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Nanobiocatalysis for protein digestion in proteomic analysis

Jungbae Kim[1,*], **Byoung Chan Kim**[2], **Daniel Lopez-Ferrer**[3], **Konstantinos Petritis**[4], and **Richard D. Smith**[3]

[1] Department of Chemical and Biological Engineering, Korea University, Anam-dong, Seongbuk-gu, Seoul 136-701, Republic of Korea

[2] Institut Pasteur Korea, 696, Sampyeong-dong, Bundang-gu, Seongnam-si, Gyeonggi-do, 463-400, Republic of Korea

[3] Pacific Northwest National Laboratory, Richland, WA 99352, USA

[4] Translational Genomic Research Institute, Phoenix, AZ, 85004, USA

Abstract

The process of protein digestion is a critical step for successful protein identification in the bottom-up proteomic analysis. To substitute the present practice of in-solution protein digestion, which is long, tedious, and difficult to automate, a lot of efforts have been dedicated for the development of a rapid, recyclable and automated digestion system. Recent advances of nanobiocatalytic approaches have improved the performance of protein digestion by using various nanomaterials such as nanoporous materials, magnetic nanoparticles, and polymer nanofibers. Especially, the unprecedented success of trypsin stabilization in the form of trypsin-coated nanofibers, showing no activity decrease under repeated uses for one year and retaining good resistance to proteolysis, has demonstrated its great potential to be employed in the development of automated, high-throughput, and on-line digestion systems. This review discusses recent developments of nanobiocatalytic approaches for the improved performance of protein digestion in speed, detection sensitivity, recyclability, and trypsin stability. In addition, we also introduce the protein digestions under unconventional energy inputs for protein denaturation and the development of microfluidic enzyme reactors that can benefit from recent successes of these nanobiocatalytic approaches.

Keywords

Enzyme coating; Nanobiocatalysis; Nanostructured materials; Protein digestion; Trypsin stabilization

1 Introduction

In a typical shotgun proteomics study, proteins are digested into peptides by a protease such as trypsin prior to the MS analysis of peptides. The smaller peptides facilitate protein identification by tandem MS and also allow coverage of proteins that would be problematic due to e.g. solubility and heterogeneity. However, the present practice of protein digestion in solution is long, tedious, and difficult to automate. The development of a rapid, recyclable and automated digestion system has been a topic of many research trials, such as enzyme immobilization, employment of protein-denaturing conditions, and development of enzyme

[*]Corresponding Author: Jungbae Kim, Tel) +82-2-3290-4850, Fax) +82-2-926-6102, jbkim3@korea.ac.kr.

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columns as well as other digestion platforms on chips or plates. However, a broadly useful approach has not been defined, and one key reason for this appears to be the poor stability of trypsin through the various sample manipulations. As an example, while several companies have tried to develop trypsin digestion columns, their success has been limited by the short lifetime of column due to the poor stability of trypsin in the column [1,2].

Recent successes with nanobiocatalytic enzyme stabilization have indicated great potential for improving trypsin stability [1]. Nanobiocatalytic approaches have used various nanostructured materials, such as nanoporous materials, nanoparticles, nanofibers and nanotubes, as a host for enzyme immobilization and stabilization [3]. Nanostructured materials have several advantages over conventional solid support materials for enzyme immobilization. First, they provide larger surface areas for the immobilization of enzymes, leading to improved enzyme loading and apparent enzyme activity per unit mass or volume of immobilization host. Second, they have uniform and well-controlled size distribution that enables the systematic enzyme activation and stabilization based on the synergetic interactions between enzyme immobilization techniques and nanostructures. Third, they retain useful properties, such as magnetism and conductivity, which can be effectively employed in improving various enzyme applications [1,3,4].

These features of nanostructured materials have been employed in immobilizing and stabilizing trypsin for rapid and recyclable protein digestion. Various nanobiocatalytic approaches have been developed by using various enzyme immobilization techniques, such as enzyme adsorption, covalent attachment, enzyme crosslinking, and combinations of the aforementioned methods. They have shown utility for activation, stabilization, recycled uses, magnetic separation, and proteolytic resistance of nanobiocatalytic trypsin systems. Especially, the recent success of nanobiocatalytic trypsin stabilization in a form of enzyme coating [5] has demonstrated potential for use in rigorous denaturing conditions and for online digestion columns.

In this review, we discuss recent developments of nanobiocatalysis for the improved performance of protein digestion, together with its status and potentials to be used in various conditions and platforms, such as microwave, ultrasound, high pressure and microfluidic reactors. It must be noted that most of nanobiocatalytic approaches cannot be directly employed for in-gel digestions because they generated large-sized enzyme immobilizations that might be unable to penetrate into the gel matrix. However, once proteins are eluted from the gel matrix via various elution techniques such as electroelution [68,69], passive elution [70], or ultrasound assisted elution [71], nanobiocatalytic systems can be used for the follow-up protein digestion in solution.

2 Nanostructured materials employed in protein digestion

The process of protein digestion has been improved by using three different nanostructured materials, such as nanoporous materials, nanoparticles, and nanofibers. Figure 1 shows various immobilization approaches using these nanostructured materials for efficient protein digestion. Nanoporous materials have been used as nanoscale reactors for expedited protein digestion by simply adsorbing trypsin and proteins into nanometer scale pores, which enables much better contact between trypsin and proteins due to their high concentrations and proximity in a confined environment of nanopores (Section 2.1). Nanoparticles, especially magnetic nanoparticles, have been widely used for an easy recycle of covalently-attached trypsin on nanoparticles (Section 2.2). Finally, the trypsin coating on nanofibers has demonstrated its unprecedented success in stabilizing the enzyme activity by showing no activity decrease under recycled uses for one year and impressive proteolytic resistance (section 3) [5].

2.1 Nanoporous materials

Nanoporous materials have attracted a lot of attention as a host for enzyme immobilization due to their controlled porosity and high surface area. Various approaches have been reported for the immobilization of enzymes into nanoporous materials, including adsorption, covalent attachment, and 'ship-in-a-bottle' approaches [3]. However, only a simple adsorption approach has been used in immobilizing trypsin for the improvements of protein digestion in proteomic analysis. On the other hand, nanoporous materials have also been used for the enrichment of protein substrates and peptide products to improve the protein digestion and the detection sensitivity, respectively. In this section, we discuss the uses of nanoporous materials not only as nanoscale reactors for protein digestion, but also as pre-concentrators for the enrichment of digested peptides.

