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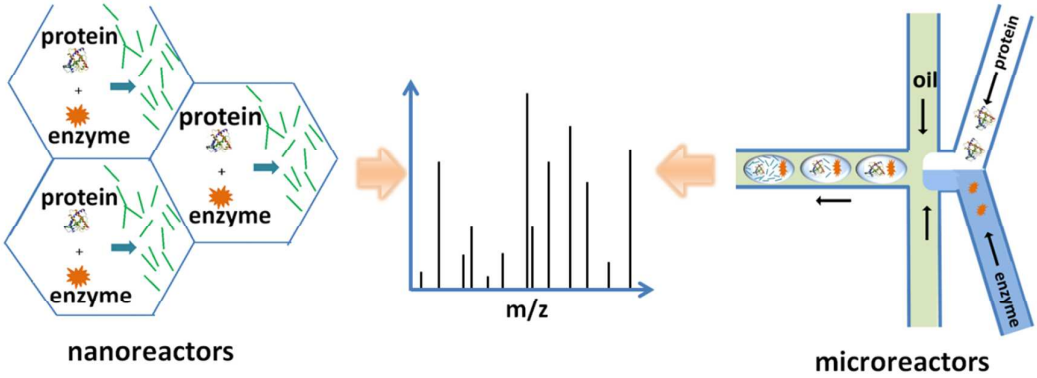
Graphical Abstract

This article reviews the recent advances regarding the development of nanomaterial-based nanoreactors and microfluidic droplet reactors and their applications in protein analysis.



[1]High-Efficiency Nano/Micro-Reactors for Protein Analysis

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Abstract

Protein analysis is always one of the key fields researchers focus on. Recent years has witnessed the rapid development of novel nanomaterials and microfluidic droplets as ideal reactors for protein analysis. This article reviews the recent advances regarding the development of nanomaterial-based nanoreactors and microfluidic droplet reactors and their applications in protein analysis, especially in proteomics. The categories, features and applications of various lately developed micro/nano-reactors have been summarized and future directions and challenges in this field have also been discussed.

1. Introduction

Proteins, macromolecules which comprise one or more long chains of amino acid residues with specific spatial conformations, are involved in diverse biological processes in living organisms. It's noted that proteins execute important functions at the cellular-level, including providing energy, maintaining cell structures, catalyzing metabolic reactions, responding to stimuli and tissue repair.[1] Therefore, protein analysis regarding their compositions, structures, identification, quantification, interactions and functions have been seen to provide valuable insights into the macromolecules themselves as well as the key biological processes they participate. However, protein analysis is still far from ideal owing to the great sample complexity, very low abundance,

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nanoscaled molecule size and limited capability of the existing techniques, especially in micron or nanometer scale. To address the above challenges, enabling protein analysis in small scale makes great sense since it not only reduces the sample consumption, but more importantly set up desirable conditions close to those *in vivo*.

Proteomics, which focuses on large-scale analysis of proteins and contributes greatly to basic understanding of gene function in the post-genomic era,[2] is attracting growing attention across the globe nowadays and urgently needs advanced analytical methods and techniques for proteins in micro-/nano-scale. To date, the most challenging hotspots of protein analysis in micro-/nano-scale include 1) pivotal proteins with post-translational modifications (PTMs) and their functions, such as glycoproteins,[3, 4] membrane proteins[5, 6] and phosphatidylinositol transfer proteins[7]; 2) new proteolysis technologies with better efficiency using capillary electrophoresis (CE) and microfluidic devices[8-17] and 3) novel functional materials as reactors for enzyme immobilization and protein extraction.[14, 18-25]

Proteins may undergo thousands of reactions including protein synthesis, protein proteolysis and enzymatic catalysis while carrying out their biological functions, which makes it necessary to introduce reactors in micro-/nano-scale for protein analysis. In this review, we summarize typical categories, features and applications of recent developed micro-/nano-reactors for protein analysis on two main aspects: 1) various types of nano-/nanoporous materials with huge surface area for enzyme immobilization and nano-confinement effect towards accelerating protein proteolysis; 2) microfluidic droplets which are fit for kinds of protein reactions with high throughput and speediness. Moreover, prospects on micro-/nano-reactors have also been proposed.

2. Functional nanoparticles based enzymatic reactors

It could be announced that the speech of Feynman in California Institute of Technology in 1959 was one of the milestone events that marked the rise of nanotechnology. Since then, this technology has attracted much attention and developed rapidly because of its broad applications. Some of the most representative works should come from Mirkin et. al. who have developed many kinds of useful nanomaterials based on gold nanoparticles (AuNPs) for biological analysis due to the good stability of Au-S bond for modification and the excellent biocompatibility of AuNPs.[26-49]

As is well known, proteomics provides evidence to elucidate virtual roles of proteins, analyzes their PTMs and discovers potential biomarkers in biological process.[1, 50-55] Sample preparation for proteomics is fundamental to the sensitivity, accuracy and robustness of overall analysis. Standard procedures of sample preparation include protein

extraction, solubilization, separation, denaturation, reduction and alkylation of cysteines and proteolysis. Among these procedures, proteolysis is one of the key procedures prior to identification for mass spectrometry (MS) based proteomics workflow. Traditional proteolysis includes in-gel digestion and in-solution digestion. In-gel digestion at 37 ℃ overnight after 1-/2-dimensional polyacrylamide gel electrophoresis is a universal applied method coupling extraction and identification by MS; in parallel, conventional in-solution digestion also requires no less than 12 hours incubation at 37 ℃ to achieve high efficacy. The main problem of both methods is the slow speed, which affects their application in large-scale protein identification. Besides the commonly used enzymes (e. g. trypsin) may induce artificial modification on substrate proteins during the long lasting digestion process.[56]

Due to their intrinsic properties such as large surface to volume ratio and good biocompatibility, functional nanoparticles are normally used as the substrates for enzyme immobilization and can be effective to overcome the shortcomings of conventional proteolysis methods. Further it's easy to modify the surface of nanoparticles with functional groups, which can form covalent bonding with enzyme. In recent years, many enzyme immobilized reactors based on functional nanoparticles have been designed and applied in proteolysis of real-case bio-samples.[57-64].

