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An easily regenerable enzyme reactor prepared from polymerized high internal phase emulsions

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ABSTRACT

A large-scale high-efficient enzyme reactor based on polymerized high internal phase emulsion monolith (polyHIPE) was prepared. First, a porous cross-linked polyHIPE monolith was prepared by in-situ thermal polymerization of a high internal phase emulsion containing styrene, divinylbenzene and polyglutaraldehyde. The enzyme of TPCK-Trypsin was then immobilized on the monolithic polyHIPE. The performance of the resultant enzyme reactor was assessed according to the conversion ability of N_{α}-benzoyl-L-arginine ethyl ester to N_{α}-benzoyl-L-arginine, and the protein digestibility of bovine serum albumin (BSA) and cytochrome (Cyt-C). The results showed that the prepared enzyme reactor exhibited high enzyme immobilization efficiency and fast and easy-control protein digestibility. BSA and Cyt-C could be digested in 10 min with sequence coverage of 59% and 78%, respectively. The peptides and residual protein could be easily rinsed out from reactor and the reactor could be regenerated easily with 4 M HCl without any structure destruction. Properties of multiple interconnected chambers with good permeability, fast digestion facility and easily reproducibility indicated that the polyHIPE enzyme reactor was a good selector potentially applied in proteomics and catalysis areas.

1. Introduction

Recently, protein digestion has been studied in many research areas including proteomics, bioengineering and food industry, and the high-throughput and high proteolytic enzyme reactor is expected for efficient protein digestion. Comparing with the traditional protein digestion in solution suffered some drawbacks, such as enzyme autodigestion, low efficiency, extended incubation time and sample loss or contamination, [1], the immobilized enzymatic reactors (IMERs) had great advantages in the aspect of protein digestion [2–4]. Therefore, many IMERs have been studied by immobilizing enzymes on different materials such as polymeric membranes and nanofibers, [5,6], core–shell magnetic nanoparticle, [7,8], graphene oxide nanosheets [9] and organic-inorganic hybrid monolith [10]. These rapid and low carry-over IMERs were successfully applied to protein digestion and proteomic analysis [11].

Among these IMERs, the monoliths, composed of porous solid with small-sized skeletons and relatively large through-pores, could offer fast mass transfer and high enzyme binding capacity [12,13]. Generally, monolithic supports were prepared as silica-based monoliths [14,15] and organic polymers [16]. Silica-based monoliths had silica skeletons and through-pores via a sol–gel process or hybrid organic-inorganic process [17,18]. which made them high permeability, high mechanical strength, and good organic solvent tolerance [19]. But, the preparation of these monoliths was complicated, sometimes leading to poor reproducibility [20]. Another problem was that the nonspecific adsorption of the silica-based monolith was obvious due to the residual silanols, and the uncontrollable polymerization procedure lead it to an irregular pore size and complicated absorption/desorption behavior at the surface of the monolith. In comparison, with good porosity, mild biocompatibility and high-throughput conveying capacity, [21], [22] organic polymer materials had been widely applied in many research areas

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like scaffolds for in vitro 3D cell culture, [23], protein separation and phosphopeptides enrichment, [24], biomedical sensor support, [25], biocatalysts [26] and biomacromolecules trapping [27]. But the swelling of organic solvents might lead to the change of pore structure and the decrease of mechanical stability.

Notably, polymerized high internal phase emulsions (polyHIPEs) have been developed for extensive application in biocompatibility substrates [28,29]. After polymerization, polyHIPEs were formed and the droplets of dispersed phase were removed yielding a highly interconnected network pores with quite well defined diameter. Depending on dispersed phase in emulsion, polyHIPEs had adjustable meso- or macropore skeleton which avoided the problems of nanoparticle agglomerating, low permeability and surfactants demanding [30]. Moreover, the inherent meso- and macropore of polyHIPEs were benefit for specific in-situ absorbents of biological macromolecules when some functional group reagents were added in the polymerization procedure [31].

Here, we developed an easy-preparing and regenerable polyHIPE based IMER for trypsin immobilization and protein digestion. Our studies suggested that the developed IMERs not only have high trypsin immobilization and bioactivity in protein digestion but also have strong regeneration capacity and long-term durability. According to our knowledge, this is the first time report that using polyHIPEs as immobilized enzyme reactor support for protein digestion research.

