

A Recyclable Poly(ionic liquid)s Enzyme Reactor for Highly Efficient Protein Digestion

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ABSTRACT: One of the most significant tasks for proteomic research and industrial applications, is the preparation of recyclable enzyme reactor. Herein, a novel recyclable enzyme reactor has been developed based on monodispersed spherical poly(quaternary ammonium ionic liquid)s particles immobilized trypsin. A new quaternary ammonium ionic liquids functional monomer was first synthesized. The ionic liquids functional monomer was then copolymerized with ethylene glycol dimethacrylate by precipitation polymerization. The resultant monodispersed spherical particle showed a large surface area (231 m²/g) and high binding capacity for trypsin (200 mg/g) due to the large surface area and strong interaction. The polymer microsphere loaded trypsin was used as an enzyme reactor for the digestion of standard protein, semi-complex samples and skim milk, respectively. The results indicated that the enzyme reactor exhibited highly efficient protein digestion and excellent stability. The digestion time of the present ionic liquids enzyme reactor for the digestion of protein, the solution could be reduced to even 5 min. The ionic liquids enzyme reactor showed excellent reusability and could be reused for more than four times. When it was kept at 4 °C for 12 d, and used for skim milk digestion, the obtained MALDI-TOF score could also reach 88 with 29 matched peptides.

KEYWORDS: Enzyme reactor; Ionic liquids; Poly(ionic liquids); Protein digestion.

INTRODUCTION

Ionic Liquids (ILs) are usually consisted of a discrete cation and anion pair and have been considered as green chemicals due to their excellent solubility, negligible volatility, thermal stability, tunable hydrophilic and hydrophobic property by simple changing counter anions or counter cations [1-3]. In the past few years, ILs have attracted much attention in the fields of materials chemistry, catalysis (including chem- and bio-catalysis), electrochemistry, separation science, and organic synthesis as solvent [4-10]. Especially in the fields of bio-catalysis fields,

stability, reaction activity and stereoselectivity of the enzyme could be remarkably improved in ILs medium [11-15]. However, the liquid state of ILs limits the recycling, recovery, and further use. Poly(ionic liquid)s materials, which have both solid- and ILs-like properties, are new kind of functional materials obtained by copolymerization of a ILs functional monomer with a cross-linked monomer. The resultant poly(ionic liquid)s materials have been widely used as functional materials in the fields of thermo-responsive materials, catalysis, separation, adsorption, energy harvesting,

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and bio-related field [16-21]. However, to the best of our knowledge there is no report on employing poly(ionic liquid)s in the field of enzyme catalysis.

In bottom-up proteomics approach [22], enzymatic digestion of protein prior to analysis by mass spectrometry plays a very important role for their successful identification. Both in-gel and in-solution digestion protocols have been employed in a wide range of applications for routine analysis. However, they both also suffered from several disadvantages such as low activity, long digestion time, interference due to protease autolysis, non-reusability of the protease used and poor reproducibility. To circumvent these problems, the development of faster and more efficient digestion approaches has been invested many efforts [23-27]. One of many modern approaches to speed up protein digestion, which has received considerable interest, is immobilized enzyme reactor. A proteolytic enzyme is usually attached to a solid material via affinity binding, adsorption, covalent linkage or entrapment/encapsulation [28-29]. In order to obtain biocompatible supporting materials to improve microreactor performance, many novel materials including inorganic nanocrystals, metal-organic frameworks, organic micro- and nanoparticles have been performed as carrier supports for proteolytic enzymes immobilization [30]. For example, polydopamine coated magnetic Fe₃O₄ and polydopamine coated magnetic graphene nanocomposites have been reported as carriers for the immobilization of trypsin [31-33].

Herein, a recyclable enzyme reactor has been developed based on binding trypsin onto a novel poly(quaternary ammonium ionic liquid)s materials, which was obtained by copolymerization of 4-vinyl benzyl triethylammonium chloride ionic liquids monomer and ethylene glycol dimethacrylate (EGDMA) via precipitation polymerization. The most remarkable characteristic of our work is the one-step synthetic method for the poly(ionic liquid)s material. The following process of trypsin immobilization is much more simple and facility. The resultant enzyme reactor showed excellent enzymolysis ability. Compared with traditional digest method, our new enzyme reactor displayed much more rapid enzymolysis rate and high activity.