Liu et al. developed a gold nanoparticle (AuNPs)/ poly(diallyldimethylammonium chloride) (PDDA) multilayered microfluidic enzymatic reactor.[62] By using layer-by-layer self-assembling technique, gold nanoparticles with negative charges can interact with positively charged PDDA to form a multilayer network. Given the good biocompatibility, AuNPs/PDDA network preserved enzyme bioactivity. Therefore, such a system can be utilized in highly efficient proteolysis. Using this microchip reactor, down to femtomole proteins were digested and successfully identified by matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF MS/MS). Combined with liquid chromatography-electrospray ionization- tandem mass spectrometry (LC-ESI-MS/MS), the AuNPs/PDDA multilayered enzymatic reactor has been applied in protein extracts from mouse macrophages. The experimental data show that 497 proteins have been successfully identified. Another type of important functional nanomaterials is magnetic nanoparticles. Due to their magnetic features, magnetic nanoparticles allow the ease in immobilization and separation. Zhang's group fabricated a magnetic silica microspheres-based protease microreactor.[57] By the application of strong magnetic field, magnetic silica microspheres were locally packed into the microchannel. Copper ion could couple with carboxyl group, and enzyme was then adsorbed onto the copper ion. The proteolytic microreactor was applied in the fast digestion of cytochrome c (Cyt-c) and bovine serum albumin (BSA) as model proteins. The digestion products were

characterized using MALDI-TOF/TOF MS with sequence coverage of 77% and 21% observed, respectively. This microreactor was also applied to the analysis of one RPLC fraction of rat liver extract. After a database search, 23 unique peptides corresponding to 7 proteins were identified when one RPLC fraction of rat liver extract was digested by the microreactor. This opens a route for its future application in top-down proteomic analysis. Given the low efficiency of conventional tryptic method in case of highly complex samples, Qin et al. established a tryptic reactor, by entrapping trypsin in hairy noncross-linked polymer strands, which covered magnetic nanoparticle core.[63] The preparation of hairy noncross-linked polymer chains hybrid magnetic nanoparticles (PHMN) is illustrated in Fig. 1. The initiator, 3-(2-bromoisobutyramido)propyl(triethoxy)-silane (BIBAPTES) was first synthesized, then the magnetic nanoparticles reacted with the triethoxysilane group of BIBAPTES. Surface initiated atom transfer radical polymerization (SI-ATRP) grafting was carried out between immobilized initiator and N,N,N',N'',N''-pentamethyldiethylenetriamine. Crude products were treated with ethylenediamine to obtain aldehyde groups on PHMN. At last, trypsin was immobilized on PHMN. Using multiple reaction monitoring (MRM) method based on PHMN-trypsin, absolute protein quantification of enolase was realized with increasing digestion efficiency compared to nanoparticles under conventional preparation procedures. For complex protein samples from *Thermoanaerobacter tengcongensis* (TT), obviously increased digestion efficiency and completeness were demonstrated by 27.2% and 40.8% increase in the number of identified proteins and peptides as well as remarkably reduced undigested proteins residues compared with that obtained using conventional free trypsin digestion. Li et al. demonstrated a regenerable protease microreactor by immobilizing enzyme through chelated adsorption with metal-ion on chip.[64] The metal chelating agent was immobilized onto the surface of magnetic silica microsphere (MS microspheres). A strong magnetic field was utilized to pack the MS microspheres into the microchannel. Model proteins Cyt-c, BSA, and myoglobin (MYO) were characterized with MALDI-TOF/TOF MS. The results were summarized in Table 1.

3. Nanoporous material based enzymatic nanoractors

Spatial effect is an important factor affecting the efficiency of chemical reactions, majority of which are carried out at the micro-/nano-scale. To meet with the practical needs, efforts have been committed to the development of various kinds of new materials to improve the reaction efficiency. To our best knowledge, the first works demonstrating such novel application based on spatial effect were from Qu et. al. and Wu et. al.. They developed sol-gel based functional microfluidic devices as enzymatic reactors. Qu et. al. designed and synthesized crafted

copolymer to obtain hydrophobic PMMA microchannel for entrapping enzymes.[65] Then this reactor was used for proteolysis with high efficiency. By using poly(dimethylsiloxane) (PDMS) casting technique, Wu integrated stainless steel tubing (SST) with microchannels to fabricate the microfluidic devices based enzymatic reactor with feasibility of on-line protein analysis thanks to the replaceable tip and the embedded SST electrode.[66] Furthermore, they also developed such reactors with titania and alumina modification.[67] All these reactors were suitable for high-throughput protein identification. In recent years, porous nanomaterials emerged and quickly became one of the hotspots of contemporary scientific research. For example, Zhao et. al. were famous for their representative porous silica materials, such as SBA series[68-94] and FDU series[95-99]. Most biological applications of these materials are in the field of proteomics research. Porous materials have been of widespread use because of the features of high surface areas, tunable surface chemistry, ordered and well-controlled size distribution as well as chemical stability.[100, 101] Consequently, enzymes could be trapped in the cores via physical adsorption instead of chemical coupling, to retain high catalytic activity. The basic working principle of porous materials based enzymatic reactor can be illustrated in the Fig. 2.[102] Due to the fast enrichment of proteins and enzymes into the nanopores, the reaction efficiency can be greatly improved benefiting from highly increasing local concentrations. Various types of porous materials, including mesoporous silicates[103-122] and hydrogel[123] are developed as new enzymatic reactors. These porous structures offer possibility for enzymes or other molecules to be entrapped.[67, 124]

According to the definition from the International Union of Pure and Applied Chemistry (IUPAC), solid materials can be divided into three categories according to the pore size, including microporous materials with diameter less than 2 nm; mesoporous materials with aperture between 2-50 nm; macroporous materials with pore size of greater than 50 nm. Our previous work studied the surprising phenomenon that both preload of proteases and protein digestion process performed with fast kinetics in nanoporous materials.[113] A model of modified sequential proteolytic mechanism was developed to imitate the kinetics of proteolysis with MYO as an example, while angiotensin 1 and ACTH (1-14) as model peptides. The results of our research showed that the generated peptides in proteolysis could be trapped within a confined space formulated by the pores of nanomaterials and prepared for further digestion to the final products. Meanwhile, proteins into the porous cavity during the digestion process would have an impact on the first step of proteolysis. The present model has the potential to be used for the study of enzyme reactions in cells.