2. Materials and methods

2.1. Materials and chemicals

Reagents including styrene (STY), divinylbenzene (DVB), sorbitan monooleate (Span80), TPCK-trypsin (Type I, >10 000 units/mg N_α-benzoyl-L-arginine ethyl ester hydrochloride, TPCK treated), N_α-benzoyl-L-arginine ethyl ester (BAEE, 98%), N_α-benzoyl-L-arginine (BA, 98.5%), Tris (Hydroxymethyl)aminomethane (Tris), iodoacetamide (IAA), dithiothreitol (DTT), Cytochrome C (Cyt-c) and bovine serum albumin (BSA) were purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). Potassium persulfate ($K_2S_2O_8$, AR), Glutaraldehyde (GA, 25%, w/v aqueous solution) were obtained from Xilong Chemical Reagent (Guangzhou, China). Methanol and acetonitrile were of HPLC grade from Dikma Co., Ltd. (CA, America). All other reagents were of analytical grade, such as sodium hydroxide, hydrochloric acid and ammonium acetate. The water was deionized by an arium® 611 system (Sartorius, Germany) with resistance ≥18.2 MΩ/cm. All solutions and deionized water were filtered through 0.22 μm filter membrane before HPLC and LC-MS analysis.

Before using, STY and DVB were washed with 5% NaOH aqueous solution and then with water to remove the polymerization inhibitor. After washed to pH 7.0 with water, the obtained solution was dried with $MgSO_4$.

2.2. Preparation of polyHIPE monolith

Firstly, the dispersed phase of PGA and $K_2S_2O_8$ solution was prepared as following: polyglutaraldehyde (PGA) was obtained with GA solution reacted with 1.0 M NaOH at pH 10.5 for 30 min and then the solution was adjusted to pH 7.0 by 1 M HCl [32]. 6.531 mL 0.02% (g/mL) $K_2S_2O_8$ solution was mixed with 0.417 mL PGA solution as dispersed phase for the preparation of HIPE.

0.772 mL continuous phase containing STY, DVB and Span 80 was added into centrifugation tube and rotated in a vortex mixer (IKA MS3 basic, Germany) at 3000 rpm for 30 s, then 6.948 mL dispersed phase was added drop-wise to the continuous phase under condition of gentle stirring. The emulsion was finally stirred for 3 min to form a uniform emulsion. About 1.5 ± 0.2 mL emulsion was transferred into 2.5 mL sealed syringe and polymerized at 50 °C for 24 h. The synthesized polyHIPEs was washed with deionized water and ethanol-water solution (1:1 V/V). Finally, the monoliths were washed and soaked with ammonium acetate buffer (AAB, 0.1 mol/L, pH 8.0). It was measured that a dried polyHIPE monolith was about 0.7 ± 0.1 g.

The freshly prepared emulsions were placed for several hours to observe the stability and variation then the two phases was observed under the microscope in 400 and 1000 times. After polymerization, deionized water was used to investigate the mechanical strength and flow velocity of the monoliths at a pressure of 70 kPa. The morphology and cavity size distribution of monoliths were determined by SEM image analysis (S4800 field emission scanning electron microscope, Hitachi, Japan). Nitrogen adsorption/desorption measurements were valuated on a Micromeritics® ASAP 2020 adsorption apparatus using a BET model for surface area evaluation. To valuate the immobilization probability, the experiments of FTIR spectrum (Nicolet iS10, Thermo Fisher, USA) and elemental analysis (EA2400II, PerkinElmer, USA) were carried out.

2.3. Enzyme immobilization on polyHIPE monolith

For enzyme immobilization, the prepared monolith was connected to a constant flow pump (HL-2D, Shanghai Huxi Analysis Instrument Factory Co., Ltd) and kept at 37 °C. 2.0 mL TPCK-trypsin solution (0.2 mg/mL) was slowly injected and immobilized naturally onto the monolith for 10 min. The residue trypsin solution was pumped out and the reactor was washed with 2 mL AAB solution. After the procedure of enzyme immobilization, the prepared IMER was stored at 4 °C. For long-term storage, the IMERs were soaked in AAB and stored at −20 °C in fridge.

2.4. Investigation of monolithic polyHIPE based IMER

The TPCK-trypsin immobilization rate was calculated by conversation rate of BAEE to BA. Also, the conversion rate of BAEE to BA was used to optimize the synthesis conditions of monolith and assess the properties of IMER [33]. In brief, the prepared monolithic polyHIPE based IMER was heated in a cabinet drier at constant temperature of 37 °C, and then 0.650 mL solution of Tris−HCl and BAEE (0.3 mL of 0.1 mol/L Tris−HCl and 0.35 mL of 5.0 mg/mL BAEE) was pumped through the IMER and reacted for 10 min at a constant velocity. After that, 1.95 mL water was used to wash out the BAEE and BA and deionized water was used to clean the IMER. All eluents were respectively collected and the solution was filtered through 0.22 μm filter membrane before HPLC analysis.

To confirm the limitation of reusability of IMER, it was used many times. Once the enzyme activity of IMER was dramatically decreased, 15 mL AAB was injected to monolith to clean up and 20 mL HCl (4 mol/L) was used to regenerate the monolith for breaking the −C═N− bond between the TPCK-trypsin and PGA based on the Schiff base reaction. The remnant HCl was washed out by 6 mL deionized water and followed by 15 mL AAB. After that, TPCK-trypsin was immobilized on monolith once again and the regenerated IMER was reused.

