Recent advances in immobilized enzymatic reactors and their applications in proteome analysis

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Immobilized enzymatic reactors recently have drawn much attention because of the striking advantages, such as high substrate turnover rate and ease in coupling with the separation and detection systems. Carrier materials, which have great effects on the development of the immobilized enzymatic reactors, have always being the focus of study. In this paper, the contributions, mainly in the last 5 years, on the enzymatic reactors and their applications in proteome study are reviewed, with some newly developed inorganic and organic carriers for enzyme immobilization described in details. Moreover, the hyphenation of immobilized enzymatic reactors with the separation and identification systems is also summarized. By reviewing these achievements, it could be seen that enzymatic reactors have very bright future, especially in proteome analysis.

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\textbf{1. Introduction}

Proteomics, the study of all the proteins expressed by a genome, is now one of the most appealing subjects in the post-genomic era [1,2]. Compared to the genome analysis of a certain species, the proteome study is rather challenging because of the relatively large number, the wide dynamic range, the variety of complexes, and the continuous change with time and space. Therefore, it is not surprising that proteome research needs the multidisciplinary effort to confront the formidable difficulties. Obviously, in order to enable breakthroughs in proteomics, high efficient separation, high sensitive characterization and high throughput analysis are urgently required.

Up till now, 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) [3]. Another 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 [4]. 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 traditional digestion performed in solution suffers the drawbacks, such as enzyme autodigestion, low efficiency, extended incubation time and manual operation [5–7]. To avoid these shortcomings, recently more and more attention has been paid to the immobilized enzymatic reactors [8–12]. Besides the improved digestion efficacy, such reactors could be easily coupled with separation and detection systems, leading to the high throughput analysis of proteomes.

The immobilization of enzymes onto solid materials could be traced back to the 1950s [13,14]. In the last decades, numerous of enzyme immobilization methods have been developed [15,16]. In brief, protolytic enzymes could be covalently bonded, trapped, or physically adsorbed onto different carriers, which in turn greatly affect the properties of the immobilized enzyme. In this review, the immobilized enzymatic reactors are classified according to the carriers, and their hyphenations with the separation and identification systems are introduced as well.

2. Inorganic carriers for enzyme immobilization

2.1. Inorganic particulate materials

Among various inorganic carriers, silica materials, which have been widely used as the stationary phases for HPLC, play an important role for enzyme immobilization. For the first time, Wainer and co-workers immobilized trypsin on silica particles modified by hydrophilic polymers and glutaraldehyde in sequence [17]. The results showed that the catalytic activity of the immobilized trypsin could be well maintained. Bonneil et al. proposed an immobilized trypsin reactor with porous glass beads (80–120 mesh; 700 Å average pore size) as the carrier [18,19], by which trypsin was bonded via disothiocyanate. With their microreactor, good reproducibility and high enzymatic activity were obtained. To improve the sensitivity of peptide mapping, they further integrated an immobilized trypsin reactor with solid phase extraction before CE analysis, by which the protein digestion, concentration, separation and detection could be finished within 4 h [19].

2.2. Inorganic monolithic materials

Inorganic monoliths, a large category of advanced materials, have not been revived until the late 1990s [20]. The conventional silica-based monolithic columns are usually prepared by sol–gel approach, in which a porous silica rod could be formed by the hydrolytic polycondensation of alkoxysilane. Due to the existence of μm-size flow-through pores constituting a macroporous network and nm-size mesopores on the skeleton, as shown in Fig. 1 [21], silica-based monoliths have various merits, such as low back pressure drop across the column, good permeability and fast mass transfer kinetics [21–24]. More recently, silica-based monoliths have been adopted as the carriers for enzyme immobilization.

Sakai-Kato et al. did some excellent work on the development of immobilized enzyme reactors based on monolithic silica. They developed a simple in situ encapsulation procedure to prepare the immobilized trypsin reactor [25]. After mixed with a fully or partially hydrolyzed silane, trypsin could be well encapsulated in the hydrogel after several days. The enzymatic activity of the resultant monolithic reactor was about 700 times higher than that in free solution. It was noteworthy that, by immobilizing trypsin in the monolith located at the upstream of a separation capillary, the authors enabled the enzymatic digestion and CE separation in a single capillary. Thereafter, they improved this technique by coating trypsin-containing gel onto a porous silica monolith [26], which was subsequently fitted into a 96-well plate for high throughput proteome analysis. It was found that the encapsulated trypsin within the gel matrix could offer high catalytic turnover rate due to the large surface area of monoliths.