EXPERIMENTAL SECTION

Chemicals and materials

Bovine Serum Albumin (BSA), β -casein and Trypsin were purchased from Beijing J&H Chemical Co., Ltd

(Beijing, China). 4-vinyl benzyl chloride, 1,4-dithiothreitol (DTT) and Iodine Acetamide (IAA) were brought from Sigma-Aldrich (St. Louis, MO). Acetonitrile, EGDMA, azobisisobutyronitrile (AIBN), triethylamine, ethyl acetate, methylene chloride, and methanol were obtained from Shanghai biological engineering Co. Ltd (Shanghai, China). Other solvents and chemicals were at least analytical-grade. Skim milk was gained from a supermarket. The buffer system was prepared as shown below: an appropriate amount of Tris base was dissolved in deionized water and adjusted the pH to 6.8 with 12 M HCl, and finally added enough deionized water to obtain the desired volume. Hepes-NaOH buffer was prepared by a similar approach.

Instruments

¹H-NMR spectra obtained through a Bruker AVIII spectrometer (Bruker, Bremen, Germany). FT-IR spectra were recorded on a Vector 22 spectrometer (Bruker, Bremen, Germany). Thermogravimetric analysis (TGA) was carried out through a SDTQ600 (TA, USA) under N₂ flow with a heating rate of 10 °C min⁻¹ up to 800 °C. Scanning electron microscopy (SEM) images was obtained with a Hitachi SU-1510 (Hitachi, Japan). The micropore size and surface area were determined using an AUTOSORB-1-MP (Quantachrome, USA). All MALDI-TOF mass spectra were gained through a Bruker FLEXTM time of flight mass spectrometer (Bruker, Bremen, Germany).

Synthesis of 4 - vinyl benzyl triethylammonium chloride ionic liquids functional monomer

Acetonitrile (3 mL) and 4-vinyl benzyl chloride (1 mL) were mixed in a round bottom flask under the magnetic stirring. Then, 1 mL of triethylamine was added dropwise to the mixture, and the solution was kept under the magnetic stirring for 4 h. When the reaction finished, most of the solvent was removed by evaporation. Subsequently, ethyl acetate was added into the bottom flask, and the white solid appeared immediately. Finally, the product was filtered, washed with 5 mL of ethyl acetate three times, and dried under vacuum conditions for 24 h. The yield of the product is 87.6%.

Preparation of monodispersed spherical poly(4 - vinyl benzyl triethyl ammonium ionic liquid)s particles

The monodispersed spherical poly(ionic liquid)s particles were prepared via precipitation polymerization.

Methanol (60 mL) and 4-vinyl benzyl triethyl ammonium chloride ionic liquids (30 mg) were added into a round bottom flask. Thereafter, EGDMA as the crosslinking monomer and AIBN as the initiator were also added. The mixture was ultrasonicated at room temperature for 5 min and then purged with nitrogen gas for 10 min. The above pre-polymerization system was put into the water bath at 60 °C for 24 h. Then the obtained polymer particles were collected by centrifugation and washed three times with methanol to remove residual unreacted reagents. Finally, the monodispersed sphere particles were dried at a vacuum oven to a constant weight at 40 °C.

The adsorption capacity of poly(quaternary ammonium ionic liquid)s particles to Trypsin

The poly(quaternary ammonium ionic liquid)s particles (5 mg) were added to the buffer solution containing trypsin at different concentrations (20 mM Tris-HCl, pH=6.8, 0.1-0.8 mg/mL). After the system was incubated for 12 h, the materials were collected by centrifugation, and the concentration of trypsin in the supernatant was detected by UV-Visible absorption spectrophotometry.

Adsorption kinetics of poly(quaternary ammonium ionic liquid)s particles to trypsin

A series of 0.6 mg/mL protein solutions were prepared by dissolving trypsin into 20 mM Tris-HCl buffer solution (pH = 6.8). Then the poly(quaternary ammonium ionic liquid)s particles (5 mg) were added to the enzyme solution. These mixtures oscillated gently and the supernatant was then taken out at different intervals of 15, 30, 60, 360, 720, 1080, 1440 min at room temperature to measure the concentration of trypsin with UV-visible absorption spectroscopy.