2.1.1 Nanoporous materials as nanoscale reactors for protein digestion—The nanometer scale pores of nanoporous materials have been used as enzyme reactors to improve the detection sensitivity as well as expedite the speed of protein digestion. Two different approaches have been used as shown in Figure 2. The first approach is to initially adsorb trypsin into cyano-functionalized nanoporous silica (CNS), and then incubate the trypsin-adsorbed CNS (trypsin-CNS) in the protein solution (Fig. 2A). Trypsin-CNS showed much more efficient performance of protein digestion than in-solution digestion, by successfully identifying 2 ng/μL proteins after digestion for 20 min. The trypsin-CNS digestion of biological complex sample was also successful by identifying 165 proteins out of a sample extracted from the cytoplasm of human liver tissue. The improved performance of protein digestion was explained by the nanoscopic confinement and enrichment of protein substrates within nanopores [6].

The second approach is to initially adsorb proteins into nanoporous silica, such as SBA-15 or FDU-12, and then incubate the nanoporous silica enriched with proteins in the trypsin solution (Fig. 2B) [7–9]. This approach was first proposed by using SBA-15, consisting of one-dimensional nanochannels with a pore diameter of 8 nm that are packed in a two-dimensional hexagonal structure. The trypsin digestion of myoglobin for 10 min led to the identification of eight peptides covering 58% of the protein sequence with an intense signal, while the conventional overnight in-solution digestion under identical conditions resulted in the generation of only three peptides representing the sequence coverage of 27%. This improved performance of protein digestion in nanopores was explained by the enrichment of protein substrates followed by the trypsin adsorption within nanoporous silica channels [7]. This successful protocol was further investigated and optimized for the digestion of two model proteins, myoglobin and cytochrome c, and the higher peptide sequence coverage of 98% for myoglobin could be achieved by increasing the time spans of both enrichment and digestion from 10 min to 15 min [9].

In an extended study, new nanoporous silica, called FDU-12, has been used to achieve the desired features of protein digestion, such as rapid digestion, larger sequence coverage, and high sensitivity. FDU-12 consists of highly ordered three-dimensional interconnected nanopore networks, in which large nanocellular pores (27 nm) are connected by expanded entrances (17 nm). In a comparative study with the other nanoporous silica such as SBA-15 and MCF, FDU-12 showed much better performance in the nanopore digestion of various model proteins and biological complex sample of nuclear protein fraction from mouse liver cells. From the Raman spectroscopy results, the mechanism for the 'reagent-free' denaturation of protein substrates was proposed when enriched in the nanopores of FDU-12. In other words, the unique three-dimensional nanopore structure of FDU-12 has facilitated a three-step process: substrate enrichment, reagent-free protein denaturation, and efficient proteolytic digestion of denatured protein substrates in the nanopores of FDU-12. The

overall process of in-nanopore digestion took less than 15 min due to high surface area, favorable interactions of FDU-12 nanopores with protein substrates, and a highly-permeable structure for trypsin, protein substrates and protein digests. The 3-D interconnected pore structure of FDU-12 allowed better performance than the 2-D pore structure of SBA-15 by improving the protein sequence coverage of myoglobin from 31% to 84%. The use of FDU-12 enabled substrate enrichment, efficient proteolysis in nanopores, and facile peptide release after digestion, leading to high sensitivity by detecting protein digests at low femtomoles using MALDI-MS/MS[8].

As described above, the approaches of using nanoporous materials as nanoscale reactors have generated successful results in expediting the protein digestion and improving the detection sensitivity. However, there are still some issues to be addressed for the realization of automated and high-throughput process of protein digestion. The use of nanoporous materials requires centrifugation and tedious handling to recover them after each step of protein immobilization and enrichment. Nanoporous materials need to be excessively washed before their recycled uses in order to prevent any problem of sample carry-overs. The time span of in-nanopore digestion should be further reduced for the successful on-line digestion in an automated and high-throughput way. It is anticipated that the incorporation of nanoporous materials into columns or microfluidic systems can possibly solve these potential problems in the system development for efficient protein digestion.

2.1.2 Nanoporous materials for enrichment of digested peptides—Nanoporous materials provide uniform and well-controlled nanopores with large pore volume and high surface area, which can be used for the enrichment of proteins and peptides. Protein profiling and peptidome analysis of biological samples could be successfully achieved by using functionalized nanoporous silica with different pore sizes and structures [10–13]. In a similar approach, the protein digestion was followed by the selective enrichment of protein digests before the MS analysis, leading to the great improvement of detection sensitivity. Zou and coworkers immobilized Fe^{3+} on the nanopore surface of nanoporous silica, called MCM-41, with the particle size of 600 nm and the pore size of 3 nm, and used the Fe^{3+}-immobilized MCM-41 to selectively enrich phosphopeptides from the tryptic digests of α-casein and β-casein by taking advantage of the strong affinity between the Fe^{3+} ion and the phosphate group. Abundant non-phosphopeptides were effectively removed, and the detection sensitivity of phosphopeptides was improved by more than one order of magnitude [14]. In an extended study, they functionalized MCM-41 with titanium phosphonate to selectively capture phosphopeptides from the tryptic digests of β-casein, and the detection limit of as low as 1.25 fM phosphopeptides could be achieved based on the MALDI-TOF MS analysis [15].

Kim and coworkers reported the use of magnetically-separable nanoporous carbon foams, called Mag-MCF-C, for the efficient enrichment and desalting of protein digests in the MALDI-TOF MS analysis [16]. The quick capture of Mag-MCF-C using a magnet enabled the easy and simple enrichment and desalting process, comprising three steps of adsorption, washing, and separation. The detection sensitivity could be improved by generating distinct MALDI mass spectra of peptides even at a peptide concentration as low as 50 pM, while the sequence coverage for protein identification was also significantly improved when compared to other conventional methods [16]. Han et al. synthesized nanoporous Fe_2O_3 microspheres with the particle size of 3 nm and the large inter-particle pores of 48 nm, and used them for the selective enrichment of phosphopeptides from the tryptic digests of caseins [17]. High sensitivity, selectivity and capacity of phosphopeptides could be achieved under a mild condition in a relative short time.

