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Trypsin immobilization in ordered porous polymer membranes for effective protein digestion

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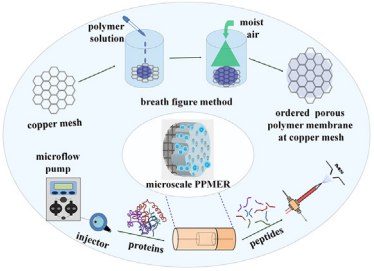
HIGHLIGHTS

- A porous polymer membrane enzyme reactor was developed.
- Breath figure method was used for the fabrication of porous polymer membrane.
- The enzyme reactor was coupled to nLC-ESI-MS/MS for proteins on-line digestion.

GRAPHICAL ABSTRACT



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ABSTRACT

Fast and effective protein digestion is a vital process for mass spectrometry (MS) based protein analysis. This study introduces a porous polymer membrane enzyme reactor (PPMER) coupled to nanoflow liquid chromatography-tandem MS (nLC-ESI-MS/MS) for on-line digestion and analysis of proteins. Poly (styrene-*co*-maleic anhydride) (PS-*co*-MAn) was fabricated by the breath figure method to make a porous polymer membrane in which the MAn group was covalently bound to enzyme. Based on this strategy, microscale PPMER (μPPMER) was constructed for on-line connection with the nLC-ESI-MS/MS system. Its capability for enzymatic digestion with bovine serum albumin (BSA) was evaluated with varied digestion periods. The on-line proteolysis of BSA and subsequent analysis with μPPMER-nLC-ESI-MS/MS revealed that peptide sequence coverage increased from 10.3% (digestion time 10 min) to 89.1% (digestion time 30 min). μPPMER can efficiently digest proteins due to the microscopic confinement effect, showing its potential application in fast protein identification and protease immobilization. Applications of on-line digestion using μPPMER with human plasma and urinary proteome samples showed that the developed on-line method yielded equivalent or better performance in protein coverage and identified more

Abbreviations: PPMER, porous polymer membrane enzyme reactor; nLC-ESI-MS/MS, nanoflow liquid chromatography-tandem MS; PS-*co*-MAn, poly (styrene-*co*-maleic anhydride); BSA, bovine serum albumin; Cyt C, cytochrome C; RAFT, reversible addition-fragmentation chain transfer; DATB, S,S'-bis (α, α'-dimethylacetic acid) trithiocarbonate; AIBN, azo-bis-isobutryonitrile; BAEE, N-*a*-benzoyl-L-arginine ethyl ester; BA, N-*a*-Benzoyl-L-arginine; SEM, scanning electron microscope; PDI, polydispersity index; GPC, gel permeation chromatography; DTT, dithiothreitol.

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membrane proteins than the in-solution method. This may be due to easy accommodation of hydrophobic membrane proteins within membrane pores.

1. Introduction

Proteolysis is a vital and well-established preliminary step in mass spectrometry (MS) based protein analysis [1,2]. The typical in-solution digestion method utilizes low concentration proteases to avoid sample contamination and self-digestion of the enzyme. In-solution proteolysis is generally slow and inefficient at digesting proteins that are in low concentrations. To resolve this problem, enzyme immobilization methods have been widely developed with on-line enzymatic reactors. These methods are based on protease immobilization on solid substrates such as microchips [3–5], membranes [6,7], nanoparticles [8–15], porous glasses [16,17], and monolith [18,19] or on entrapment of enzymes within hollow fibers for proteolysis or deglycosylation [20,21].

Nanoporous materials, including carbon nanotubes [22,23], and mesoporous silica [24–26], have attracted interest for their biological applicability. Advantages of these materials include their large specific surface area and tunable pore size. These pores can accommodate proteases from small globular proteins with diameters of a few nanometers to large enzyme molecules [27]. The method increases the reaction efficiency of enzymes immobilized by porous materials. The enhanced efficiency originates from local increases in the concentration of immobilized enzymes compared to that of in-solution digestion processes. The time-consuming protein-enzyme processes of in-solution digestion are replaced with a rapid process of protein entrapment in nanopores followed by proteolysis.