Preparation procedures of monodispersed spherical poly(quaternary ammonium ionic liquid)s particles enzyme reactor

The enzyme reactor was obtained by adding the monodispersed spherical poly(quaternary ammonium ionic liquid)s particles (5mg) to 0.6 mg/mL of trypsin solution (20mM Tris-HCl, pH=6.8). After kept the system for 12h, the materials loaded trypsin were collected by centrifugation, and washed three times with water and NH_4HCO_3 (50mM, pH=8.0) buffer solution, respectively, to remove unbound enzyme.

Digestion of standard protein solution with poly(quaternary ammonium ionic liquid)s particles enzyme reactor

Standard protein (1 mg/mL, BSA or β -casein) was added to the solution of NH_4HCO_3 (1 mL, 50 mM, pH=8.0), and denatured for 2 h. Thereafter, DTT (10 mmol) was added. After the mixtures remained for 2 h, IAA (20 mmol) was also added. The mixtures were kept under the dark condition for 30 min. The prepared solution was used for digestion by 5 mg of the poly(ionic liquid)s particles loaded enzyme at 37 °C. For comparison, the traditional in-solution digestion was carried out in NH_4HCO_3 (50 mM, pH=8.0) solution (the quantity of the enzyme was identical to the loading quantity of the synthesized material) with the substrate-to-enzyme ratio of 40:1 at 37°C.

Digestion of skim milk solution with poly(quaternary ammonium ionic liquid)s particles enzyme reactor

Skim milk (10 mL) and 50 mmolL NH_4HCO_3 solutions containing 8 M urea (10 mL) were mixed and denatured for 2.5 h. Then DTT (10 mmol) was added, and the mixtures remained for 2 h. After, IAA (20 mmol) was added, and the mixtures were maintained under the dark condition for 30 min. In order to make the urea concentration below 1 M, 50mmolL NH_4HCO_3 solution was used to dilute the sample. The prepared sample (1.0 mL) was then digested by 5 mg of the poly(ionic liquid)s particles loaded enzyme at 37°C. For comparison, the prepared sample was also treated by the traditional in-solution digestion.