2.2 Nanoparticles

Magnetic nanoparticles are nanostructured materials that have been most frequently used for trypsin immobilization because they enable the easy recovery of enzyme immobilization systems after protein digestion. In most cases, trypsin was immobilized onto magnetic nanoparticles via a conventional approach of covalent attachment. High trypsin loading on magnetic nanoparticles, due to large surface area of nanoparticles for trypsin immobilization when compared to micrometer-sized magnetic beads, enabled effective protein digestion within a short time ranging from 10 s to 5 min [18–20]. Covalently-attached trypsin on magnetic nanoparticles showed good stability, leading to a good performance of protein digestion even at elevated temperature such as 57 °C [18]. Trypsin-immobilized magnetic nanoparticles have been employed mostly for on-chip digestion [20–22], and an easy recovery via simple magnetic capture played a key role in the facile separation of peptides as well as recycled uses of immobilized trypsin.

Li et al. developed an approach for on-plate digestion by using trypsin-immobilized magnetic nanoparticles [23]. The solution of protein substrates was firstly loaded onto the plate, followed by the addition of trypsin-immobilized magnetic nanoparticles. The plate was kept in a humidifier chamber at 50 °C. After the protein digestion was completed within 5 min, a magnetized needle was used to remove magnetic nanoparticles from the plate. Finally, the matrix solution was added, and the samples were subsequently analyzed by MALDI-TOF-MS. Within only 5 min digestion, the peptide sequence coverage was higher than or equal to that of traditional in-solution digestion that took place for 12 h. This on-plate digestion was successful in analyzing the RPLC fractions of the rat live extract as well as model proteins such as myoglobin and cytochrome c. This approach has a great potential to be used for the automated and high-throughput processes of protein digestion.

Protein digestion on a microchip was performed by packing the capillary channel with trypsin-immobilized magnetic microparticles or nanoparticles [20–22,24]. From a comparative study of particle sizes in their digestion efficiency, sub-micrometer particles (500–1000 nm) were selected and packed into the channel for on-chip digestion of five model proteins [21]. In another study, magnetic nanoparticles of 50 nm in diameter were used for successful digestion of an RPLC fraction of the rat liver extract as well as three model proteins [22]. In more detail, trypsin was covalently attached onto magnetic nanoparticles, and the trypsin-immobilized magnetic nanoparticles were then packed into a capillary column of microchip by applying a magnetic field. Finally, the protein solution was added and allowed to flow through the reactor, during which the proteins were digested. On-chip protein digestion with high sequence coverage could be completed in a short time such as 10 s under a flow rate of 5 μL/min. This rapid digestion could be achieved due to enhanced contact between trypsin and protein substrates through much smaller pores between magnetic nanoparticles when compared to magnetic microparticles. The controlled use of magnetic field enabled the new packing of trypsin-immobilized magnetic nanoparticles on a chip in less than 1 min. The successful on-chip protein digestion by these recyclable and easily-replaceable trypsin microreactors on a chip has opened up the path to the automated and high-throughput processes of protein digestion.

3 Nanobiocatalytic stabilization in a form of enzyme coating

Interestingly, not many research efforts have been dedicated to the stabilization of trypsin activity in the field of proteomics even though the poor stability of trypsin is one of the most serious hurdles against automating the process of protein digestion. As an example, the present failure of trypsin digestion column mostly results from the poor stability of trypsin in rigorous conditions of operation and/or the proteolytic digestion of trypsin by the proteases in proteomic samples. The addition of protease inhibitors in the samples can be a

partial solution, but the activity of immobilized trypsin is decreased or eventually shut off in the presence of trypsin inhibitors. Recent developments of nanobiocatalytic enzyme stabilization have opened up the route to an eventual solution for automated on-line digestion by generating the promising results, such as high enzyme loading, activity, stability, recyclability, and resistance against proteolysis [1,5]. During the last few years, several review papers have been published on the subject of nanobiocatalytic approaches for the stabilization of enzyme activity [1,3,4,25]. Therefore, this section is dedicated to the in-depth discussion on one of those approaches, called the 'enzyme coating' approach, which can possibly revolutionize the process of protein digestion in proteomic analysis.

3.1 Enzyme coating approach

The improvement of protein digestion has been achieved by immobilizing trypsin in nanoporous materials or on nanoparticles via conventional techniques of adsorption and covalent attachment. These two approaches can marginally stabilize the trypsin activity, but the ultimate stability of trypsin for repetitive uses could not be accomplished, particularly, in the presence of proteolytic activities. The approach of enzyme coating carefully combines the two approaches of covalent attachment and enzyme crosslinking, which led to an unprecedented success in enzyme stabilization [5]. The fabrication of enzyme coating consists of two steps (Fig. 3). In the first step, the seed enzyme molecules are covalently attached to functionalized surfaces of nanostructured materials, leading to monolayer coverage of enzyme molecules. In the second step, after the addition of a highly concentrated enzyme solution, the enzyme crosslinking via the glutaraldehyde (GA) treatment is performed to fabricate the enzyme aggregate coating onto the covalently-attached seed enzyme molecules on nanostructured materials.

The approach of enzyme coating has been successful in the stabilization of trypsin, chymotrypsin, lipase, and glucose oxidase on various nanostructured materials, such as electrospun polystyrene-based nanofibers, polyaniline nanofibers, magnetic nanoparticles, and carbon nanotubes [5,26–30]. This suggests that the success of enzyme coating is neither enzyme-specific nor nanomaterial-specific, but rather versatile in developing nanobiocatalytic systems with stabilized enzyme activity. The enzyme coating of sub-micrometer thickness would place much less mass-transfer limitation in digesting protein molecules than the crosslinked enzymes of micrometer or millimeter size. In addition, the fabrication of enzyme coating on nanomaterials provides opportunities to employ the advantageous features of nanomaterials, such as durability of polymer nanofibers, magnetic separation of magnetic nanoparticles, and conductivity of carbon nanotubes. For these reasons, the enzyme coating approach has great potentials to be used in various enzyme applications, such as bioconversion [28], biosensors, and biofuel cells [30], as well as protein digestion in proteomic analysis [5].