Since porous materials enhance digestion efficiency, a number of porous materials have been explored. Proteases immobilized on nanoporous membranes can digest protein in a few minutes and lead to high peptide coverage compared to the in-solution method. Several kinds of porous membranes have been utilized for enzyme immobilization, including nylon membranes [28–30]. Electrostatic interactions between enzyme and membranes were commonly utilized for enzyme immobilization. While the inner surface of nanopores can be used for electrostatic adsorption of enzyme, this approach is relatively inefficient due to continuous bleeding of enzymes [31]. Covalent linkage is a suitable alternative used to immobilize proteases at the surface of nanoporous membranes and avoid bleeding enzyme molecules. Membranes with ordered nanopores containing multifunctional groups for covalent enzyme immobilization are excellent candidates.

The recently developed breath figure method exhibits a few advantages, such as simple preparation of ordered polymeric porous membranes using polymers dissolved in volatile organic solvent exposed to moisturized air flow [32,33]. A variety of polymers [32] have been employed in the formation of ordered microporous films with pore diameters ranging from 100 nm to 20 μm, including comb-like copolymers, star polymers, linear homopolymers, hyperbranched polymers, and rod-coil/coil–coil block copolymers. Importantly, these porous polymers possess multifunctional groups that readily react with enzymes. To the best of our knowledge, porous polymer membrane enzyme reactors fabricated by the breath figure method are not frequently reported because they are fragile, with poor durability and tenacity.

This study introduces a novel strategy for overcoming the shortcomings of microporous membrane based enzyme reactors fabricated by the breath figure method. First, a block copolymer made of poly styrene-*co*-maleic anhydride (PS-*co*-MAn) was utilized to construct polymer membranes with MAn groups that easily react with enzyme (i.e. trypsin) in moderate conditions. The breath figure method was effective for fabricating ordered porous polymer membranes due to the presence of both hydrophilic and hydrophobic segments in the PS-*co*-MAn framework. Copper mesh was utilized as a solid substrate of the membrane enzyme reactors to overcome the weakness of poorly tenacious membranes and increase their durability for repeated usage. The PS-*co*-MAn based porous polymer membrane enzyme reactor (PPMER) developed in this study was integrated as a microscale reactor prior to nanoflow liquid chromatography-tandem mass spectrometry (nLC-ESI-MS/MS) for on-line high speed protein digestion and proteomic analysis. The performance of microscale PPMER (μPPMER) for proteolysis was evaluated with bovine serum albumin (BSA) and cytochrome C (Cyt C) by examining protein sequence coverage, minimum amount of protein handled, and reproducibility with nLC-ESI-MS/MS. Finally, the developed on-line method was applied to pooled human plasma to compare its performance with that of the in-solution digestion method. The present study demonstrates great potential for μPPMER applications in rapid and effective proteolysis of a small amount of proteins.

2. Experimental

2.1. Chemicals and materials

Styrene (St) and MAn monomers were obtained from Sinopharm Chemical Reagent Beijing Co., Ltd (Beijing, China) and Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China), respectively. The chain transfer reagent for reversible addition–-fragmentation chain transfer (RAFT) polymerization, S,S'-bis (α, α'-dimethylacetic acid) trithiocarbonate (DATB), was synthesized according to the reference [34]. The RAFT polymerization initiator, azo-bis-isobutryonitrile (AIBN), was obtained from Shanghai Chemical Plant (Shanghai, China). N-*a*-benzoyl-L-arginine ethyl ester (BAEE) was obtained from Acros Organics (Fair Lawn, NJ, USA). N-*a*-Benzoyl-L-arginine (BA) was purchased from Aladdin Reagents Industrial Co., Ltd (Shanghai, China). Analytical reagent grade organic solvents, including 1,4-dioxane and ether, were obtained from Beijing Chemical Factory (Beijing, China). Trypsin, cytochrome C, and BSA were purchased from Sigma (St. Louis, MO, USA). Copper mesh (50 μm in thickness) was obtained from Huawei hardware store (Beijing, China). Water used throughout the experiments was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA). Human plasma and urine samples were obtained from 3 healthy adults of Severance Medical Center (Seoul, Korea), and each group was pooled together.

2.2. Apparatus

A model S-4800 scanning electron microscope (SEM) from Hitachi Co. (Hitachi, Japan) was used to examine the morphology of copper mesh and ordered porous membrane made by the breath figure method.

The molecular weight and polydispersity index (PDI) of PS-*co*-