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Review

Microscale immobilized enzyme reactors in proteomics: Latest developments

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ABSTRACT

Enzymatic digestion of proteins is one of the key steps in proteomic analyses. There has been a steady progress in the applied digestion protocols in the past, starting from conventional time-consuming in-solution or in-gel digestion protocols to rapid and efficient methods utilizing different types of microscale enzyme reactors. Application of such microreactors has been proven beneficial due to lower sample consumption, higher sensitivity and straightforward coupling with LC–MS set-ups. Novel stationary phases, immobilization techniques and device formats are being constantly developed and tested to optimize digestion efficiency of proteolytic enzymes. This review focuses on the latest developments associated with the preparation and application of microscale enzyme reactors for proteomics applications since 2008 onwards. A special attention has been paid to the discussion of different stationary phases applied for immobilization purposes.

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1. Introduction

During the last decade, miniaturization of analytical systems has rapidly developed as a result of advanced microfabrication techniques. The breadth of applications based on integrated microfluidic systems has considerably expanded within the last few years from genomics and proteomics [1,2] to medical diagnostics [3], drug discovery [4], and many other research fields as well. The studies focused on the characterization of different biomolecules have gone through a ground-breaking transition based on miniaturization – an idea borrowed from the natural living systems, efficiently operating at microflow rates with high surface area and surface-to-volume ratios [5]. Biological samples often contain very low amounts of analyte(s) of interest with high level of heterogeneity. Sometimes the enrichment of certain type(s) of analytes becomes crucial like in the case of phospho- or glycopeptides. In these situations, downscaling of analytical devices can provide easy sample processing with improved sensitivity. Microscale devices such as capillary columns [6], microfluidic chips [7], microfabricated ESI interfaces [8,9] and miniaturized enzyme reactors [10] have been developed as powerful alternatives to the conventional analytical systems. These miniaturized systems provide considerably faster analysis, greatly reduced sample and

Abbreviations: ESI, electrospray ionization; IMER, immobilized enzyme reactor; RPLC, reversed-phase liquid chromatography; MSP, monolithic stationary phase; NP, nanoparticle; GNP, gold nanoparticle; MOF, metal-organic framework.

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reagent consumption, improved detection limits, easy automation and increase in the sample throughput [11].

In proteomics studies, characterization of complex protein mixtures from biological matrices requires improved analytic strategies. In the conventional approach, proteins are separated by 2D-gel electrophoresis and proteolytically cleaved in-gel prior to mass spectrometric (MS) analysis. However, in-gel digestion has considerable drawbacks such as long analysis times with multiple processing steps, limited dynamic range and incomplete extraction and/or non-specific adsorption of some peptides. To overcome these drawbacks, an alternative approach, termed shotgun proteomics, has been developed. It involves in-solution digestion of all proteins in a mixture and separation of the resulting peptides by HPLC, followed by MS and/or tandem mass spectrometric (MS/MS) analysis and database search to identify individual proteins [12]. This approach generally requires the use of high-resolution instrumentation, having MS/MS capabilities. Efficient digestion of proteins in shotgun proteomics approach remains a key step for their successful identification. Undoubtedly, both in-gel and in-solution digestion protocols still have a wide range of applications for routine analysis. Alas, they both suffer from several disadvantages such as long digestion times, low activity, non-reusability of the protease used, interferences due to protease autolysis as well as poor reproducibility and difficult automation. To circumvent these problems, several efforts have been made to the development of faster and more efficient digestion procedures. A recent review by Capelo et al. summarized several modern approaches to speed up protein digestion [13].

Microscale immobilized enzyme reactors (IMERs) or simply microreactors have received a considerable interest in recent years due to various advanced features. Immobilization requires a stationary phase/support onto which a proteolytic enzyme is attached via covalent linkage, adsorption, affinity binding or entrapment/encapsulation [14]. An IMER can handle sample quantities down to a few nanoliters. Most of the IMERs can operate in a flow-through manner; hence they can be coupled online to nano/micro-LC–MS for high-throughput analysis. IMERs can be prepared in various formats with different stationary phases for repeated operation, hence offering a cost-effective solution in case of expensive proteases [15]. Furthermore, higher amounts of proteases can be loaded onto the support because there are virtually no interfering peptides originating from protease autolysis. This results in a significant increase in the digestion efficiency, operational and long-term stability, and more importantly, considerably decreased digestion times [16]. In recent years, various types of stationary phases have been employed in enzyme immobilization. Here, we present a critical review of the recent developments associated with the preparation and application of microscale immobilized enzyme reactors for proteomics applications since 2008 onwards. Additionally, a few recent reports involving rapid protein digestion by microreactors without enzyme immobilization will also be briefly reviewed.