Recently, Zare’s group introduced a novel approach to prepare inorganic monolithic enzymatic reactors by photo-initiated polymerization [27,28]. One major advantage of this approach is the lack of dryness at high temperature so that the cracking of the monolith could be avoided. Later, they enhanced the activity of enzymatic reactor by covalently bonding trypsin onto such monolithic silica via Schiff chemistry at room temperature, in which an alkoxysilane reactant with an aldehyde functional group links to an inactive amine on trypsin to form an imine bond, as shown in Fig. 2 [29]. The results suggested that the proteolytic activity of such an immobilized trypsin was increased by 2000-fold compared to that obtained in solution.

Calleri et al. reported the immobilization of trypsin on the silica-based monoliths via epoxy groups, and investigated the kinetic characteristics of the trypsin bioreactor and the effects of various parameters on enzymatic activity [30,31]. After the column was activated by 3-glycidoxypropyltrimethoxysilane in toluene, trypsin reacted directly with the epoxy groups via its nucleophile functionalities. With such an immobilized enzyme reactor, the same group achieved the hyphenation of on-line digestion with HPLC via a switching valve. It was found that the cleavage efficiency (aminoacidic recovery, %AA) achieved in 20 min by the on-line protocol was at least comparable, or even better than the conventional off-line 4 h consuming method of digestion. By using the on-line system, protein digestion and genetic variant identification in serum samples were performed by the same group, and mutation sites in beta-lactoglobulin A and B variants were successfully located [32].

Brennan and co-workers recently prepared protein-doped monolithic silica columns for immobilized enzyme reactors, which allowed the screening of enzyme inhibitors with MS as the detector [33]. In their work, the enzyme was entrapped within a bimodal meso/macroporous silica network prepared by a sol–gel process. It was found that such columns were advantageous for the entrapment of soluble proteins for bioreactor chromatography.

More recently, Ota et al. developed one kind of elegant trypsin-immobilized monolithic silica with pipette-tip formula for high throughput protein digestion [34]. The silica-based monolith was first chemically modified by 3-aminopropyltrimethoxysilane, and then fixed into a 200 μL pipette tip by supersonic adhesion. After the carriers were activated by disuccinimidyl suberate (DSS), trypsin was finally immobilized. The tip enabled the digestion of reduced and alkylated protein within 20 times operation, and the enzymatic activity of the immobilized trypsin tip was about 50 times higher than that of the conventional in-solution format, indicating its potential for a rapid and high throughput analysis.

Besides silica-based monolith, some other inorganic monolithic materials have also been exploited for enzyme immobilization. Yi et al. developed a novel immobilized trypsin reactor with titania monolith as the carrier [35]. The material was prepared from biocompatible precursors using aqueous processing conditions involving the formation of a glycerol-titania composite sol and titania condensation. By the addition of poly(ethylene oxide), macroporous titanio monolith was obtained. In their experi-
Fig. 1. SEM-picture of the typical porous structure of monolithic silica columns (a), the mesoporous structure of the silica skeleton (b), and the macropores or through-pores (c) (from reference [21] with permission).

Fig. 2. Strategies for the functionalization of the PSG-PEG monolith with trimethoxysilylbutyraldehyde linker and the attachment of trypsin (from reference [29] with permission).
ment, γ-glutamyl transpeptidase, a clinically relevant protease, was entrapped in the monolithic network, which retained up to 90% of its in-solution $k_{\text{cat}}$ value, although the $K_m$ value also increased, leading to an overall catalytic efficiency of 23–45% of that performed in solution.