3.2 Trypsin coatings on electrospun nanofibers

The approach of enzyme coating was employed to develop the stable and robust trypsin coating on electrospun polymer nanofibers (Fig. 4) [5]. First, polymer nanofibers were prepared by electrospinning the mixture of polystyrene and poly(styrene-co-maleic anhydride). Trypsin molecules were covalently attached to electrospun polymer nanofibers through the reaction between the amino groups of trypsin and the maleic anhydride groups of poly(styrene-co-maleic anhydride). The enzyme crosslinking via the glutaraldehyde treatment in a highly concentrated trypsin solution resulted in the trypsin-aggregate coated nanofibers. The SEM images of trypsin-coated nanofibers vividly showed the thick but still sub-micrometer scale coating of trypsin aggregates on nanofibers, while the covalently-attached trypsin could not be observed in the same scale of SEM images (Fig. 4). The activity of trypsin coating was 300 times higher than that of covalently-attached trypsin

because the thick coating significantly increased the enzyme loading when compared to the approach of covalent attachment.

Trypsin coating on nanofibers resulted in an unprecedented stability by showing no decrease in activity even after repeated uses for one year. This highly stable form of trypsin immobilization enabled the repeated digestions of bovine serum albumin over 40 days and successful peptide identification by LC-MS/MS, revealing the resistance of trypsin coating to autolysis. Trypsin-coated nanofibers also showed good resistance to the proteolytic activity of chymotrypsin, and were successfully employed in digesting the yeast proteome extract with high reproducibility and within a shorter time frame than in-solution digestion. Biocatalytic nanofibers with trypsin aggregate coating, which retain high enzyme activity and stability, are resistant to autolysis and proteolysis, and successfully digest proteins in a reproducible and rapid manner, have demonstrated a great potential for repeated and automated protein digestion in proteomic analysis [1,5].

4 Protein digestions under ‘non classical conditions’

Protein digestion is one of the most important, yet time-consuming, steps in proteomic workflows, in which trypsin is most widely used to transform proteins into peptides. Because the protein digestion is generally slow, this portion of the proteomic workflow determines the overall rate of protein identification and often limits the throughput of the analysis. One of the strategies involves increasing the temperature to speed the kinetics of protein denaturation and digestion. However, increased temperature can also denature the trypsin, which is a key reason for interest in thermo-stable organisms (i.e. to understand and engineer more heat-stable enzymes [31,32]). These efforts have resulted in the production of enzymes that can function at higher temperature, where the sample proteins of interest can be completely denatured allowing the easy access of heat stable protease to the cleavage sites of protein substrates. Using this approach, Havlis and coworkers demonstrated that protein digestions within 30 min could be achieved [31]. Nevertheless, 30 min may be excessive for many applications involving very high sample numbers or ‘on-line’ applications. For this reason, there is a strong focus on looking at other energy inputs in order to accelerate the protein digestion. In this section, we discuss these other energy inputs for rapid protein digestion, which can be called ‘non-classical conditions’ (Fig. 5) to differentiate them from the classical condition of high temperature. It should be pointed out that various approaches of nanobiocatalytic enzyme stabilization can contribute to the improved performance of protein digestion in rigorous conditions, including these non-classical conditions, by stabilizing the trypsin activity as exemplified in the case of trypsin-coated nanofibers.

4.1 Microwave-assisted digestion

Microwave irradiation has been broadly explored to digest several proteins in gel or complex protein extracts in solution [33]. There are two main hypotheses to explain the accelerated protein digestion under microwave irradiation. One assumes that it is just a transfer of heat based on traditional conduction/convention heating. The other hypothesis, pointing to a nonthermal effect, is based on non-ionizing radiation that interacts with ion pairs and organic molecules retaining a dipole. In this case, the microwave energy is directly transferred to these species by a different means. To dissect the effect of microwave irradiation, Pramanik et al. used a microwave apparatus to control the level of microwave irradiation, and maintained the reaction temperature at 37 °C by using a reaction chamber. They investigated the effect of microwave irradiation in assisting the digestion of proteins such as cytochrome c, ubiquitin, lysozyme, and myoglobin. According to this study, heat by itself does not accelerate the reaction [34]. Microwave-assisted digestion was performed to speed up the in-gel reaction by digesting proteins that were previously separated by the SDS

gel [35]. The protocol of microwave-assisted digestion was further optimized either by using different organic co-solvents or by applying microwave energy to clinical samples [36,37].

4.2 Ultrasound-assisted digestion

In recent years, ultrasonic energy has also been explored for accelerating protein digestion [38] for either in-gel or in-solution digestion of single proteins and complex protein mixtures. Effectively complete digestion could be achieved in 1 min, with similar results as conventional protocols. Interestingly, the addition of organic co-solvents such as acetonitrile showed no influence on digestion yields.

Ultrasound-assisted digestion was also demonstrated for discovery proteomics [39]. In an extended study, accurate protein quantification could be achieved in investigating the differences in mouse macrophage proteomes after low dose irradiation exposure [40].

Ultrasonic energy generates the mechanical phenomena of microjetting and microstreaming, leading to the cavitation effect when the bubbles collapse. This cavitation effect produces local regions of extremely high pressure and temperature, which can aid mixing between the enzyme molecules and the proteins in the sample. Ultrasound increases the temperature slowly in equilibrium with the surrounding solution while the temperature increases very quickly under microwave irradiation. This fact is especially important in proteomics because temperature is a key factor in maintaining the selectivity of the method and avoiding the loss of enzyme activity. Ultrasound-assisted digestion can be performed in an automated and high-throughput way by using robotic stations with an array of probes.

4.3 Pressure-assisted digestion

In 2008, Lopez et al. reported a method for ultrafast digestion by using high pressure in the process of trypsin digestion [41]. This method used the pressure cycling technology, which had been developed as an efficient method for cell lysis. The protein digestion was performed at the pressures ranging from 5000 to 35,000 psi, and the pressure effect on various parameters such as addition of organic solvents, number of cycles, and digestion time were investigated. Pressure-assisted digestions could be successfully conducted at room temperature, which is advantageous for limited chemical artifacts ascribed to heating. Carbamylation of cysteines as well as methionine oxidation are the most common modifications found in typical proteome analyses. The system was coupled to LC-MS systems to demonstrate fast on-line digestion steps for both individual proteins as well as complex protein mixtures [42]. It was found that reductively-methylated and TPCK-treated trypsin seems to retain its activity under pressures as high as 20,000 psi, allowing better protein coverage than a conventional digestion procedure.