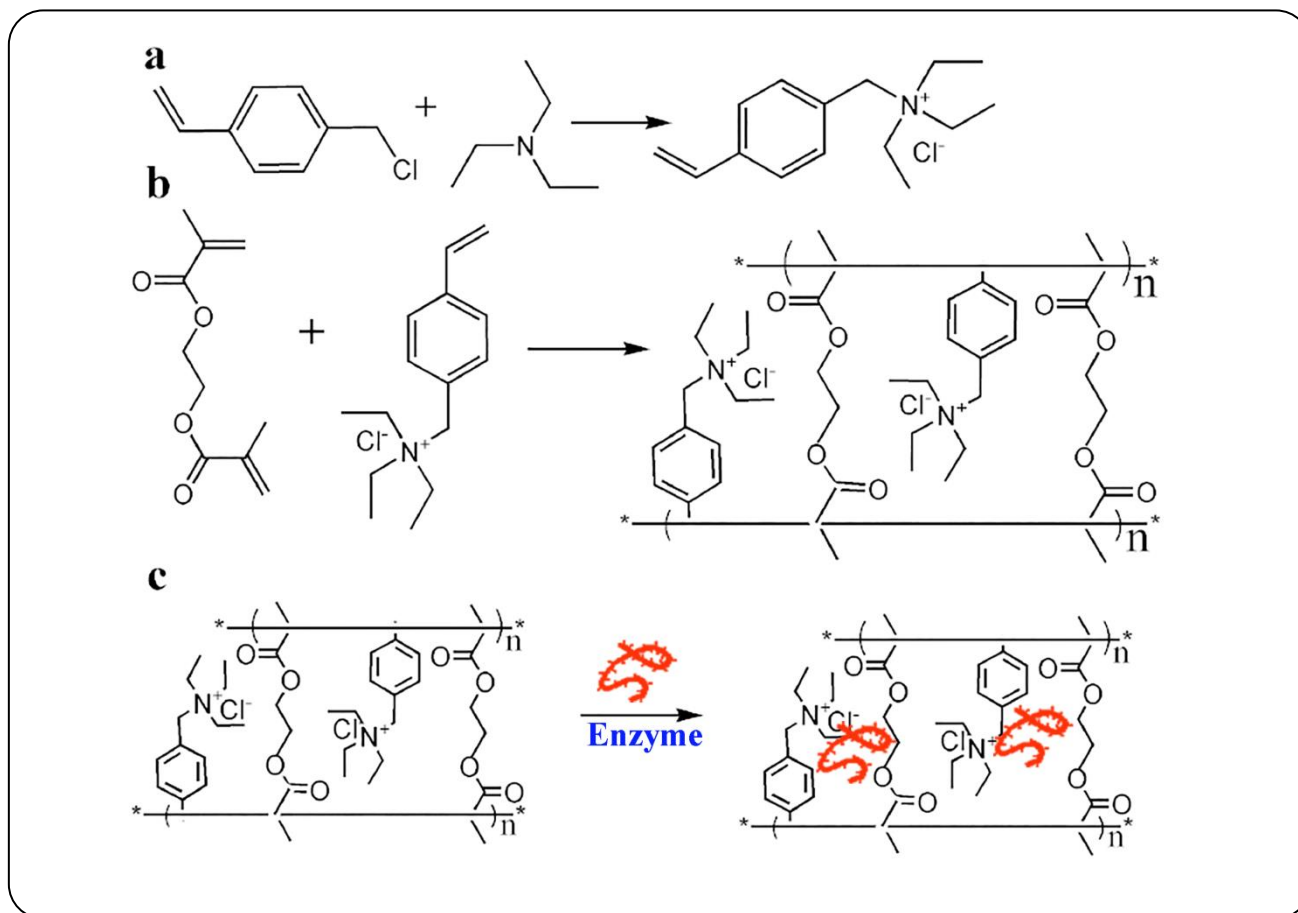
Stability and reusability of the enzyme reactor

The identical enzyme reactor was employed to digest the skim milk sample. When the digestion was finished, the materials loaded trypsin was collected by centrifugation and washed with purified water (3 mL) three times. Then the recovered enzyme reactor was used to the next milk sample. In order to investigate the stability, the poly(ionic liquid)s particles loaded enzyme were stored at 4 °C for 12 d. Then, the particles enzyme reactor was used to digest the skim milk sample at 37 °C.

RESULTS AND DISCUSSION

Preparation of poly(ionic liquid)s

In order to prepare the monodispersed spherical poly(quaternary ammonium ionic liquid)s particles,



Scheme 1: The preparation process of ionic liquids monomer (a), poly(ionic liquids) (b) and the interaction between the polymer and the enzyme (c).

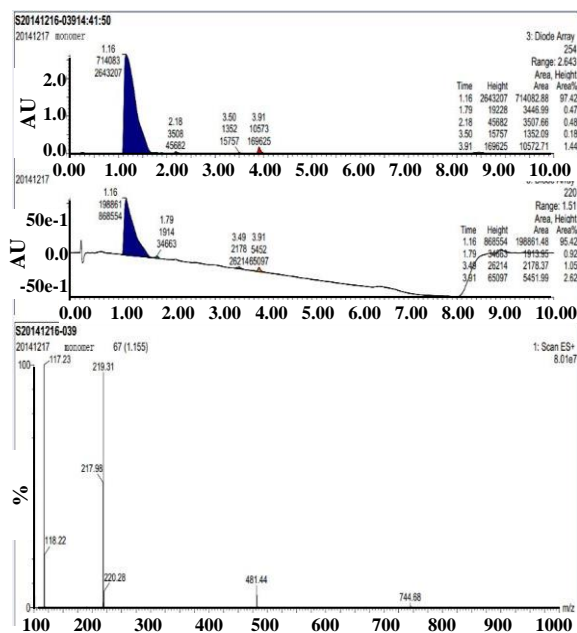


Fig. 1: HPLC-MS spectra of the quaternary ammonium ionic liquid functional monomer.

a novel 4-vinyl benzyl triethylammonium chloride ionic liquids functional monomer was first synthesized by the reaction of between triethylamine and 4-chloromethylstyrene via a simple one-step procedure. The synthesis process has been shown in Scheme 1a. The purity of the monomer was checked by HPLC-MS. The results indicated that there was the main peak at 1.16 min and the yield of the product reached 97.42%. Moreover, the characteristic ionic existed at the m/z 219.31, which was identical to the cationic molecular weight of the monomer (Fig. 1).