2.3. Open tubular capillary

Using the inner surface of a fused silica capillary as the direct carrier for enzyme immobilization is a simple but smart idea. One sort of such enzymatic reactors was developed by Kuhr and co-workers [36–38], in which trypsin was immobilized to the inner wall of a capillary via biotin–avidin–biotin coupling. The immobilized enzyme could offer good stability and high activity for the complete digestion of picomole quantities of protein. Zou’s group described a nl. microreactor with trypsin immobilized on the inner wall of a capillary [39]. The capillary was first treated with 3-aminopropyl-triethoxysilane, followed by glutaraldehyde activation and trypsin immobilization. Using such a microreactor, about 0.1 pmol and even femtomole proteins could be digested for peptide mapping analysis. Zhao et al. recently integrated a similar microreactor with nanoelectrospray emitter for the on-line digestion and fast peptide mass mapping from dilute protein samples [40]. Because the inner wall of the capillary was modified by covalent chemical bonds, the adsorption of peptides and proteins was suppressed. When 1 nM cytochrome c was digested, the sequence coverage up to 90% was obtained. Innovatively, Zou’s group prepared an immobilized metal-ion chelating capillary microreactor [41], which showed advantages, such as ease in regeneration, good reproducibility and less consumption of samples. In addition, enzyme immobilization on a portion of a silica capillary was achieved by photo-initiated polymerization by Bossi et al. [42].

2.4. Nanomaterials

In recent years, one of the significant advances in material science is the capability to prepare materials with well-defined designs and sizes in the nanometer regime. With the rapid development of nanotechnology, the immobilization of enzyme was well performed on various nanomaterials, such as nanoparticles, nanofibers, mesoporous materials and single enzyme nanoparticles [43], which could offer large surface areas, pore sizes tailored to protein molecule dimensions, functionalized surfaces, multiple sites for interaction or attachment and reduced mass transfer limitation.

Yang and co-workers did a lot of outstanding work on the immobilized enzyme reactors with nanomaterials. They reported protein digestion inside the nanoreactor channels of mesoporous silica SBA-15, and evaluated the performance by peptide mass mapping [44]. After 10 minutes’ 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 only 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 [45]. As shown in Fig. 3, magnetic microspheres were first modified with tetraethyl-orthosilicate (TEOS), and then reacted with GLYMO-IDC, and then reacted with GLYMO-IDC, followed by the reaction of glycidoxypropyltrimethoxysilane (GLYMO) and

\[ \text{GLYMO} + \text{IDA} \xrightarrow{\text{Addition}} \text{GLYMO-IDC} \]

\[ \text{TEOS, } \text{NH}_2\text{OH, } \text{H}_2\text{O and ETOH} \xrightarrow{\text{CuSO}_4} \text{GLYMO-IDC} \]

\[ \text{Trypsin solution} \xrightarrow{\text{Immobilization}} \text{Trapsin} \]

Fig. 3. Process of Cu-IDC-GLYMO-MS microspheres preparation and trypsin immobilization (from reference [45] with permission).
iminodiacetic acid (IDA). Subsequently, the copper ion and trypsin were 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. Taken a RPLC fraction of protein extracted from rat liver as the sample, the digestion could be finished within 5 min, and 23 unique peptides, corresponding to 7 proteins, were identified, demonstrating the potential application in proteome analysis. More recently, 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 [46]. The nano-structured coating was assembled via a layer-by-layer electrostatic binding of poly(diallyldimethylammonium chloride) and gold nanoparticles (AuNPs), as shown in Fig. 4. 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.

3. Organic carriers for enzyme immobilization

Organic materials display great variability and good biocompatibility regarding to the physical and chemical characteristics. Up to now, various methods have been developed for enzyme immobilization on such materials, spanning from binding on prefabricated materials to incorporating into in situ prepared carriers [15].

3.1. Organic particulate materials

PerSeptive Biosystems Inc. (Framingham, MA) manufactured a new material, Poros®, consisted of cross-linked poly(styrene-divinylbenzene) with large through pores and small diffusive pores, by which enzyme could be immobilized, commercialized under the trademark of Poroszyme® by Applied Biosystems Inc. The bulk media and cartridge (30 mm × 2.1 mm ID) of this product enabled rapid on-line tryptic digestion of proteins in flow-through environment [47–52].