For protein digestion, 1 mL of 10 mg/mL BSA and Cyt-c were respectively dissolved in 1 mL of 50 mM Tris−HCl (pH 8.1) containing 8 M urea and then the solution was reduced via 0.1 mL of 0.1 M DTT for 20 min at 50 °C. After cooling to room temperature, BSA and Cyt-c were alkylated in the dark with 0.1 mL 0.1 M IDA for 15 min at room temperature. The pretreated BSA and Cyt-C sample (0.2 mg/mL) was pump through the IMER by using the peristaltic pump at 37 °C in cabinet drier for 10 min. After digestion, the

enzymatic hydrolyzate was flushed out by 2 mL AAB solution and collected for peptides analysis with LC-MS/MS.

2.5. HPLC analysis and LC-MS/MS analysis

HPLC (Shimadzu LC-20A, Kyoto, Japan) with photodiode array detector (PAD) were employed to analyze BAEE and BA. Luna 5μ C18 (2) 100A (250 mm × 4.6 mm, 5 μm, Phenomenex, USA) column was used and the operation parameters were optimized. The main determining wavelength was 253 nm. Gradient elution program was set as follow: solution A: deionized water with 0.1vol% fomic acid (FA), solution B: acetonitrile with 0.1 vol% FA. The flow rate was 0.80 mL/min gradient conditions: 10% B for 5 min, to 50% B in 15 min, to 10% B in 5 min and kept at 10% B for 5 min. 10 μL of sample was injected.

Protein and peptides analysis were carried out with LC-ESI-MS/MS (LTQ-Orbitrap, Thermo-Fisher, San Jose, CA, USA). Aeris PEPTIDE XB-C18 column (250 mm × 4.6 mm, 3.6 μm, Phenomenex, USA) was used with the flow rate of 300 μL/min. The separation condition was optimized and water containing 0.1vol% formic acid (A) and CH_3CN (B) were used to generate a 60 min gradient, set as follows: 5% B for 1 min, to 40% B in 33 min, to 95% B in 6 min, kept at 95% B for 6 min, to 5% B in 10 min and kept at 5% B for 4 min. 10 μL of sample was injected for LC-ESI MS^3 analysis at positive ion mode. The spray voltage was 3.0 kV, and the heated capillary temperature was 300 °C. The MS was operated in the data-dependent mode, in which a survey full scan MS spectrum (from m/z 100 to 2000) was acquired in the Orbitrap with a resolution of 30,000 at m/z 400. This was then followed by MS^2 scans of the most abundant ions and the MS^3 scans of first, second and third most abundant ions from MS^2. The resulting fragment ions were recorded in the linear ion trap. The acquired mass spectrometric raw data was processed using MassMatrix. The mechanism of searching could be explained in details in the published papers of Xu et al. [34].

3. Results and discussion

3.1. Effect of contents in high internal phase emulsion

Variation in the chemistry of the polymerization mixture was expected to change trypsin immobilization and mechanical strength, and to some extent to influence the diameter of pore around immobilization active sites. It was reported, [25], the monolith composed of porous solid with facileness of functionality and relatively large through-pores, could offer high rates of mass transfer and high enzyme binding capacity. In our study, the polyHIPE based IMER were prepared with styrene as monomer, divinylbenzene as crosslinker and span80 as emulsifier. Meanwhile, in the water phase, polyglutaraldehyde was used as functional additive for TPCK-trypsin immobilization. Particularly, compositions and proportions of high internal phase emulsion greatly influence the morphology and porosity of polyHIPE, which would finally affect the permeability and mechanical strength of IMER. The monoliths were designed with different phase ratios of STY/DVB (v/v) in oil phase. Five IMERs were first prepared and different STY/DVB (v/v) ratio from 2:1 to 6.5:1 was investigated (Fig. S1, Supplementary Material). The obtained results showed that lower ratio of STY/DVB (2:1) in continuous phase developed larger oil vacuoles and the excessive DVB made the monoliths compactness with hardly impermeable and the 6.5:1 ratio of STY/DVB made the monolith loose with the bubble-liking cavities obviously. The monoliths had an ideal mechanical strength and permeability when the STY/DVB (v/v) ratio was 3:1. Magnified electron microscope dyed photograph of emulsion showed that the emulsion was well formed and highly stable for more then 72 h when the STY/DVB (v/v) ratio was 3:1 (Fig. 1).