3.1. Microporous materials

Zeolites, molecular sieves and activated carbon are typical representatives of microporous materials. Such materials tend to have uniform and ordered pores, continuous network skeleton, high surface to volume ratio and a large number of acidic active sites distributed on the surface of the channels. In recent years, the composition of micropores molecular sieve has evolved from inorganic components to organic ingredients. Huang et al.[125] modified poly(methyl methacrylate) (PMMA) microchannels with zeolite nanoparticles after introducing silanol functional groups for the first time. Then trypsin could be stably immobilized in the channel, which was attributed to silica sol-gel matrix. The gel network was fabricated through silicon-oxygen-silicon bridged to silanol groups. Standard proteins such as BSA and Cyt-c were used for a less than 5 s proteolysis reaction and MALDI-TOF MS identification to evaluate the performance of this microreactor. The results were summarized in Table 2. The microreactor could be used repeatedly and preserved for 1 month at 4 ℃.

3.2. Mesoporous materials

Represented by SBA-15[104-112], mesoporous materials are more conducive to the entry of proteins and other biological macromolecules, as well as modification of various types of active groups, compared to microporous materials.[126, 127] Thus, mesoporous materials own broader application prospects. Li et al. synthesized rod-like SBA-15 mesoporous materials to study the adsorptional performance and catalytic activity of Porcine pancreatic lipase (PPL).[109] The experimental data clearly showed that PPL was adsorbed into the channels of SBA-15 and the ordered mesoporous structure of SBA-15 after adsorption of PPL was retained. The immobilized PPL within SBA-15 showed excellent adaptability at higher pH and better thermal stability comparing with free PPL (see Table 3). Qiao et al. described a mesoporous silicates based nanoreactor for efficient proteolysis.[118] With cyano-functionalization, trypsin displayed a preference of adsorption. Compared to conventional in-solution digestion, this nanoreactor displayed a much higher tryptic digestion efficiency owing to the mesoporous structure for nanoscopic confinement and local enrichment (Fig. 3).

Mesoporous materials can not only act as the site for enzymatic reaction but also be used for functional proteins enrichment. Functional proteins exhibit great significance in biological processes. Many diseases are associated with the functions and abundance of especially modified proteins. Also, these vital proteins play important roles in cell related functions. For example, acetylated proteins can precisely adjust and control various pathways within cells to execute the instruction of genes; methylated histone owns the basic functions of the formation of

heterochromatin, X chromosome inactivation, gene imprinting and transcription regulation. However, almost all of the functional proteins are in low abundance in cells. Thus, it's difficult to quantify and identify these proteins. For example, glycosylation of protein, as attachment of glycans to asparagine (N-linked glycans) and serine or threonine (O-linked glycans), is an important posttranslation modification interrelated with a number of biological processes. However, glycoproteins are difficult to be identified due to the low abundance of glycopeptides and the ion suppression effect caused by the nonglycopeptides.[128-135] Mesoporous silica has the advantages of large accessible porosity and high surface area so that can be used as an enzyme reactor after entrapping specific protease. Using boronic acid functionalized mesoporous silica, Xu et al. presented a nanoreactor for detection of glycopeptides degradated from glycoproteins by enriching the glycopeptides on the functionalized materials for the first time, which could be explained that there was chemo-affinity between glycopeptides and boric acid species.[136] In comparison to direct (traditional) analysis, this novel method enabled 2 orders of magnitude improvement in the detection limit of glycopeptides and and the recovery of the enriched glycopeptides is up to 83.5%. Phosphorylation is also an important protein modification, which is a reversible reaction to regulate a majority of biological processed, involving cellular growth and metabolisms. However, the very low concentrations of many phosphoproteins make it tough to analyze these proteins.[137, 138] Han et al. synthesized mesoporous Fe_2O_3 microspheres with uniform particle size at around 3 μm while inter-particle pores at 48 nm.[139] It was the first time that this kind of material was applied for the enrichment of phosphopeptides with MALDI-TOF MS detection. Phosphopeptides can be selectively detected with high intensity in MALDI-TOF mass spectrometry. Elimination of "shadow effect" was observed by using mesoporous Fe_2O_3 microspheres, and the detectable limitation is 5×10^{-10} M.

3.3. Macroporous materials

Macroporous materials have attracted growing concern of scientists by virtue of its excellent mass transfer performance and good protein adsorption capacity. For example, fiberglass-based nanoreactors present advantages of regenerability, feasibility and well performance.[140-143] Bao et al. coated a piece of glass fiber with graphene oxide and chitosan.[140] Then the prepared trypsin-immobilized glass fiber was inserted into microchip channel to obtain porous enzymatic reactors. The bioreactor was used for tryptic digestion of series of standard proteins within less than 10 s and then identified by MALDI-TOF MS. The performance of this novel bioreactor was comparable to the conventional 12 h in-solution digestion. Fan et al established a microchip based bioreactor for rapid microfluidic

proteolysis. A poly(urea-formaldehyde)-coated fiberglass core was first prepared, trypsin was covalently bonded to the core via condensation reaction. Then the trypsin-immobilized fiber was inserted into the main channel. Using the established platform, standard proteins of hemoglobin and lysozyme were digested efficiently and feasibly.[82] Qian et al. developed a phospho-directed nanoreactor with alumina-functionalized macroporous ordered silica foams (Al-MOSF).[144] The large pore size and high pore volume provided possibility for rapid in situ digestion. Moreover, thanks to the reaction between Al species and phosphorous groups, phosphopeptides, specific products of the proteolysis, could be isolated from the nonspecific peptides by enrichment on Al-MOSF reactor. Consequently, this strategy has the potential to be applied in the identification of phosphoproteins in complex biological samples.

Above all, various enzymatic reactors have been developed as an effective way to overcome the shortcomings of the traditional proteolysis methods. Here, we summarize the advantages and disadvantages of both novel enzymatic reactors and conventional enzymatic reaction as shown in Table 4.

4. Droplet-based microreactors

Microfluidics handles fluids that are geometrically constrained to a small, typically sub-millimeter scale. In 1990, Manz et al.[147] first proposed the definition of miniaturized total analysis system (μ-TAS), which aims to integrate multiple components of a room-sized laboratory, including sample pretreatment, separation, detection and other systems into one chip. Since then plenty of microfluidic platforms with low consumption of samples and laboratory space, well control of flow characteristic[148] have been established to provide solutions in fields of DNA analysis[149], capillary electrophoresis[150], cellular analysis[151] and so on, greatly pushing forward the development of microfluidics.