The ionic liquids functional monomer was further confirmed using the ^1H NMR spectrum, and the data were listed as follows. ^1H NMR ($\text{CDCl}_3\text{-d}_6$, 0): 1.409-1.510 (9 H, t, $J=40.4$); 1.911 (2 H, s, $J=764.4$); 3.451-3.506 (6 H, m, $J=22$); 5.373-5.401 (1 H, d, $J=11.2$); 5.811-5.855 (1 H, d, $J=17.6$); 6.684-6.755 (1 H, m, $J=28.4$); 7.286-7.554 (4 H, m, $J=40.8$). We could conclude from the data that, within the permitted error,

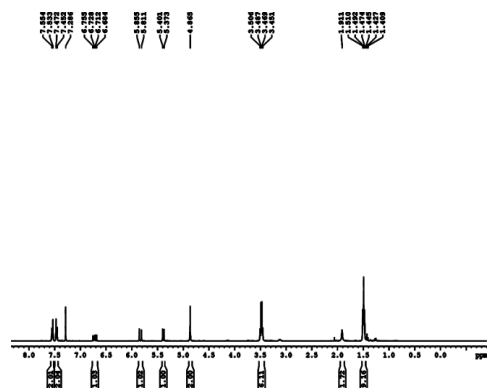


Fig. 2: ^1H NMR spectra of the quaternary ammonium ionic liquids functional monomer

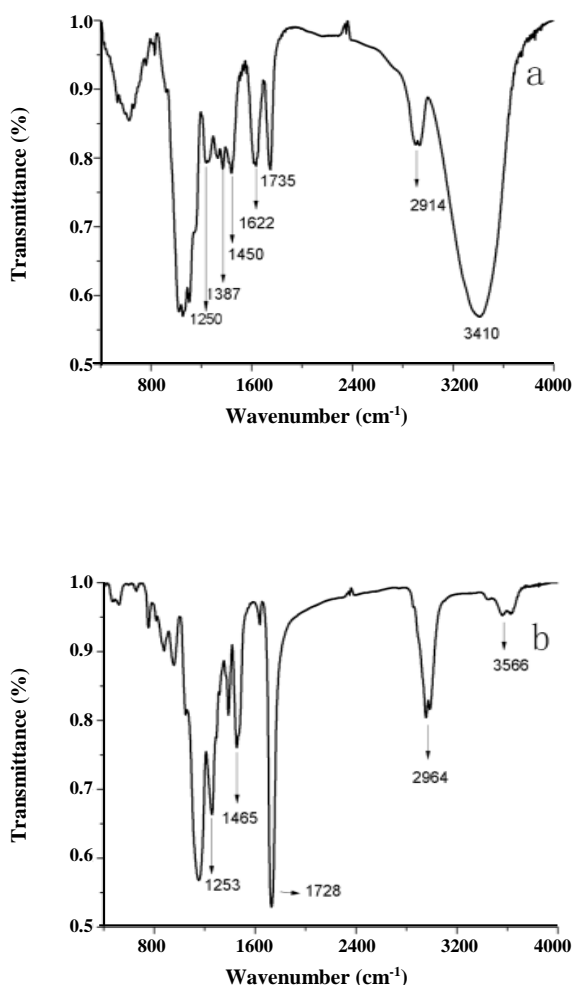


Fig. 3: FT-IR spectra of the synthesized monomer (a) and the poly(ionic liquid)s (b).

the number of hydrogen atoms in each corresponding displacement was consistent with the synthetic functional monomer. Beyond that, there existed no extra impurity peaks in the ^1H NMR spectra (Fig. 2).