Based on the commercial Poroszyme® together with its fittings, Regnier and co-workers developed several automated proteome analysis systems with the combination of immobilized enzyme columns and multidimensional chromatography [47–49]. A typical automated five-column chromatography system, as shown in Fig. 5, consisting of an immunoaffinity column, a buffer exchange column, an immobilized trypsin reactor column, a capture and desalting column and a reversed-phase column coupled to MS. By using such a system, the separation and the subsequent identification of hemoglobin proteins from human plasma proteins were achieved, and protein structure variants were easily recognized. Slysz and Schriemer recently developed an integrated protein analysis system, in which the digestion column, packed with Poroszyme immobilized trypsin beads, was attached at the downstream of a capillary C4 reversed-phase column, and a pH adjustment part was added to improve the compatibility of buffers, as illustrated in Fig. 6 [7]. When proteins were passed through the system, the resulted peptides were analyzed by ESI-MS/MS. Later, they further improved the system by applying a novel mirror-gradient unit to supply optimal conditions for the real-time tryptic digestion [53]. For standard proteins, peptide mass fingerprinting with high sequence representation was easily achieved, with detection limits down to 5 fmol (85 pg myoglobin).
Hedstrom et al. developed another kind of immobilized trypsin reactor by packing 150 μm C18 bead (Röhm-Pharma GmbH, Darmstadt), one kind of epoxy-activated polyacrylic matrix, into an HPLC column, and connected it to a gel filtration column in a microLC system, followed by ESI-MS/MS [54]. In one step, the proteins were concomitantly desalted, separated, digested and identified within 40 min, and the sequence coverage of 78–95% was achieved.

3.2. Organic monolithic materials

As another kind of important materials, organic polymer monoliths emerged about 15 years ago, and were at first used as the stationary phase for HPLC [55,56]. Differed from the inorganic counterpart, the preparation of organic polymer monoliths is often simple and straightforward, which involves mixing monomers, initiators, cross-linkers and porogenic solvents in a mould and exposing to UV light or heat to initiate the polymerization. Due to the large variety, organic polymer monoliths have been widely used in proteome analysis [57–60]. Moreover, since such materials could offer high permeability, good biocompatibility, and fast mass transfer, recently they have also been employed as the carriers for enzyme immobilization.

3.2.1. Polyacrylamide-based monoliths

Palm and Novotny proposed a simple and rapid method to fabricate a monolithic polyacrylamide enzymatic reactor in a capillary [61]. Trypsin solution was first added into the polymerization mixture, consisting of acrylamide, methylenebisacrylamide and N-acryloxysuccinimide dissolved in a buffer, together with poly(ethylene glycol), N,N,N,N′-tetramethylethylenediamine and ammonium persulfate, and then the mixture was polymerized at room temperature for 30 min. The permeability of such a monolithic carrier was so excellent that the flow rate reached up to 33 μL min⁻¹, corresponding to a linear flow of 17 mm s⁻¹, by which the digestion of BSA, cytochrome c and myoglobin could be finished within 52 s, with the sequence coverages of 32.2, 47.1, and 29.4%, respectively. With the same method, they further immobilized a peptide-N-glycosidase F on monolithic polyacrylamide to remove N-glycans from small- and medium-sized glycoproteins, such as ribonuclease B, asialofetuin, α1-acid glycoprotein and ovalbumin [62]. It was found that a residence time of 3.5 min was sufficient for deglycosylation with the immobilized enzyme reactor, while about 12 h was required when performed in solution. In addition, the enzyme activity of the microreactor could be maintained for at least 8 weeks even stored at room temperature.

3.2.2. Polymethacrylate-based monoliths

Poly(glycidyl methacrylate-co-ethylene dimethacrylate) (poly(GMA-EDMA)) polymers were first used to immobilize trypsin by Turkova et al. in the late 1970s [63]. Their experiments showed that the coupling reaction between the epoxide group and the carboxyl group was practically pH-independent. Trypsin was attached to a greater extent in acidic than in alkaline media. Petro et al. immobilized trypsin onto monolithic polymethacrylate in a 50 mm × 8 mm ID stainless steel column by a four-step method, as shown in Fig. 7 [64], and prepared a series of enzymatic reactors based on various monoliths, such as rigid macroporous monoliths consisting of 2-vinyl-4,4-dimethylazlactone, ethylene dimethacrylate and acrylamide or 2-hydroxyethyl methacrylate, as well as porous poly(butyl methacrylate-co-ethylene dimethacrylate) monoliths with reactive poly(2-vinyl-4, 4-dimethylazlactone) chains [65–68]. Furthermore, some on-chip microreactors were also developed by the same group, by which myoglobin was digested with a residence time of 11.7 s and sequence coverage of 67% [66]. In addition, by a microchip based reactor with a length of 10 cm, the digestion of eight proteins, with MW ranging from 2848 to 77,754 Da, could be finished in less than 1 min. Moreover, it was found that the sequence coverage decreased with the increase of MW [67].