Volume fraction of dispersed phase (content of water phase in emulsion, V/V) resulted polyHIPE monoliths differences at permeability and mechanical strength (Fig. S2, Supplementary Material). The lowest volume fraction of dispersed phase in HIPE resulted in the hardest and the worst permeability for polyHIPE monolith. As shown in Table 1, the IMER with different dispersed phase had different permeability and enzyme immobilization efficiency. The lower amount of dispersed phase resulted in a small through-pore structure of monolith with bad permeability (columns 1 and 2) and lower enzyme capacity as well as enzyme bioactivity. Only when the dispersed phase volume fractions reach to 89% and 91%, the TPCK-trypsin capacities both are 0.36 mg and the TPCK-trypsin bioactivity reaches the highest (columns 4 and 5). In terms of the permeability and mechanical strength of polyHIPE monolith, 90% dispersed phase value fraction was selected. In summary, suitable materials with well-organized framework, well permeable, large enzyme capacity and high enzyme bioactivity are helpful for enzyme immobilization and protein digestion. Full pretreatment with the material, such as ordered, regular and porous structure at controllability of synthesis process would benefit for protein enzymatic hydrolysis [35,36].

At the point of most promising monolith, the IMER should be easily prepared with the higher immobilization rate of trypsin on the surface of monolithic porous polymers. It was reported that the porous support covalent with PGA immobilized enzyme by both absorption and covalent binding due to the presence of PGA functional groups which could reacted with amino, thiol, imidazole and phenol groups of proteins [37]. More PGA adding in emulsions seems benefit for not only the permeability of polyHIPE monoliths, but also for the TPCK-trypsin immobilization capacity and bioactivity. Different concentrations of PGA in dispersed phase (V/V: 0%, 3%, 6%, 9%, 12%) were investigated. Table 2 shows the effects of PGA concentration in dispersed phase. Except the 12% of PGA in dispersed phase could not synthesis a homogeneous monolith after

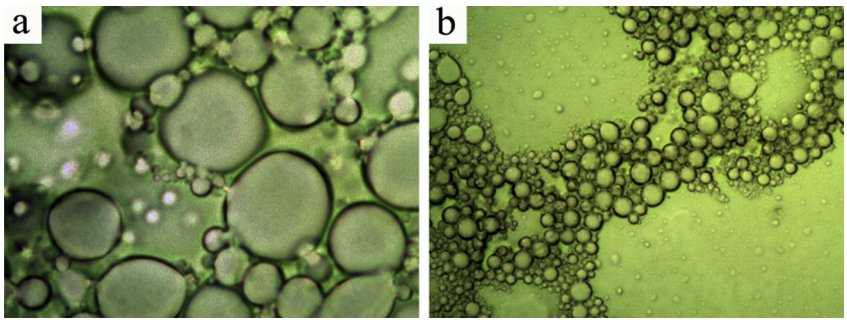
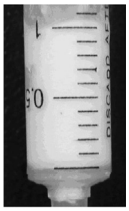


Fig. 1. Magnified electron microscope dyed photograph of high internal phase emulsions at STY/DVB ratio of 3:1 (v/v) and dispersed phase of 90% (v/v). a.1000 times; b. 400 times.

Table 1
Effects of different volume fraction of dispersed phase on preparation of polyHIPE monoliths[a].

Column	1	2	3	4	5
STY-DVB(V:V = 3:1)/mL	0.772	0.772	0.772	0.772	0.772
dispersed phase/mL (%[b])	2.131 (73)	2.841 (79)	4.025 (84)	6.392 (89)	8.168 (91)
PGA/mL (9%[c])	0.185	0.247	0.350	0.556	0.710
Morphology Hardness[d]	 very hard	 hard	 hard-medium	 medium	 soft
Flow velocity[e]/μL·s^{-1}	–	0.7	34.8	45.5	71.4
Trypsin capacity[f]/mg	0.30	0.28	0.32	0.36	0.36
Trypsin bioactivity[g]/%	52.53	58.86	68.31	71.95	70.03

[a] The continuous phase contained 0.445 mL of STY, 0.150 mL of DVB and 0.177 mL of Span 80 and the columns 1, 2, 3, 4, 5 were polymerized with 1.5 mL emulsion in 2.5 mL plastic syringe at 50 °C for 24 h.
[b] Dispersed phase volume fraction (%) = $V_{dispersed\ phase}/(V_{STY-DVB} + V_{dispersed\ phase})$*100.
[c] Concentrations of PGA in the dispersed phase (%) = PGA value/dispersed phase value.
[d] The monoliths had several shape changes at pressure from tube handle. Very hard: no change; Hard: subtle compression; Hard-medium: little compression but full resilience after withdrawing handle force; Medium: a little compression but full resilience; Soft: some compression but a little resilience.
[e] Flushing with 1 mL deionized water at backpressure of 70 kPa; column: 15 mm × 5.4 mm I.D., 10 mm length. "-": it was too hard to pump water.
[f] TPCK-trypsin capacity was determined with the conversation rate of BAEE to BA with free TPCK-trypsin which was washed from the monolith. The total given amount of TPCK-trypsin was 0.4 mg.
[g] TPCK-Trypsin bioactivity was calculated with the conversation rate of BAEE to BA at TPCK-trypsin immobilized polyHIPE monolith comparing with 2 mL 0.2 mg/mL free TPCK-trypsin solution.

