreatment time of ~3.5 min (including 1 min of thermal denaturation and reduction and ~2.5 min of microreactor digestion). Proteins with concentrations ranging from 50 ng/mL (~6 fmol) to 1 mg/mL (~120 pmol) were positively identified by the online system. Such a platform was further suc-
cessfully applied for analyzing the soluble fraction of mouse liver extract. The results demonstrate that the online integrated platform is of great promise for high-throughput proteome profiling and improved identification capacity for low-abundance proteins with a minute sample amount.

3.3 Three-dimensional chromatography techniques

Two-dimensional chromatography could not be good enough to separate all peptides in complicated proteome samples. Ultra high resolution/peak capacity is always significant to identification of more proteins, especially those low-abundance proteins. Although organelle proteome analysis is an efficient way to identify more proteins as conducted by Zhang et al. for nuclear proteome in C57 mouse liver tissue [42], a prefractionation is usually demanded for low-abundance protein identification. A three-dimensional (3D) chromatography in coupling to MS/MS system was reported using SEC as prefractionation prior to online SCX-RPLC-ESI-MS/MS [43]. Normal human liver protein extracts were trypically digested before and after SEC pre-separation. This SEC-SCX-RPLC method has been demonstrated to have great improvements in both proteome coverage and protein identification confidence compared to 2D-LC-MS. Many mid- and low-abundance proteins as well as proteins with extreme MW or pI were found in this work. Both pre- and post-proteolytic separations were studied to reveal that 3D chromatography was more effective for complex proteome analysis. Both strategies were recommended to use for complex samples, and the post-proteolytic procedure was more efficient in protein identification.

As a bottom-up approach, shotgun strategy has been demonstrated to possess moderate/high peak capacity and resolution as well as the compatibility with MS. MDLC techniques have been widely used due to their complete automation, low-cost, and high sensitivity. This is the main reason for multidimensional protein identification technology widely applied to current analysis of mouse liver plasma membrane proteins [44], mitochondria proteome of human fetal livers [45], subcellular fractions from rat livers [46], SARS associated corona virus proteins [47], and liver proteome [48]. The bottom-up approach could also be used for bio-marker discovery or functional proteome research and proteome expression profiling. In conclusion, multidimensional separation of peptides combined with mass spectrometry is a significant and prospective area of proteomic research.

3.4 Chromatography array technology

Array-based electrophoretic strategies such as protein chips and capillary electrophoresis arrays have been applied in genome and proteome research with their high throughput and rapid analysis. Chromatography array technology has challenges because of the complexity and high requirements for HPLC systems. However, much progress has been made in this area. Horn et al. [49] presented a high-throughput procedure for proteome research of native constituents. Human serum sample was first separated with SEC column in a mild condition and collected in a 96-well microplate. 96 Anion exchange columns (AEC) were arranged just as 96-well microplates to separate SEC fractions in a parallel way. All fractions were submitted for quantitative assay and MS identification. The use of microplates as a medium made it possible to fulfill a high-throughput array separation. This could even increase the system resolution by the adoption of a third separation dimension. Zhang et al. developed a capillary array RPLC-based multi-dimensional separation systems coupled with MALDI-TOF-MS/MS for high-throughput proteome research [50, 51]. It includes a SCX column as the first dimension and 10 parallel capillary RPLC columns as the second dimension [50]. A multiple-channel interface was fabricated. Digested proteins extracted from liver cancer tissues were analyzed by the system. Over 1202 proteins were found with one-tenth time needed in common 2DLC system. An automated and optimized system was further developed [51]. Capillary SCX chromatographic column was used as the first dimension and 18 parallel capillary RPLC columns were integrated as the second separation dimension (Figure 4). Peptides bound to the SCX phase were eluted using gradient elution. Effluents were sequentially transferred onto each subset of precolumns. After salt fractionation peptide fractions were concurrently back-flushed from the precolumns and separated simultaneously with 18 capillary RP columns. LC

Figure 4 The schematic diagram of the array-based 2D-LC-MS/MS system. From Ref. [51] with permission.
effluents were directly deposited onto the MALDI target plates for subsequent MALDI experiments. An 18-fold increase in throughput compared with serial-based 2D-LC system was realized by this new system. The effectiveness of array-based MDLC/MS platform for separation and identification of a complex proteome sample demonstrated that it would be an effective high-throughput way for large-scale proteome research.

4 Protein and peptide separation in top-down approaches

One of the disadvantages in separating thousands and even millions of digest peptides is the increased complexity of samples. Although peptide level proteome technologies offer better resolution and less sample discrimination than 2D-PAGE, bottom-up approaches provide very limited “true” molecular information of intact proteins, particularly for proteins with posttranslational modifications (PTMs). While utilizing top-down approaches, the overall sample complexity could be significantly reduced, and quantitative results and the PTMs could be potentially obtained. A large portion of disease biomarkers is regarded as low-abundance proteins. Peptides of the low-abundance proteins are always covered by plenty of other peptides in the shotgun approach. Therefore, top-down approaches have great potentials to enhance the proteomic research in biomarker discovery.