4.4 Nanobiocatalytic systems in unconventional digestion conditions

It is expected that the use of nanobiocatalytic systems can further improve the performance of in-solution protein digestion in unconventional conditions described in the former sections. Microwave energy has been extensively applied to several proteomic studies and proven to be a powerful means to reduce the time span of protein digestion down to a matter of seconds. Lin and coworkers [43–45] have demonstrated that trypsin-immobilized magnetic microspheres and nanoparticles resulted in a successful digestion under microwave irradiation. The process was optimized by using standard proteins, and the method was evaluated by digesting the protein extract from rat liver. All digestions were performed within 15 sec, and resulted in similar number of identified proteins when compared to in-solution digestion at 37 °C for 16 h. Lopez-Ferrer et al. have demonstrated that the combination of trypsin-immobilized silica microspheres with ultrasound irradiation is a very promising technology [46]. This approach was employed for the quantitative analysis in

combination with ^{18}O labeling, and showed a high degree of reproducibility, as well as very efficient digestions using a minimum amount of enzyme. The simplicity of this approach further makes it amenable to automation and large-scale multiple sample analyses.

5 Fabrication and application of microfluidic enzymatic reactors

Immobilized enzymes have been used for several decades to fabricate large-scale reactors for several industrial processes [47]. The introduction of proteomics has renewed interest for enzyme immobilization and enzymatic reactors, but this time in the micro/nano scale. Several papers have been published on the subject most of which have been summarized in several excellent review papers that have been published in the past ten years [48–52]. The application of enzymatic reactors in proteomics hold the promise of faster digestions, resistance to autolysis, repeated use, cost minimization, ease of automation, compatibility with very small amount of sample, and high digestion reproducibility.

Several techniques and number of supports have been used for the fabrication of enzyme reactors. These reactors can be prepared either using the ‘in batch’ approach where the enzyme is firstly immobilized on the support and then is packed into the column using a slurry packing technique or the ‘in situ’ approach where the enzyme is directly immobilized on the pre-packed column. Comparison of those two approaches by Massolini et al. [53] favors the ‘in situ’ approach due to the loss of catalytic activity during the ‘in batch’ packing process. As a result, the ‘in situ’ immobilization technique has been adopted by most groups working in the field of enzymatic reactors. Among the different immobilization supports used for enzymatic microreactors, monolithic supports were found to be the most suitable, enabling the fabrication of highly active enzymatic reactors, especially in the capillary column format. The major advantage of monolithic supports is the outstanding mass transfer they enable due to the convective flow through the pores.

Enzyme reactors used for protein characterization are usually positioned pre- or post-separation column (or capillary electrophoresis device), and coupled on-line or off-line with some type of mass spectrometers. The majority of those applications use the reactor pre-column in order to digest proteins into peptides, followed by trapping/pre-concentration and separation of these peptides before analysis by mass spectrometry [54–60] (Fig. 6A). Most of the reports demonstrate the performance of the reactors with peptide mapping of small standard proteins and only a few papers have demonstrated results from a whole microorganism cell extract [57]. In addition to peptide mapping, post-column digestion has also been used in order to deglycosylate proteins and analyze the glycans by mass spectrometry [61].

Unlike pre-column/separation enzymatic reactors, very few reports have been published on post-column enzymatic reactors for proteome analysis [62]. This is due to several challenges such as (i) band broadening caused by post-column volumes, connections, etc., (ii) the need to modify the experimental conditions for the separate step such as pH, solvent, temperature, etc., to those required for an enzymatic post-column reaction and analysis by mass spectrometry, and (iii) separation of complex mixture of proteins that presents its own difficulties. Although challenging, such an approach could be very powerful in proteomics as the protein chromatographic separation information is maintained (i.e., peptides from each protein are eluted under a very narrow window, identical to the protein chromatographic peak). This can significantly increase the confidence of peptide identifications or even allow integration between top-down and bottom-up proteomics by performing two chromatographic runs, with and without post-column digestion (Fig. 6B). In 2006, Petritis et al. demonstrated an approach for protein/peptide identification by separating peptides, digesting them post-column to their smaller homologues, and subsequently

analyzing them with high resolution mass spectrometry [63]. By obtaining accurate mass information of the undigested and digested peptides it was possible to identify them without the use of tandem mass spectrometry due to the increased specificity of the approach. Most recently, Petritis et al. demonstrated single injection top-down and bottom-up proteomics on-line integration by using a device post-column called ReplayTM (Advion, Ithaca, NY, USA) [64]. Briefly, a protein mixture was separated and split into two separate streams of post-columns. One was directed towards the mass spectrometer for analysis while the second was digested by pepsin and captured in a long capillary. When the LC-MS analysis of the first stream (intact proteins) was finished, a valve was switched directing the second stream of protein digests for analysis. As the separation was maintained in the capture capillary, the two chromatograms of the eluted intact proteins and their corresponding digest peptides were similar, allowing the correlation between them.

Although enzymatic reactors have attracted significant attention in the academic circles, which is evidenced by the hundreds of relevant published papers, they are still not commercially available. This is likely due to the short life-span of enzymatic reactors when complex proteomic mixtures such as plasma or whole cell lysate are used. One of the key issues would be the activity of proteases present in those samples, which can digest the immobilized trypsin. When simpler protein mixtures with no proteases are used, satisfactory reactor life-span is observed. Bynum et al. demonstrated an integrated microfluidic LC/MS chip for the rapid on-line deglycosylation and characterization of N-glycans from recombinant IgG antibodies [65]. The chip was re-usable for at least 300 hundred times (Kevin Killen, personal communication) when simple mixtures of antibodies were used as samples. When considering the complexity of most proteome samples containing various proteases, the new immobilization technique of enzyme coating with high stability and good proteolytic resistance [5] and offers new promise for applications with more complex and difficult mixtures.