Despite the conventional continuous-flow microfluidics, droplet microfluidics, which involves droplet arrays formed by two immiscible fluids of a volume from fL to nL in a microfluidic chip, was put forward in 2001.[152]Generally an aqueous phase and an oil phase are introduced into the chip via different inlets and compartmentalized into water-in-oil (o/w) or oil-in-water (w/o) droplets at a T-junction or a flow-focusing confluence owing to the surface tension and the shear force functioning together. As a subcategory of microfluidics, droplet microfluidics not only shares the inherent merits mentioned above, but also exhibits peculiar advantages as

follows:[148, 153, 154] 1) the fast mixing and mass transfer occurring within droplets helps promote reactions inside; 2) elimination of sample diffusion and dilution guarantees stable conditions; 3) dispersion of droplets in the continuous phase prevents samples from adsorbing on channel walls, thus eliminating residence time distribution and cross-contamination of samples and 4) high frequency of droplet generation enables high throughput analysis without enlarging the device. All these bright features allow microfluidic droplets to be an ideal platform for a variety of reactions. Over the last decades besides nanoparticle synthesis[155-157], chemical reactions[158] and cellular analysis[159], applications of mirodroplets in protein analysis have also verified the great potential of droplet microfluidics, such as protein digestion[160], protein crystallization[161, 162], enzyme kinetics study[163, 164], drug screening[165] and so on.

4.1. Droplet-based reactors for rapid protein reactions

When travelling through the straight channels the fluid inside microdroplets is folded and stretched by the recirculating flow, while in the winding portions reorientation of the fluid is induced by chaotic advection, accelerating the reaction among the solutes inside.[166, 167] One convincing instance is that Ismagilov et al. achieved better than millisecond resolution in measuring the rapid single-turnover kinetics of ribonuclease A, with microdroplets where the products were quickly yielded and well retained and localized for the subsequent fluorescence detection.[163] Hollfelder and co-workers applied microdroplets to analyzing the kinetics of a cell-based enzyme, alkaline phosphatase, which was expressed in Escherichia coli cells and presented in the periplasm, and observed kinetic behavior similar to that in bulk solution.[168]

Liu's group established an integrated platform (Fig. 4) for protein analysis based on water-in-oil droplets containing standard protein fractions from high performance liquid chromatography (HPLC) and enzyme solution, where the digestion process took place rapidly and the peptides were detected on-line by ESI-MS sequentially.[160] In this platform, droplets not only served as efficient microreactors which cut down the reaction time remarkably from 16 h to 2 min compared to the conventional in-solution proteolysis method, but also played the role of an interface between the separation and the detection systems, with high level of automated handling.

4.2. Droplet-based reactors associated with concentration gradient system

Generation of concentration gradients is a vital process in plenty of biochemical analysis, such as optimization of protein crystallization conditions,[161, 162] enzyme inhibitor assays,[169, 170] protein expression,[164, 171] drug screening[172]

and so on. Conventional methods dependent on stepwise dilution of a concentrated stock solution suffer from high time and reagent consumption in addition to enormous manual workload. In recent years, energetic efforts have been made to create small length resolutions of gradients in microfluidic droplets serving as biological reaction vessels, which not only conquer the limitations mentioned above, but also open up the possibility for biological mimesis thanks to the closer size of droplets to cells.[148]

In a typical protein crystallization study, water-in-oil droplets encapsulating buffer, polyethylene glycol (PEG) NaCl solution and a concentrated lysozyme solution performed as nanoliter-size reactors to achieve protein crystallization.[161] By adjusting the volumetric flow rates of the stock solutions, different compositions of the droplets, in other words, different crystallization conditions were successfully obtained. This system had a low consumption of protein samples of 4 nL for each trial as well as a high throughput of hundreds of trials with 1~2 min.

Whitesides et al. designed a pyramidal microfluidic network to generate a linear concentration gradient based on controlled diffusive mixing of laminar flow fluid by splitting, mixing and recombination repeatedly.[173, 174] They made an analogy with the fluid flow and an electronic circuit to calculate the concentration gradient at the outlet. Fig. 5 shows a network where B was defined as the order of the branched system containing n vertical channels ($B = n$) and V as the series number of the vertical channel, from V=0 to V=B -1. Upstream fluid flow splits at each branching point with a normalized splitting ratio of $[B - V]/[B +1]$, derived as below. In this network, the length of the horizontal channels (R_H) are negligible compared to the length of the vertical ones (R_V), therefore resistance to fluid flow, which scales linearly with the length of the channel in Poiseuille flow, are assumed to be contributed only by R_H approximately. Supposed that both the resistance and the volume throughput remain the same in all vertical channels within each branch, portions of the stream equal to $[B - V]/[B +1]$ and $[V + 1]/[B + 1]$ to the left and the right respectively, giving a splitting ratio at each branching pointing of $[B - V]/[B +1]$. With a 2-inlet network of n vertical channels and concentrations of inlet solutions being c_1 and c_2, a linear concentration gradient could be obtained in the nth branched system, from $c_{V=0} = c_1$ to $c_{V=n-1} = c_2$. Huck' group further developed this pyramidal gradient-generator to monitor an enzyme activity assay by coupling it with the microdroplet technique.[165] As illustrated in Fig. 5 (c) , first a linear concentration gradient of R_1, fluorescein diphosphate was yielded in four streams in section S_1, after which R_2, E. coli alkaline phosphatase was introduced into the channel to mix with R_1 in section S_2. The two reactants were then compartmentalized into water-in-oil droplets where the product fluorescein was formed and detected along section S_3. More kinetics information associated with different ratios of substrate concentration vs. enzyme concentration was available by varying flow rates of R_1 and R_2. With

less sample consumption, contamination of reagents and manual labor, values that were in close agreement with data obtained with a conventional assay in a 96-well plate were attained.