24 h, the other monoliths were all synthesized successfully but the performance of IMERs were little different in TPCK-trypsin immobilization efficiency and bioactivity. With regard to enzyme immobilization, the increase of the amount of porosity on the support was desirable to enhance enzyme/substrate ratio and thus the reaction rate [33,37]. Furthermore, the bioactivity of bound trypsin was verified for higher digestion efficiency with optimized PGA concentration. When PGA was not added in dispersed phase, the synthesized monolith could absorb 0.37 mg trypsin (about 90% given amount) but the bioactivity of loaded enzyme was only 38.85%. The substantial decrease of trypsin bioactivity suggested that the trypsin should be randomly adsorbed and strongly agglomerated on the surface of HIPE microsphere and the affinity sites of the agglomerated enzyme were not fully activated. The homogeneous distribution of the functional groups on the surface of the support would largely reduce the stereochemical hindrance, thus increasing the amount and bioactivity of immobilized trypsin. In our study, when the PGA concentration was increased to 6%, the permeability, pore volume and specific surface area of HIPE monolith was improved and the bioactivity of immobilized trypsin was 90.75% with 0.36 mg immobilized trypsin. The higher trypsin bioactivity was thought to be attributed to the lower enzyme/substrate ratio to avoid the reactant blocking. However, excessive PGA would result a soft and nonelastic monolith and it was not suitable for trypsin immobilization.

Table 2
Effects of PGA concentration in dispersed phase on preparation of polyHIPE monoliths[a].

Column	A	B	C	D
STY-DVB(V:V = 3:1)/mL	0.772	0.772	0.772	0.772
dispersed phase/mL	6.948	6.740	6.531	6.323
PGA/mL (%[b])	0.000 (0)	0.208 (3)	0.417 (6)	0.625 (9)
Average pore width[c] (nm)	12.56	10.87	18.31	14.00
Pore volume[c] (cm^3/g)	0.095	0.101	0.126	0.019
Specific surface area[c] (m^2/g)	22.13	30.26	45.11	5.48
Flow velocity[d]/μL s^{-1}	40.0	55.6	71.4	100
Trypsin capacity[e]/mg	0.37	0.37	0.36	0.17
Trypsin bioactivity[f]/%	38.85	72.70	90.75	55.90

[a] The continuous phase contained 0.445 mL of STY, 0.150 mL of DVB and 0.177 mL of Span 80 and the columns F, G, H, I were polymerized with 1.5 mL emulsion in 2.5 mL plastic syringe at 50 °C for 24 h.
[b] Percentage of PGA(%) = $V_{PGA}/V_{dispersed\ phase}$*100.
[c] Average pore width, pore volume and specific surface area were obtained from Micromeritics ASAP (accelerated surface area and porosimetry) for BET test.
[d] Flushing with 1 mL deionized water at backpressure of 70 kPa; column: 15 mm × 5.4 mm I.D., 10 mm length. "-": it was too hard to pump water.
[e] Trypsin capacity was determined with the conversation rate of BAEE to BA with free trypsin which was washed from the monolith. The total given amount of trypsin was 0.4 mg.
[f] Trypsin bioactivity was calculated with the conversation rate of BAEE to BA at trypsin immobilized polyHIPE monolith comparing with 2 mL 0.2 mg/mL free trypsin solution.

3.2. Characteristic performance of polyHIPE monolith IMER

The detailed morphological and structural features of the as-prepared monolith were characterized using scanning electron microscopy (SEM), fourier transform infrared spectrometer (FT-IR) and material elemental analysis. The SEM images of the HIPE microsphere in Fig. 2a and b indicated that they are well-dispersed porous microsphere with the average size of 10 μm which are composed of about 1.5 μm many holes side by side. After the IMER regeneration via HCl flushing, the obtained microspheres showed original porous surface and non-destructive 3D structure (Fig 2c), indicating monolith stabilization of long-term protein digestion. By comparison, the TPCK-trypsin re-immobilized microspheres with the high porosity still retain almost the same 3D structure and specific surface area as their precursor microspheres. FITR analysis provided information regarding the changes in TPCK-trypsin immobilization of the monolith. FITR images (Fig. 3) revealed the