6 Conclusion

Various nanobiocatalytic approaches, using nanoporous materials, magnetic nanoparticles, and polymers nanofibers, have improved the performance of protein digestion in speed, detection sensitivity, recyclability, and trypsin stability. Especially, trypsin-coated nanofibers demonstrated unprecedented success in trypsin stabilization with no activity decrease under repeated uses for one year and good resistance to proteolysis. The present successes of nanobiocatalytic approaches will lead to the development of automated, high-throughput, and possibly on-line digestion systems that can expedite the total process of protein identification in proteomic analysis. It is also anticipated that nanobiocatalytic enzyme stabilization can effectively improve the approaches with unconventional energy inputs and microfluidic enzyme reactors by extending the lifetime of trypsin activity in those rigorous conditions of operation. When considering various nanomaterials with advantageous features, the composites of various nanomaterials are expected to play a role for further improvement of present nanobiocatalytic successes in protein digestion. As an example, magnetic nanoparticles can be incorporated into polymer nanofibers, and the resulting magnetically-separable nanofibers can be used for the fabricating of stable trypsin coating that can be easily recovered by a simple magnetic capture. On the other direction, other nanobiocatalytic approaches of enzyme stabilization such as single enzyme nanoparticles [66] can be used for the development of a stable trypsin column because the single trypsin nanoparticles with stable enzyme activity can be further immobilized into nanoporous structures of the silica column for the development of on-line digestion systems with a long lifetime and reduced mass transfer limitation [67].

In conclusion, nanobiocatalytic approaches have manifested their uses and potentials in improving the performance of protein digestion for efficient proteomic analysis, and more innovative approaches in the next few years are anticipated to play a key role in replacing the present practice of in-solution protein digestion with automated and high-throughput digestion systems.

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Abbreviations

CNS	cyano-functionalized nanoporous silica
FDU-12	Fudan University-12
GA	glutaric dialdehyde
Mag-MCF-C	magnetically-separable mesocellular carbon foams
MCF	mesocellular siliceous foams
MCM-41	Mobil composition of matters-41
RPLC	reversed-phase liquid chromatography
SBA-15	University of California at Santa Barbara-15
TPCK	L-1-tosylamido-2-phenylethyl chloromethyl ketone

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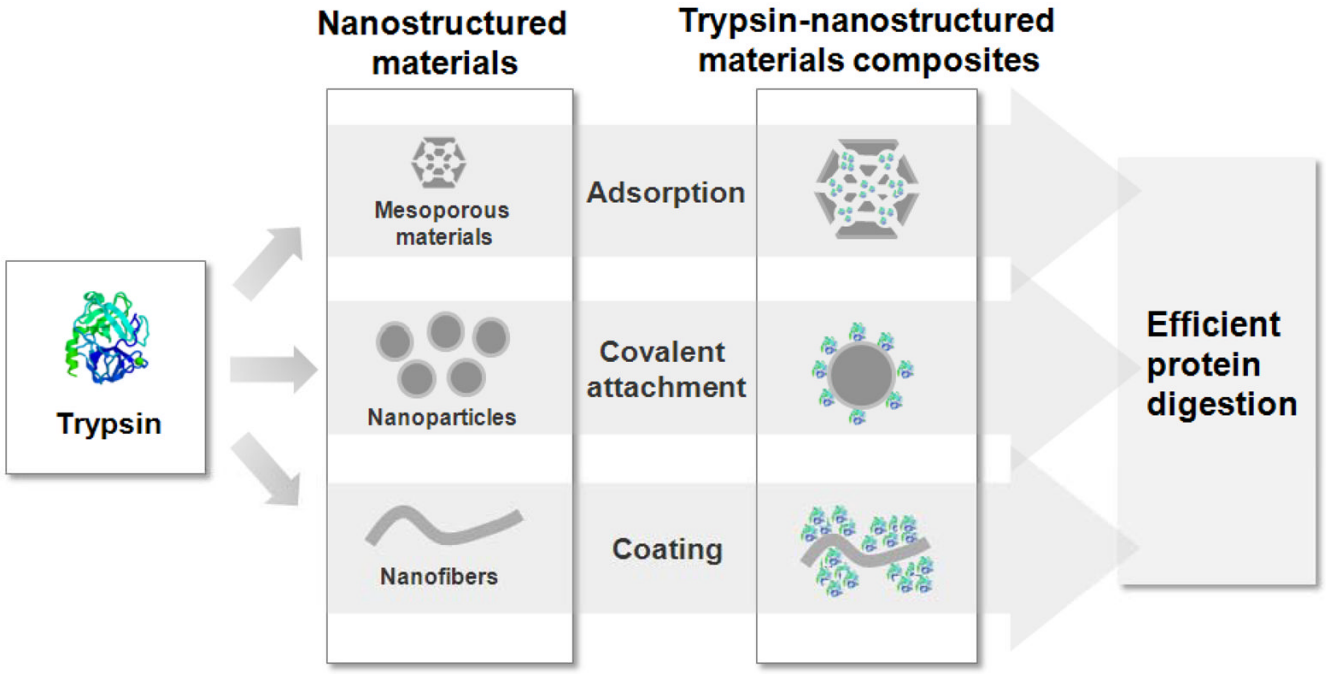

Figure 1.
Immobilization of trypsin in or on nanostructured materials such as nanoporous materials, nanoparticles, and nanofibers for efficient protein digestion.