Foret and co-workers 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 [69]. Besides, Zhang's group also did some work on poly(GMA-EDMA) based enzymatic microreactors [70,71]. 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 54.81%. Furthermore, 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 [72]. More than 20 peaks of the digest of α-lactalbumin were resolved, and the overall analysis, including on-line digestion and separation, lasted only about 16 min. Quite recently, Zou's group coupled a poly(GMA-EDMA) monolith-based nanoliter scale microreactor with μRPLC-MS/MS for proteome study [73]. 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 only by 13.3%.

Recently, Dovichi's group constructed a fully automated CE-microreactor-CE-MS/MS system for protein analysis, as shown in Fig. 8 [74]. At one end of a CE separation column, a pepsin microreactor based on the poly(GMA-EDMA) monolithic support was prepared, and coupled with a peptide separation column via a finely machined interface. A mixture of cytochrome c and myoglobin was analyzed by this system. Although the obtained sequence coverage for cytochrome c and myoglobin were only 48 and 22%, the system showed advantages, such as the fully automated operation, fast separation and high efficiency.

A commercial Monolithic Convective Interaction Media (CIM, BIA Separations, Slovenia), which was also based on rigid poly(GMA-EDMA) monolith, was used for enzyme immobilization by Benčina et al. [75]. The epoxy groups on the monolith were converted to hydroxyl groups by the treatment of H2SO4, followed by 1,1′-carbonyldimidazole before enzyme was immobilized. The kinetic study revealed that the immobilized enzyme reached its highest activity within 5 min.

3.2.3. Organic membrane materials

Organic membrane is another type of carriers that could be used for the immobilization of enzyme [76,77]. Up to date, some 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 [78].

Gao et al. constructed a miniaturized membrane reactor by fabricating the microfluidic channels on a poly(dimethylsiloxane) substrate, and then coupled it to a poly(vinylidene fluoride) based porous membrane with trypsin adsorbed [79]. In their design, proteins were pushed by a syringe pump through the membrane, and the digests were focused by transient isotachophoresis, separated by CE and identified by ESI-MS/MS. Although an ultrafast catalytic turnover rate was achieved, the dead volume of such a system was relatively large, which might affect the separation. Later, the same group developed another novel membrane reactor by placing the hydrophobic and porous PVDF membrane with trypsin adsorbed inside the commonly used capillary fittings [80,81]. The membrane-based trypsin reactor was coupled with capillary HPLC and ESI-MS/MS, in which the sample port of a micro-sample injector was connected to the outlet end of the membrane reactor, as illustrated in Fig. 9. The protein digests were hydrodynamically moved into the injection loop under the loading position, followed by the subsequent injection into a 10 cm-long capillary column packed with 5 μm porous C-18 particles. By such a system, rapid proteolytic digestion in seconds, instead of in hours, for proteins with concentration less than 10⁻⁸ M was achieved.

Tyan et al. exploited microchip based reactor by binding enzyme to the self-assembled alkanethiols monolayers of gold surfaces with N-ethyl-N-(3-dimethyl aminopropyl) carbodiimide hydrochloride and N-hydroxysuccinimide as coupling agents [82]. 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 then analyzed by 2D nano-HPLC-ESI-MS/MS system, 272 proteins were identified.

Recently, Liu et al. developed a novel microchip trypsin reactor on the basis of a layer-by-layer approach [83]. In brief, 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.
4. Conclusions and perspectives

With the acceleration of proteome research, immobilized enzyme reactors, which have the advantages of high digestion speed and ease in coupling with the separation and detection systems, have gained great popularities during the past few years. Although the advances reviewed herein are very encouraging, there is still a long way to go to make such immobilized enzymatic reactors more feasible. Carriers with superior characteristics, such as high mechanical strength, large surface area, low back pressure, high enzyme loading capacity and good bio-compatibility, are still one of the hotspots in the future research. Moreover, it is noteworthy that the robustness and compatibility of the on-line protein separation, digestion and peptide separation systems should be improved further to meet the requirements of real sample analysis. After about 10 years’ development, proteomic analysis is still full of challenges. We could predict that the development of the immobilized enzymatic reactors might be one of the key points to combine the top-down and bottom-up strategies.

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