Fang and colleagues established a DropLab system in which droplets were produced without complicated microchannel networks.[162] By rotating the slotted-vial array (SVA), a capillary tip entered and aspirated specific reagent solutions and oil stored in different vials successively, resulting in a droplet array of different aqueous compositions segmented by oil phase within the capillary. Either deposited on an oil-immersed plate or not, droplets were then incubated to conduct parallel reactions. In a case that numerous trials were to be performed, a 384-well plate might be used to substitute the SVA with other operations similar to the above ones. With the volumes precisely controlled by a program, the droplab system presented brilliant characteristics such as automatic handling, controllability of size and composition, minimized sample consumption in large scale screening in spite of relatively low throughput. In a typical trial of lysozyme crystallization, 50 12-nL droplets representing 50 crystallization conditions were assembled in a10-cm long capillary within 22.5min.

Flow injection gradient (FIG) technique, which relies on the control of axial dispersion of injected sample zone in a flowing carrier stream, was integrated with droplet-based microfluidics to generate large-scale concentration gradient in nanoliter-scale droplets by Fang's group for the first time.[169] As demonstrated in Fig. 6, after inhibitor and carrier solutions were injected into the chip by linearly moving the translational stage operated with a computer program, dispersion of the sample occurred forming a peak-shaped concentration distribution in the axial direction of the winding channel. At the confluence of the microchannels downstream, fixed amount of enzyme and substrate solutions with various concentrations of the inhibitor were compartmentalized into a series of water-in-oil droplets, where the inhibition reactions proceeded at high speed. By recording fluorescence signals of the droplets online, more than 240 enzyme inhibition concentrations spanning 3~4 orders of magnitude could be screened with an extremely low inhibitor injection volume of 16 nL. within 2.5 min, capable of high throughput and minimal sample consumption.

In a recent study, Vanapalli designed a concentration gradient generation device via dilution method.[170] As illustrated in Fig. 7, when travelling through the microfluidic channel, sample plug was trapped in the loops according to the flow-resistance mechanism and formed a static drops array (SDA). Then the diluting plug injected next flowed past the SDA and the oil film sandwiched between the two aqueous phases broke inducing coalescence, which led to exchange of diluting solution for sample. As the diluting plug moved forward, sample accumulated at its front producing a concentration gradient within the plug. With some other reagents and an oil phase injected to the device, an enzyme inhibition assay based on microdroplet reactors containing reagents of diverse concentrations

was carried out. It turned out that this system possessed not only a tunable dilution range by altering parameters such as the carrier fluid flow rate, volume of diluting plugs, and stationary drops, but also concentration gradients with high resolution and good reproducibility.

4.3. Droplet-based reactors for protein expression

Synthesis of biological molecules such as adenosine triphosphate (ATP), deoxyribonucleic acid (DNA) and proteins, which is essential for creating artificial cells, has also benefited from microfluidic droplets as a consequence of their uniform size at the micron scale and controlled conditions inside.[148] Edel's group investigated the feasibility of microfluidic droplets serving as *in vitro* compartments containing few or individual single cells and their applicability in high-throughput screening by quantifying the protein expression.[171] Variation of the cell occupancy in each droplet was enabled via regulating the flow rates of the two fluids which consisted of Luria–Bertani broth (LB medium) only and *E. coli* cells in LB medium, respectively. Another droplet-based microfluidic system combining two off-chip and six on-chip procedures with re-injection and electrocoalescence of droplets was built to study the cell-free protein expression.[164] And the capability of this system was exemplified by conducting *in vitro* transcription and translation (IVT) of *cot*A laccase genes and an activity assay of the translated protein (laccase).

5. Conclusions

In conclusion, the development of nano-/micro-reactors and their applications in protein analysis, especially in proteomics have been presented in this review. These reactors for protein proteolysis possess three outstanding advantages including 1) reducing autolysis of protease and retaining its activity; 2) enhancing enzymatic kinetics and decreasing digestion time and 3) combined with microfluidics or microcolumns, it is easy to couple these reactors with separation system for large-scale on-line MS analysis of proteins. Despite that various reactors with excellent characteristics such as ordered reaction spaces, rapid mass transfer and heat exchange have been developed to meet different research needs, there is still a long way to invent ideal carriers in the future research to obtain higher digestion efficiency and feasibility in complex biochemical reactions. What's more, in order to develop enzyme reactors at the commercial level, reaction engineering with microfluidics is needed as a solution. As for microfluidic droplets, the features of uniform size, rapid mixing, little cross-contamination, high throughput, low consumption of samples and ease of manipulation have made them ideal reactors for protein analysis, which has been evidenced by applications in the fields of protein digestion, protein crystallization, enzyme kinetics study,

drug screening protein synthesis and so on. We may expect microdroplet reactors to have a more profound impact on the protein research by being coupled to a diversity of analytical techniques and providing insights into real-case biological samples.

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Figure Captions

Fig. 1 Overview of preparation of PHMN-Trypsin. Reprinted from Ref. 63, with permission.

Fig. 2 Illustration of the glycol-directed nanodevice. Reprinted from Ref. 102, with permission

Fig. 3 a) Schematic representation of in-nanopore substrate entrapment, in situ proteolytic digestion, and subsequent MS identification. b) Pore size distribution of CNS. Reprinted from Ref. 117, with permission.

Fig. 4 Scheme of the platform integrating HPLC, droplet-based microreactor and ESI-MS for protein analysis. Reprinted with permission from Ref. 159.

Fig. 5 a) Schematic design of the pyramidal microchannels. b) Equivalent electronic circuit model of the pyramidal microfluidic network. Reprinted with permission from Ref. 172. c) The droplet-based microchannels for generating concentration gradient. Reprinted with permission from Ref. 164.

Fig. 6 (A−C) Schematic diagram of the generation of droplet array with concentration gradient by integrating FIG technique with droplet-based microfluidics. (D) CCD image of the generation of droplet array with concentration gradient taking a brilliant blue solution as a model sample. Reprinted with permission from Ref. 168.