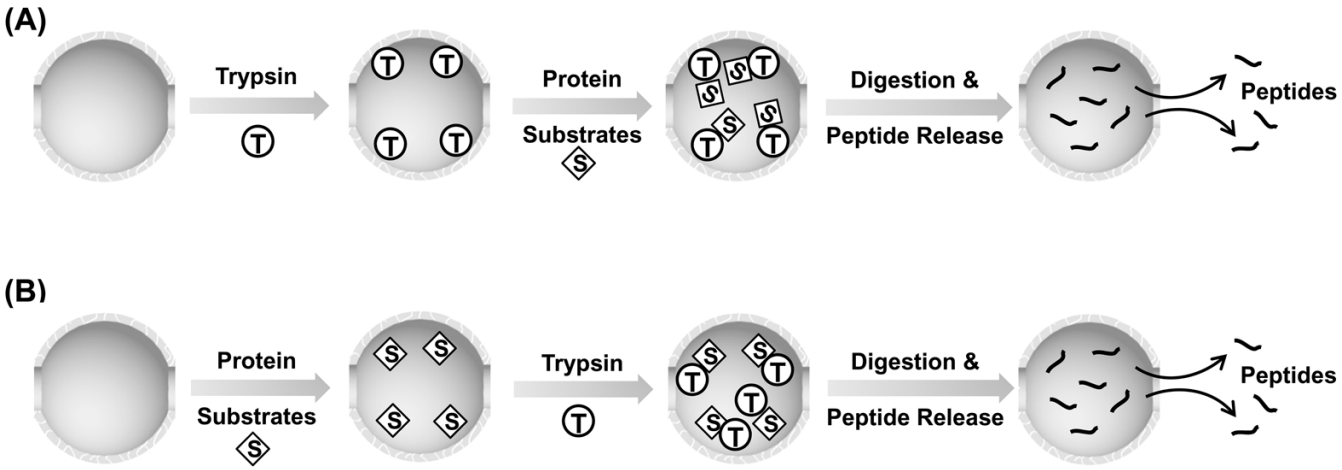

Figure 2.

Nanoporous materials as nanoscale reactors for protein digestion. (A) Trypsin adsorption was followed by the incubation in the protein solution. (B) Enrichment of proteins was followed by the incubation in the trypsin solution.

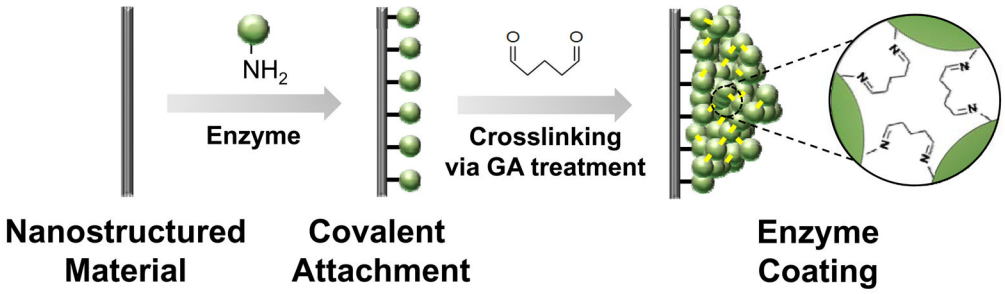

Figure 3.
Schematic for the fabrication of enzyme coating on nanostructured materials.

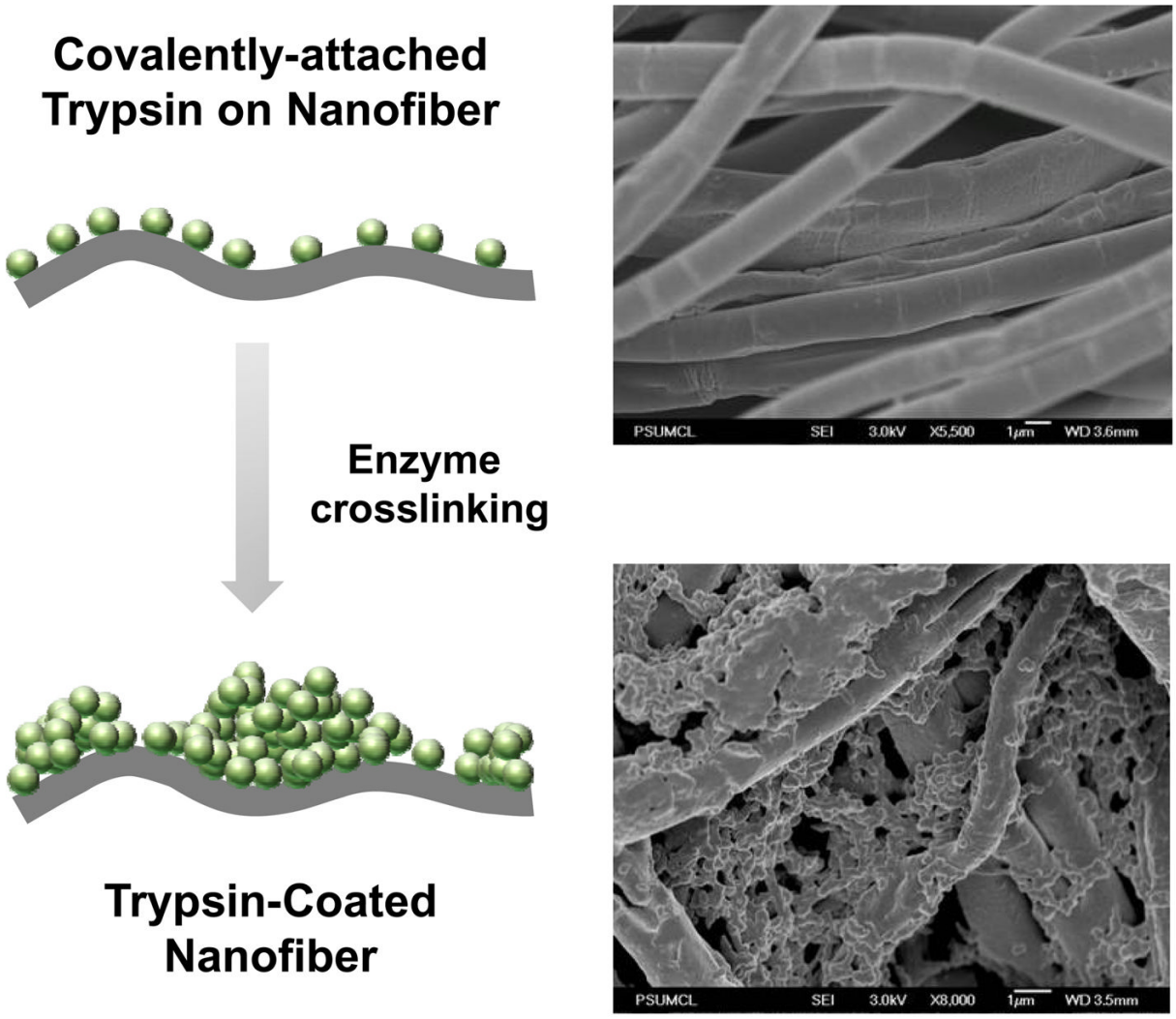

Figure 4.
Covalently-attached trypsin and trypsin coating on electrospun polymer nanofibers, and their SEM images (modified from reference [5]).