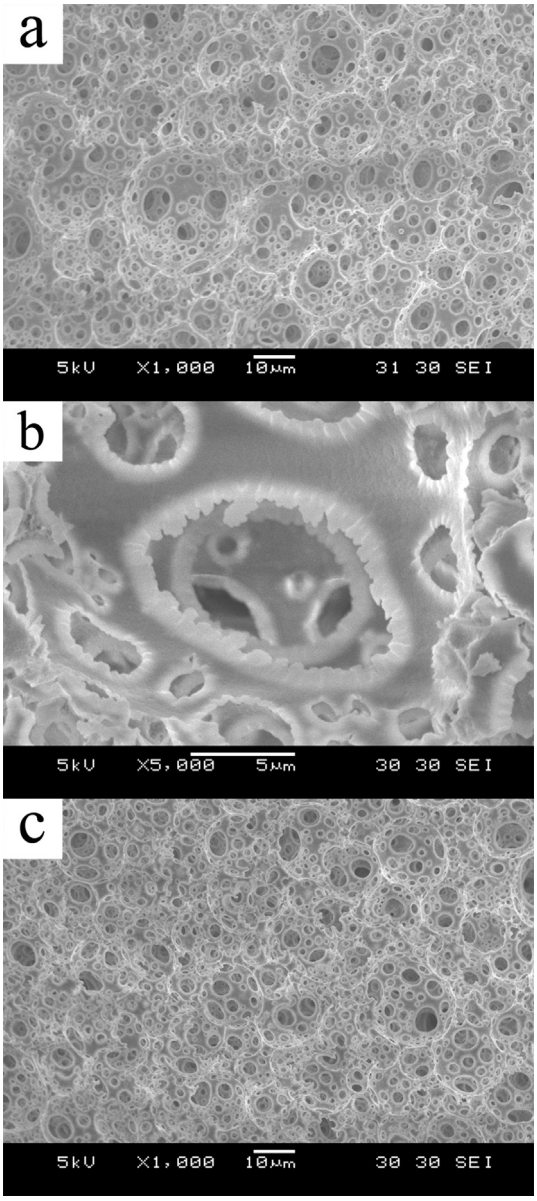
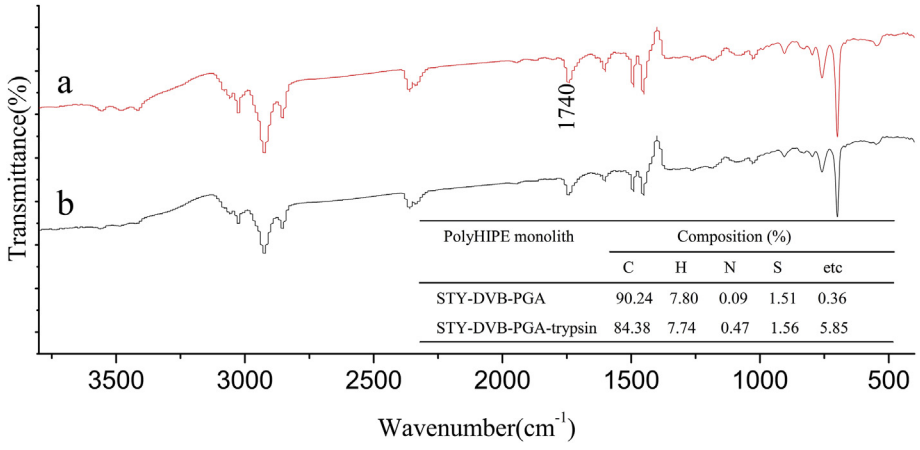


Fig. 2. SEM of PolyHIPE monoliths (a) STY-DVB-PGA-trypsin monoliths at 1000 times; (b) STY-DVB-PGA-trypsin monoliths at 5000 times; (c) regenerated STY-DVB-PGA-trypsin monoliths at 1000 times.

changes of synthesized IMER (STY-DVB-PGA) before and after conjugation with TPCK-trypsin. It can be concluded that the spectroscopy of STY-DVB-PGA monolith before conjugation with TPCK-trypsin had the main characteristic band of the C═O at 1735 cm^{-1} with weak peak. After conjunction with TPCK-trypsin, the main characteristic band of the C═O at 1735 cm^{-1} was stronger. The nitrogen compositions of two kinds of IMERs revealed that TPCK-trypsin immobilized polyHIPEs contained a higher quantity of $-NH_2$ than the monolith without TPCK-trypsin by the determination of element analysis. These results proved that the majority of TPCK-trypsin was successfully immobilized on the monolith. The SEM images shows that polyHIPE monoliths exhibited a highly crosslinked structure which has substantial three-dimensional microsphere with thin wall and interconnected small chambers (Fig. 2a and b). Also, the stable skeleton of polyHIPEs monoliths provides the proteolytic support for enzymes. The monolithic material could offer large specific surface area while the macropores and high porosity afforded better permeability, leading to increased trypsin immobilization capacity. In our study, the meso-/macroporous shell structure of the polyHIPEs monoliths not only poss high specific surface and amounts of affinity sites for the high loading capacity of trypsin but also provide large pores for protein ingress and small pores to allow diffusion of reactants and resulting digests (Fig. S3, Supplementary Material).