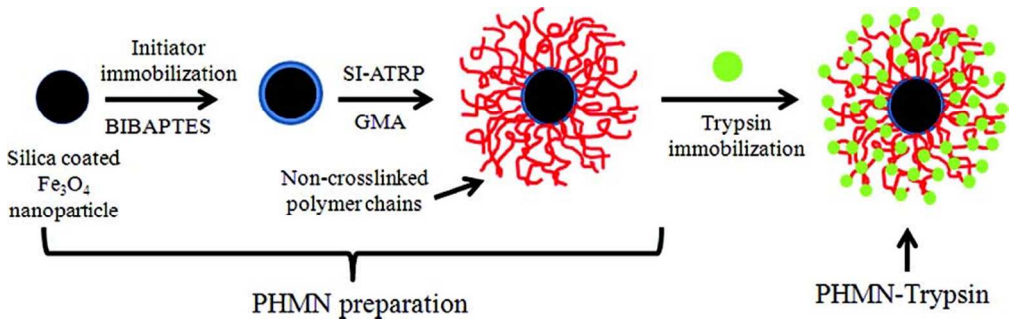
Fig. 7 Generation of concentration gradient. a) Schematic design of the microfluidic device: the red dot indicates the location where concentration of sample was measured in the mobile droplets. b) Snapshots taken in a representative experiment using black dye as sample. Reprinted with permission from Ref. 169.

Table 1. Summary of MALDI-TOF MS Results Obtained from 5-min Digestion with Trypsin-Immobilized Magnetic Nanoparticles Compared with 12-h In-Solution Digestion. Reprinted from Ref. 64 with permission.

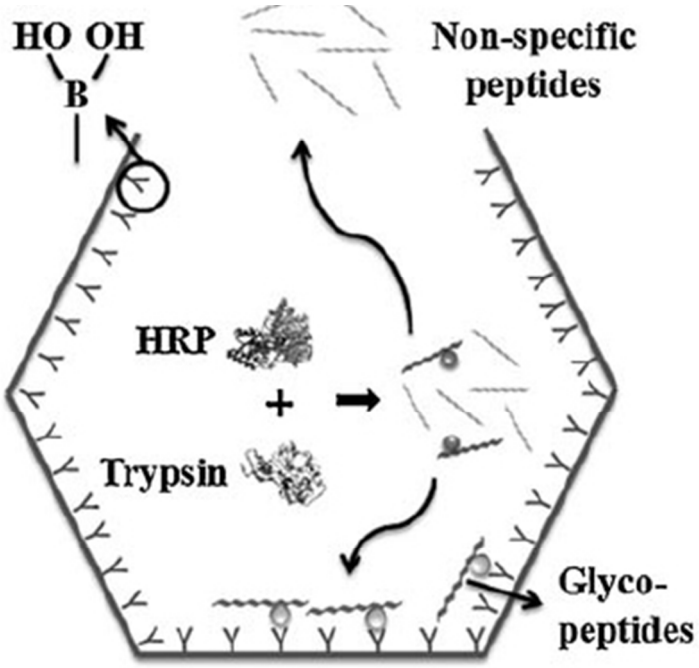
Table 2. MALDI-TOF-TOF-MS results of digests by trypsin-immobilized on-chip microreactors. Reprinted from Ref. 125 with permission.

Table 3. The activity of PPL before and after immobilization at different pH. Reprinted from Ref. 109 with permission.

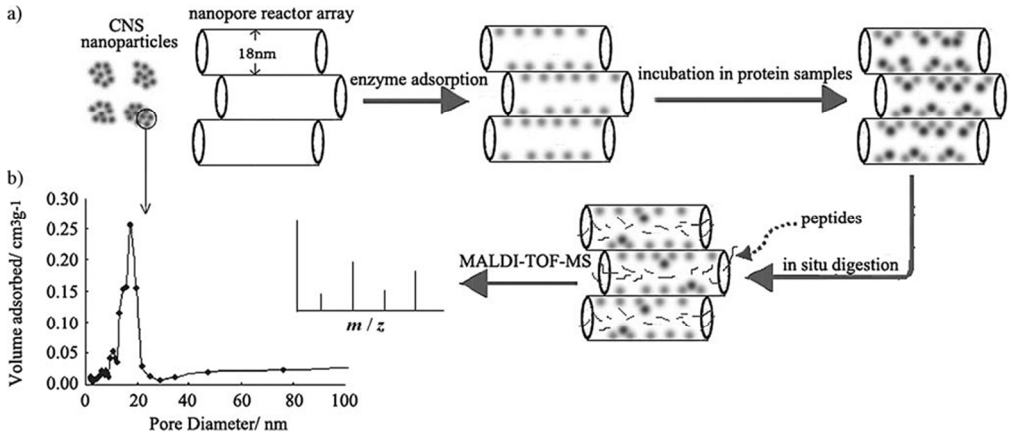
Table 4. Summary of the features of novel enzymatic reactors *vs* conventional enzymatic reaction methods.



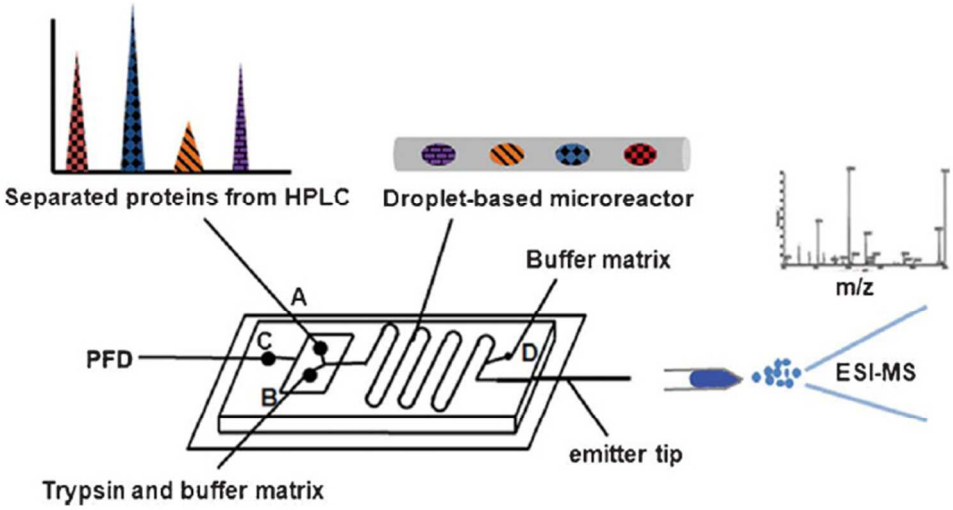
84x26mm (299 x 299 DPI)