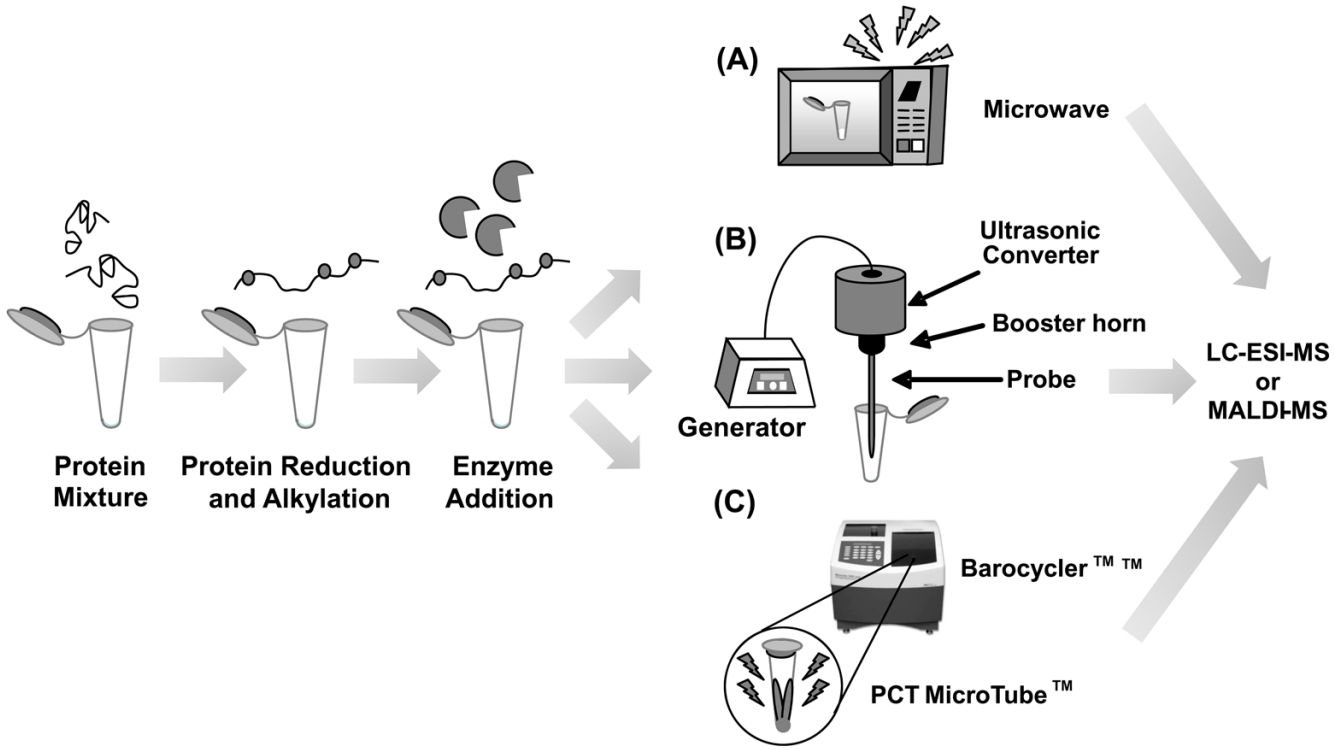

Figure 5.
Strategies for protein digestion in proteomics using ‘unconventional’ energy inputs: (A) microwave irradiation, (B) high intensity focused ultrasound, and (C) pressure-assisted digestions.

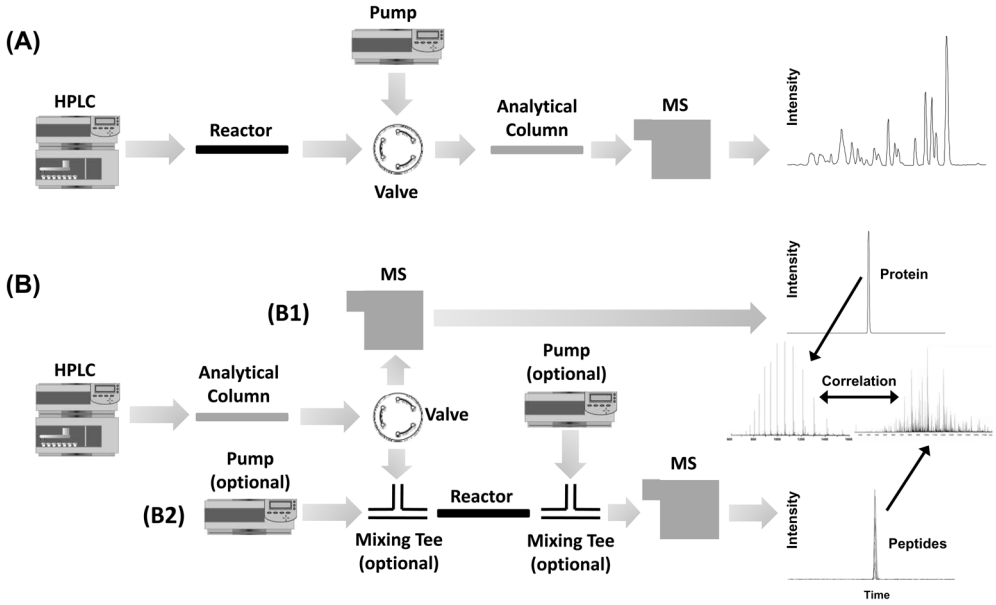

Figure 6.

(A) Pre-column immobilized enzyme reactor configuration. A protein is loaded in the reactor and digested to peptides, and then loaded in the analytical column. The valve is switched and a gradient elution is performed; peptides are separated, eluted, and detected by the mass spectrometry. (B) Post-column immobilized enzyme reactor configuration. (B1) A protein is retained in an analytical column, and then analyzed by mass spectrometry with no digestion. (B2) A protein is retained in an analytical column and digested in a post-column, and the peptides are analyzed by mass spectrometry. The protein from path B1 and its corresponding peptides from path B2 will have approximately the same retention time and elution profile enabling the correlation between them.