The maximum enzyme immobilization ability and the remained bioactivity of immobilized enzyme on the prepared polyHIPE monoliths were investigated according to the immobilization of TPCK-trypsin. Table 3 reveals that the TPCK-trypsin uploading capacity almost remains about 90% but the bioactivities of TPCK-trypsin are gradually decreased when the trypsin amount overupload over 0.4 mg. The great TPCK-trypsin uploading capacity was attributed to the substantial three-dimensional microsphere and crosslinking structure which gave sufficient space to absorb TPCK-trypsin but the IMER modified with 6% PGA (0.417 mL) could not immobilize all the TPCK-trypsin. Indeed, the suitable amount of loading trypsin should be not more than 0.4 mg.

For the regeneration of polyHIPEs monoliths, the IMER was proposed to treat with hydrochloric acid solution (HCl) to break the covalent bond between $-NH_2$ and -CHO. The IMER was successfully regenerated with HCl solution without any structure change in long-term or large scale protein digestion (Fig. 2c). The same three dimension structures and sizes as their precursor microspheres indicated that the monolith could be further reused and regenerated when the bioactivity of uploaded enzyme dramatically decreased. Actually, in our experiments, the polyHIPE monoliths still remained 57.9% TPCK-trypsin bioactivity after ten times protein digestion. The



PolyHIPE monolith	Composition (%)				
	C	H	N	S	etc
STY-DVB-PGA	90.24	7.80	0.09	1.51	0.36
STY-DVB-PGA-trypsin	84.38	7.74	0.47	1.56	5.85

Fig. 3. FTIR and Elementary Analysis of polyHIPEs. (a) STY-DVB-PGA monolith. (b). STY-DVB-PGA-TPCKtrypsin reactor.

Table 3
Trypsin immobilization and trypsin bioactivity of polyHIPE IMER.

Column	J	K(H)	L	M	N
STY-DVB(V:V = 3:1)/mL	0.772	0.772	0.772	0.772	0.772
dispersed phase/mL	6.948	6.948	6.948	6.948	6.948
Uploaded trypsin/mg	0.2	0.4	1.0	1.6	2.0
Trypsin capacity/mg	0.17	0.36	0.96	1.56	1.96
Trypsin bioactivity/%	107.63	90.20	81.10	77.20	70.80

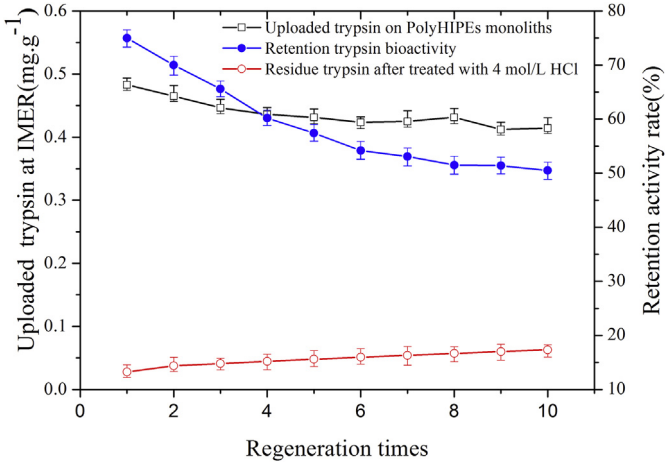


Fig. 4. Stability and regeneration ability of polyHIPE IMER.

tenth TPCK-trypsin re-immobilized IMER could hold about 50.4% bioactivity for protein digestion. Fig. 4 shows that with 4.0 M or higher concentration HCl (20 mL) treating for 10 times, the remnant of TPCK-trypsin in monolith was very little (the residual TPCK-trypsin was about 0.01 mg at Fig. S4, Supplementary Material). The high bioactivity of this regenerated IMER after facile operations for fresh enzyme uploading significantly opens up a new potential employment of polyHIPE-IMER for different enzyme immobilization and an easy renewable way to digest various proteins.

3.3. Application property of polyHIPE-IMER

The reusability of the IMER is important for routine proteolysis procedure in proteomic analysis. In this study, the STY-DVB-PGA polyHIPE-IMER monolith was successfully tested by repeated digestion of BAEE solution. Fig. 4 showed that the uploaded trypsin of IMER decreased gradually as the regeneration number increased. However, the IMER still showed a higher retainable of retention activity, where 58.3% retention activity was found after ten re-generations. This indicates that the IMER had a very operational stability for a large quantity of uploaded enzyme with high bioactivity and high recovery activity of trypsin re-immobilization.

Table 4 lists the peptide generated from BSA and Cyt-c digestion. About 59% sequence coverage with 49 peptides in BSA digestion and 78% sequence coverage with 18 peptides in Cyt-c digestion were obtained. In addition, the successful regeneration of polyHIPE-IMER for BSA and Cyt-c digestion verified that the IMER could be used as a fundamental support for recycle digestion though a long-term protein digestion (Fig. S5, Supplementary Material). The molecular weights (MW) of peptides digested from BSA and Cyt-c mainly ranged from 1000 to 1750 Da and from 750 to 1500 Da, respectively (Fig. S6, Supplementary Material). Comparing with former studies of IMER, [36], although the sequence coverages of BSA and Cyt-C were not quite high, the polyHIPE-IMER was more easily manufactured with two-step strategies (HIPE polymerization and enzyme immobilization) and had the advantages of large-scale digestion and easy regeneration. Furthermore, this monolith IMER can be tentatively applied in online fast and high-throughput protein digestion when it is coupled with other accelerated

Table 4
Peptides analysis by LC-ESI-MS/MS from BSA and Cyt-C.