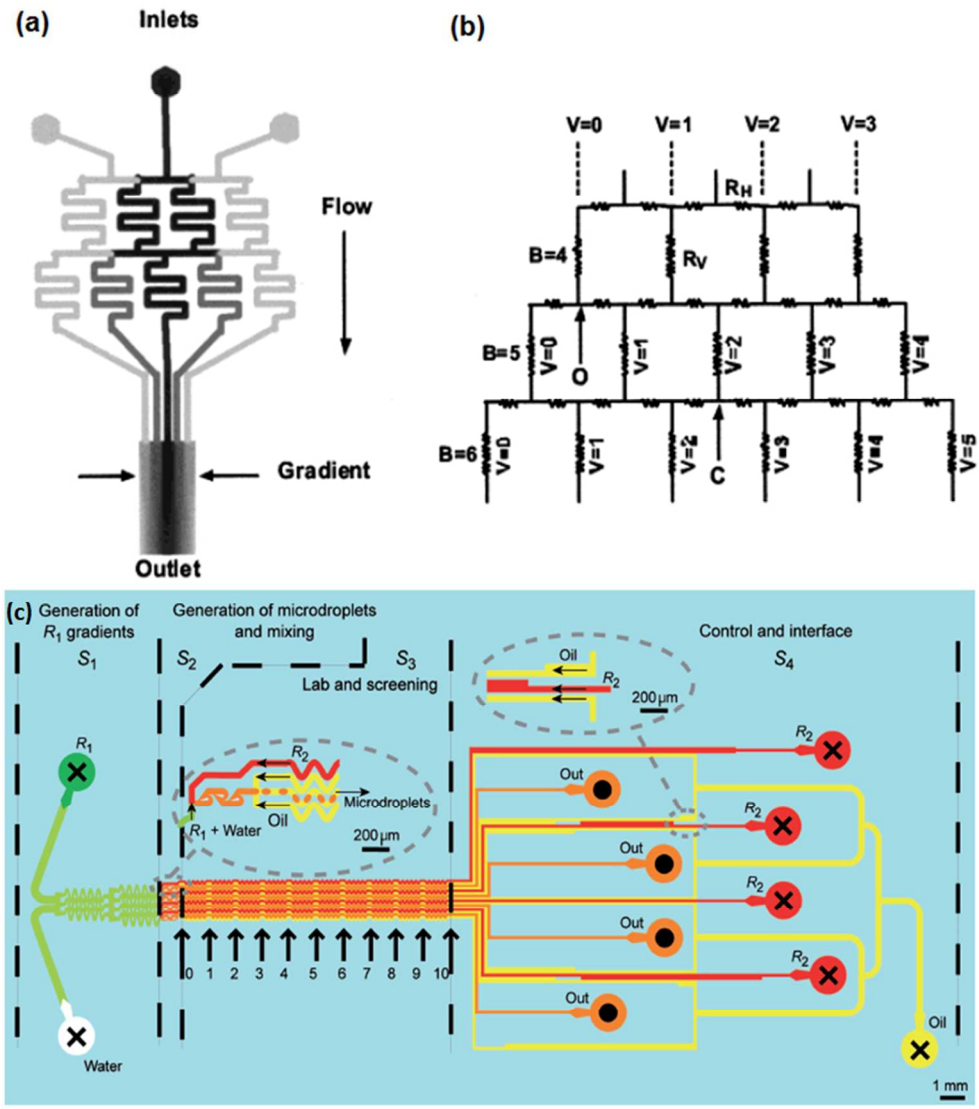
98x90mm (95 x 95 DPI)



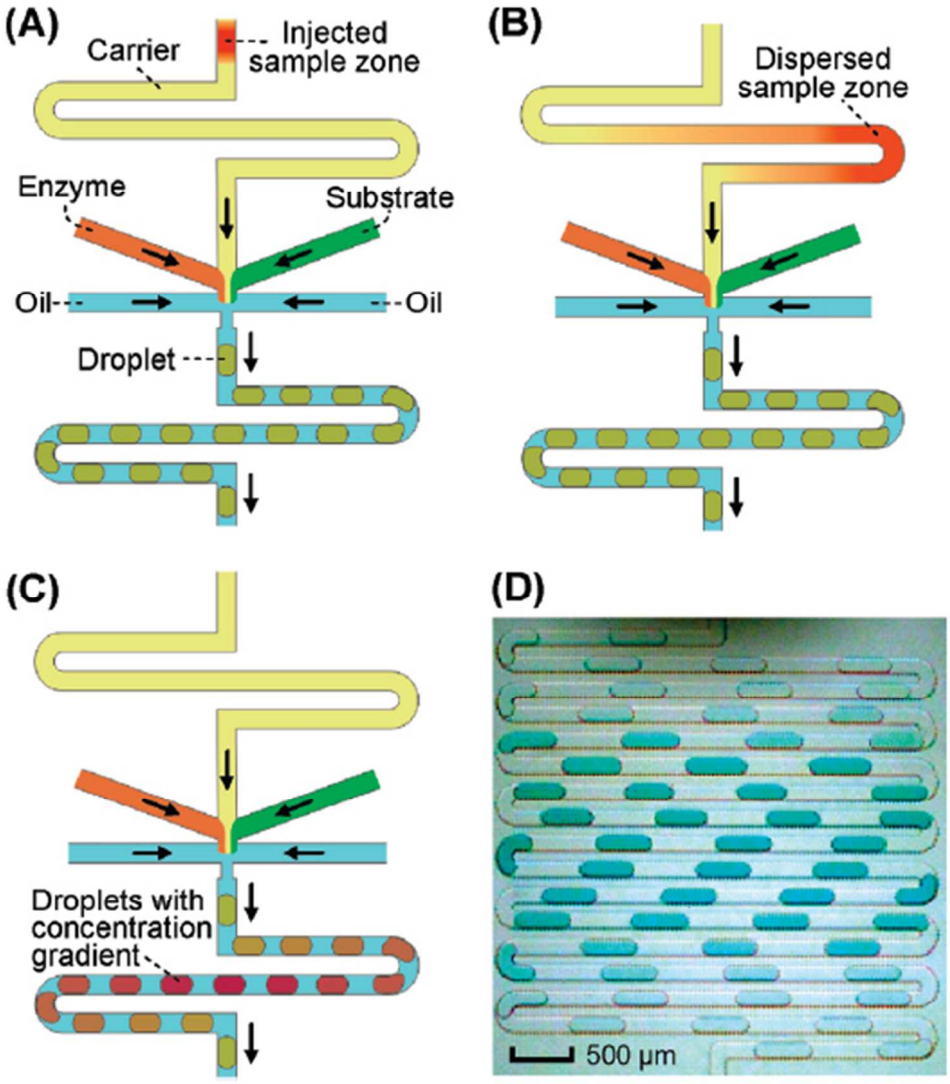
129x55mm (199 x 199 DPI)