2. Stationary phases for enzyme immobilization

Enzyme immobilization onto stationary phases is a quite old invention, dating back to the early 1970s [17]. Initial applications of immobilized enzymes were related to industrial processes, such as biocatalysis, but their use for proteolytic digestion started a couple of decades later [18,19]. Generally, development of an enzyme-immobilized system requires information about the nature of the enzyme, the nature of the stationary phase and type(s) of interactions between the enzyme and the stationary phase. In proteomic studies, trypsin is the most widely used enzyme for proteolytic digestion, having an optimal operating pH in the range of 7.5–8.5. Pepsin and chymotrypsin are also used but much less frequently, mostly due to their lower specificity. However, pepsin works best at much lower pH (optimum around pH 2–2.5), which may be desirable in some applications. Trypsin is a medium sized protease with a molecular weight of 23 kDa. It specifically cleaves proteins/peptides at the C-terminal side of lysine and arginine residues with virtually no other peptide bonds cleaved. It can be easily immobilized onto many different stationary phases. For these reasons, it has been most frequently used. The nature of the stationary phase is the most important factor in determining activity and stability of microreactors. Depending on the nature of the immobilized enzyme, the stationary phase can either stabilize or denaturate the enzyme. In the case of trypsin, hydrophilic materials help in preserving the activity, whereas hydrophobic materials are not favorable. Moreover, surface modification of unfavorable materials prior to immobilization, insertion of a linker/spacer to the surface and choice of the applied immobilization technique may also have decisive influence on the microreactor performance. Here, we focus to describe advances and limitations of different types of stationary phases recently developed to prepare IMERs for range of proteomic applications. Table 1 summarizes some of the recently prepared microreactors for rapid protein digestion, categorized based on the five different stationary phase/column types. For the description of general immobilization techniques and the factors affecting enzyme activity and IMER performance, the reader is referred to some general review articles about enzyme immobilization [14,20–23].

2.1. Enzyme immobilized reactors based on monolithic stationary phases

Monolithic stationary phases (MSPs) were introduced in the 1990s as an alternate to the conventional particle-based chromatography media [24–27]. These stationary phases became rapidly popular due to some advanced features associated with them. First, their preparation is rather simple which involves *in situ* copolymerization in confined molds. This eliminates the hassle of column packing, which is needed for particle-based stationary phases. Second, interconnected networks of micropores eliminate backpressure issues, which are often associated with particle-based phases. Third, surface modification of MSPs can be performed *in situ* depending on the type of application. Moreover, different kinds of monomers can be copolymerized to obtain tailored surface chemistry of the stationary phase. The preparation of MSPs involves mixing of one or more monomers, a crosslinker, a porogen and an initiator. A porogen is a reagent in which the monomers and the crosslinker are soluble but the respective polymer material is insoluble. The polymerization mixture can be filled into the variety of housings, such as conventional HPLC columns [26], capillary columns [28], microfluidic chips [29], or pipette tips [30]. A variety of other formats, e.g. short monolithic columns [31] or monolithic disks [32] have also been reported. The polymerization reaction can be conducted thermally or using a UV-radiation source. After the completion of the reaction, the monoliths are washed out to remove porogen, which leaves behind a porous network. Hence, by changing the composition of porogens used or the monomer-to-porogen ratio, pore sizes can be optimized as required, which is an additional benefit of MSPs. Over the recent years, several excellent reviews have been published, dealing with the preparation and properties of different MSPs [33–36].

Based on the monomers used for the preparation of MSPs, they can be divided into two classes: (i) organic monoliths, based on methacrylate monomers, and (ii) inorganic monoliths [37–39]. Both classes of MSPs have their own unique features and a variety of procedures available for their fabrication, like radical polymerization, cryogel formation, polycondensation and living