Protein	No	Sequence numbers	MW	Peptide sequence
BSA	1	25–34	1193.6026	DTHKSEIAHR
	2	35–44	1249.6219	FKDLGEEHFK
	3	66–75	1163.6309	LVNELTEFAK
	4	66–88	2608.2026	LVNELTEFAKTCVADESHAGCEK
	5	76–88	1463.5927	TCVADESHAGCEK
	6	89–100	1419.6980	SLHTLFGDELCK
	7	89–105	1946.0191	SLHTLFGDELCKVASLR
	8	101–105	545.3414	VASLR
	9	118–138	2542.1654	QEPERNECFLSHKDDSPDLPK
	10	205–209	649.3352	IETMR
	11	205–211	906.4722	IETMREK
	12	223–228	706.3555	CASIQK
	13	223–232	1195.5911	CASIQKFGER
	14	229–232	508.2523	FGER
	15	233–241	1001.5889	ALKAWSVAR
	16	236–241	689.3734	AWSVAR
	17	242–248	847.5036	LSQKFPK
	18	249–256	922.4891	AEFVEVTK
	19	257–263	789.4741	LVTDLTK
	20	286–299	1684.8227	YICDNQDTISSKLK
	21	298–309	1532.7834	LKECCDKPLLEK
	22	300–309	1291.6044	ECCDKPLLEK
	23	347–359	1567.7446	DAFLGSFLYEYSR
	24	360–371	1439.8135	RHPEYAVSVLLR
	25	402–420	2355.1430	HLVDEPQNLIKQNCDQFEK
	26	413–420	1068.4417	QNCDQFEK
	27	413–433	2529.2223	QNCDQFEKLGEYGFQNALIVR
	28	421–433	1479.7980	LGEYGFQNALIVR
	29	437–451	1639.9393	KVPQVSTPTLVEVSR
	30	452–459	817.4903	SLGKVGTR
	31	456–459	432.2600	VGTR
	32	460–468	1166.4926	CCTKPESER
	33	469–482	1724.8395	MPCTEDYLSLILNR
	34	483–489	898.4819	LCVLHEK
	35	483–495	1539.8215	LCVLHEKTPVSEK
	36	483–498	1869.0254	LCVLHEKTPVSEKVTK
	37	499–507	1138.4999	CCTESLVNR
	38	508–523	1881.9327	RPCFSALTPDETYVPK
	39	508–528	2471.1888	RPCFSALTPDETYVPKAFDEK
	40	524–528	609.2886	AFDEK
	41	524–544	2498.1908	AFDEKLFTFHADICTLPDTEK
	42	524–547	2867.4293	AFDEKLFTFHADICTLPDTEKQIK
	43	529–544	1907.9223	LFTFHADICTLPDTEK
	44	529–547	2277.1604	LFTFHADICTLPDTEKQIK
	45	548–557	1142.7143	KQTALVELLK
	46	549–557	1014.6207	QTALVELLK
	47	562–568	818.4266	ATEEQLK
	48	569–580	1399.6932	TVMENFVAFVDK
	49	598–607	1003.5800	LVVSTQTALA
Cyt-c	1	9–14	762.4872	KIFVQK
	2	10–14	634.3924	IFVQK
	3	27–39	1433.7760	HKTGPNLHGLFGR
	4	29–39	1168.6244	TGPNLHGLFGR
	5	29–40	1296.7172	TGPNLHGLFGRK
	6	40–54	1584.7674	KTGQAPGFSYTDANK
	7	40–56	1826.9027	KTGQAPGFSYTDANKNK
	8	41–54	1456.6705	TGQAPGFSYTDANK
	9	41–56	1698.8078	TGQAPGFSYTDANKNK
	10	57–73	2009.9607	GITWGEETLMEYLENPK
	11	57–74	2138.0503	GITWGEETLMEYLENPKKKYIPGTK
	12	74–80	806.4775	PGTK
	13	81–87	779.4492	MIFAGIK
	14	81–88	907.5436	MIFAGIKK
	15	90–100	1306.692	GEREDLIAYLK
	16	90–101	1434.7954	GEREDLIAYLKK
	17	93–101	1092.6327	EDLIAYLKK
	18	102–105	434.1887	ATNE

digestion devices, e.g. microwave assisted digestion to generate unique and unknown abundant peptide fragments, which would be benefited for identification of target proteins.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.03.049.

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