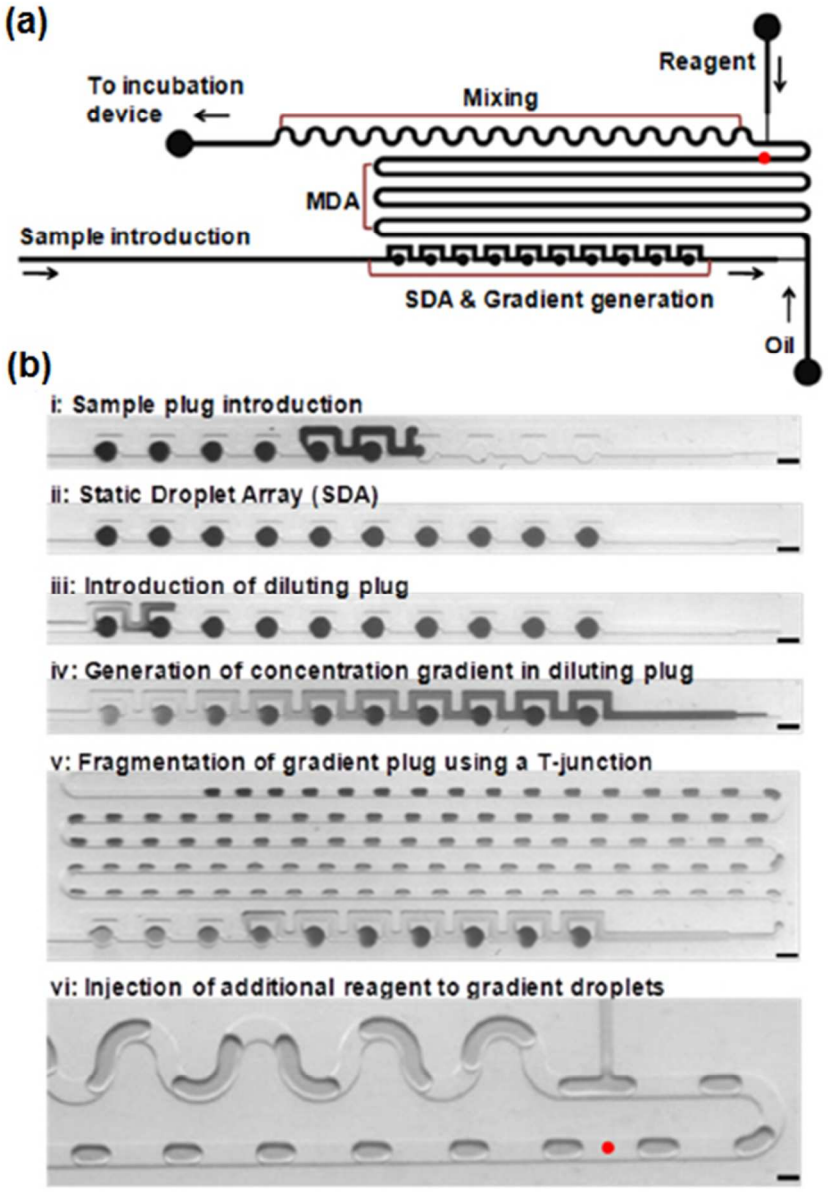
269x144mm (72 x 72 DPI)



243x271mm (95 x 95 DPI)



148x164mm (95 x 95 DPI)



120x170mm (95 x 95 DPI)

protein	MYO		Cyt-C		BSA	
	magnetic nanoparticles	in-solution	magnetic nanoparticles	in-solution	magnetic nanoparticles	in-solution
Amino acids identified	138	115	80	80	283	253
Sequence coverage (%)	90	75	76	76	46	41
Peptides matched	15	11	13	14	30	24

338x55mm (96 x 96 DPI)

Protein	Cyt-C	BSA
Amino acids identified	78	234
Total no. of fragments	22	71
Sequence coverage (%)	77	39
Digestion time	<5 s	<5 s
Peptides matched	11	38
Protein MW	12384	66000

168x57mm (96 x 96 DPI)

pH	Activity of free PPL ($U\,g^{-1}$)	Activity of immobilized PPL[a] ($U\,g^{-1}$)	Activity recovery[b] of immobilized PPL (%)
6	58.19	24.59	42.26
7	80.67	33.42	41.43
8	72.32	34.03	47.05
9	21.92	8.69	39.64

[a] PPL was incubated in the buffer solution for 24 h.

[b] The activity of the immobilized enzyme ($U\,g^{-1}$) divided by the activity of the free enzyme in solution before immobilization ($U\,g^{-1}$)

147x53mm (96 x 96 DPI)

	Novel Enzymatic Reactors					Conventional enzymatic reaction	
Categories	Functional nanoparticles based enzymatic reactors		Nanoporous material based enzymatic nanoractors				
	Gold nanoparticles[61]	Magnetic nanoparticles[57, 63, 64]	Microporous[125, 136, 139]	Mesoporous[104-112, 118, 126, 128-135, 137, 138, 145]	Macroporous[140, 142, 143, 146]	In-gel digestion	In-solution digestion
Features	Excellent biocompatibility	Easy to separate	A large number of acidic active sites	More conducive to the entry of biological macromolecules, as well as modification of active groups, compared to microporous materials		Robust for subsequently extraction and identification by MS; High efficiency of enzyme digestion	
			Uniform and ordered pores; high surface to volume ratio				
Problems	Not enough digestion efficiency for enzymatic reaction in biosystems; Limitation in complex biochemical reactions except for tryptic reaction					Slow speed; Induced artificial modification on substrate proteins by trypsin	

237x129mm (96 x 96 DPI)