4.1 Multidimensional LC separation methods

Zou et al. [52] developed a strategy for human plasma proteome research. Prefractionation of human plasma proteins using online strong cation exchange chromatography and reversed phase liquid chromatography (SCX-RPLC) was performed and followed by trypsin digestion and LC-MS/MS identification. A total of 1292 distinct proteins were identified, among which some proteins known to be present in serum at <10 ng/mL were detected. Sharma et al. [53] investigated lysates from S. oneidensis using weak anion-exchange chromatography combined with on-line RPLC-FT-ICR mass spectrometer. A set of 715 intact proteins was detected using this protocol.

4.2 Depletion of high-abundance proteins

Depletion of high-abundance proteins is the bottleneck in the area of biomarker discovery. New strategies of high-abundance protein depletion were proposed in addition to widely used immuno-affinity depletions. Gradiflow [54] is capable of separating proteins with different molecular weights and pI values to achieve the depletion of albumin in human plasma. Wasinger et al. [55] adopted the Gradiflow strategy as the prefractionation step prior to 2D LC-MS/MS analysis of human plasma proteome. Zhang et al. [56] developed a novel strategy of multi-dimensional chromatography separation in intact protein level for the depletion of high-abundance proteins existing in rat liver tissue extraction. Protein samples were first separated with a SCX column and fractions were collected every 2 min. Then SCX fractions were loaded onto RPLC column. Peak intensity of RPLC chromatogram detected by UV absorption was used to distinguish high-abundance proteins from middle- and low-abundance proteins. RPLC fractions with the peak intensity above 0.1AU were taken as high-abundance proteins and depleted. After separations, fractions of high-abundance proteins were tryptically digested and MS/MS analyzed, whereas other fractions defined as middle- and low-abundance proteins were pooled together and submitted for further RPLC-MS/MS analysis (Figure 5). The number of identified proteins was increased about 3 times compared to a 2D LC due to the depletion of high-abundance proteins. This strategy was demonstrated to be more universal and low-cost for proteome analysis.

A variety of combinations of prefractionsation for intact proteins are capable to gain better resolution and result in better analysis for samples with complexity and a wide span of a dynamic range. Qian et al. compared five approaches for the characterization of human serum proteome [57]. Six proteins with the highest abundance were depleted and the serum was further analyzed using different combinations of separation methods such as chromatography/2D-PAGE, online or offline LC-LC-MS spectrum. Different approaches bring different information of sample’s proteome. Selection of separation strategies could effectively improve system resolution. LC coupled with PAGE was evaluated by Marcus et al. in analysis of membrane protein from mouse brains [58].

Additionally, many relative LC techniques have been developed in order to facilitate protein/peptide separation, such as imprinted hydrogel with metal coordinated and macroporous thermosensitivity for protein recognition [59], monolithic columns based on macroporous polyacrylamide with immobilized pH gradient for protein analysis [60, 61], column switch recycling size-exclusion chromatography for high resolution protein separation [62], monolithic prep-column with large-bore particle-entrapped prepared by sol-gel method for proteome analysis [63], and on-column frit for µHPLC using single step sol-gel technique [64].

5 Concluding remarks

Enzymatic micro-reactors would become increasingly significant to future development and progress in proteomics. Due to the high concentration of trypsin that could be confined in the microscopic micro-cavity or channel, low levels of protein samples could be rapidly digested in the micro-reactor. Immobilized microfluidic reactors are flexible, implying that the approach could be applied in large-scale
analysis integrated with separation procedures to implement on-line protein identification. Multi-dimensional liquid chromatography has been widely used in all kinds of biological samples. MDLC coupled with MS has been playing significant roles in proteomic analysis, in both bottom-up and top-down approaches. New progressive strategies using MDLC techniques in high-abundance protein depletion and chromatography arrays would be promising in future proteomic analysis. Authors suggest that the top-down approach may be designable to separate protein mixtures first, and each protein effluent may be digested in an on-line enzymatic-reactor column before an advanced and effective MS identification.

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Figure 5 (a) The separation chromatogram of rat liver tissue lysate proteins by 1-D SCX. Fractions were automatically collected every 2 min from 3 min to 127 min, and collection time of fraction was demonstrated in the magnified illustration; (b) the RPLC elution profile of SCX Fraction 3; (c) the 3-D display of SCX-RPLC separation of rat liver protein samples. In total, 62 fractions were obtained from the first dimensional separation of SCX, and further separated by RPLC. Positions of part of the high-abundance proteins were as labeled. From Ref. [56.] with permission.
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