FT-IR spectra were also performed for the analysis of the monomer. As shown in Fig. 3a, the peaks between $2845\text{--}3050\text{ cm}^{-1}$ are attributed to the C-H stretching vibration of the methylene, methyl, ethylene and aromatic rings. The peak at 1735 cm^{-1} , is associated with the C=C stretching vibration of ethylene. The peaks at 1622 cm^{-1} and 1450 cm^{-1} are assigned to stretching vibration of aromatic rings. The peak at 1387 cm^{-1} is attributed to the absorption of methyl. The peak at 3448 cm^{-1} may be attributed to the adsorption of water. The peak at 1250 cm^{-1} is assigned to stretching vibration of C-N. All these results indicated the 4-vinyl benzyl triethylammonium chloride ionic liquids functional monomer has been successfully synthesized.

The poly(quaternary ammonium ionic liquids) materials were obtained by copolymerization of the ionic liquids functional monomer with EGDMA via precipitation polymerization. The obtained materials were characterized by FT-IR, SEM, and DSC-TGA, respectively.

The result of FT-IR was shown in Fig. 3b. We could observe that the peak at 1728 cm^{-1} is originated from stretch vibration of C=O from EGDMA. The peaks at 2964 cm^{-1} and 3025 cm^{-1} are attributed to the C-H stretching vibration of the methylene, methyl, ethylene and aromatic rings. The peak at 1465 cm^{-1} is assigned to stretching vibration of aromatic rings. The peak at 1253 cm^{-1} is assigned to stretching vibration of C-N. From the above results, the poly(quaternary ammonium ionic liquid)s materials were successfully prepared.

The SEM micrograph of poly(ionic liquid)s particles demonstrated a monodispersed spherical morphology with the diameter of about $1\text{ }\mu\text{m}$ (Fig. 4). The excellent morphology ensured the subsequent application in the enzyme reactor.

Thermo Gravimetric Analysis (TGA) was used to confirm the thermal stability of the polymer material. As shown in Fig. 5, the main quality loss of the polymer material occurred at around $250\text{--}450\text{ }^\circ\text{C}$, the cause of the phenomenon may be the pyrolysis of the poly(ionic liquid)s. And such high stability may attribute to the ionic liquids and benzene framework. Furthermore, when the material is applied to the protein digestion, the polymer structure would not be destroyed.

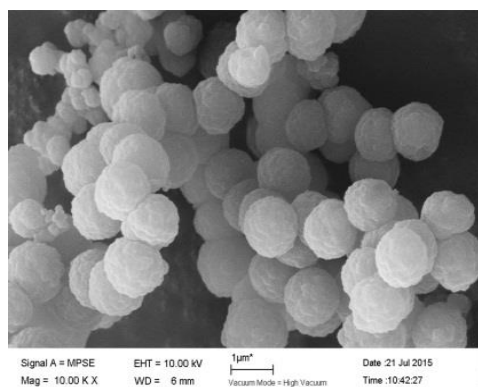


Fig. 4: SEM images of the poly(ionic liquid)s material.

Brunauer–Emmett–Teller (BET) is a significant parameter, which based on the multi-layer adsorption theory to measure the adsorption capacity of the substances. Generally, for nanoparticles, particle shape more similarity to spherical and with ordered accumulation, the polymer material can reach the higher adsorption capacity. For our synthesized material, the adsorption area is $231 \text{ m}^2/\text{g}$, which is a brilliant consequence [34]. So the material can be applied for the following experiment excellently.

Binding performance of the resultant poly(ionic liquid)s materials to trypsin

In order to prepare the enzyme reactor, the effect of the kind of buffer solution and pH value on the binding performance of the resultant poly(ionic liquid)s materials to trypsin were first investigated. Different buffers system (pH = 6.8) including Tris-HCl, HEPES-NaOH and PBS buffer solution were employed.

It could be observed that the poly(ionic liquid)s materials showed the largest binding quantity (200 mg/g) in Tris-HCl buffer system at pH 6.8 (Fig. 6a). We speculated that in this research, Tris, PBS, and HEPES were used, and only HEPES displayed a zwitterionic behavior. Zwitterionic buffer (HEPES) usually shows higher ionic strength than that of PBS or TRIS-HCl [35]. However, higher ionic strength usually weakens electrostatic interaction between the surface of the material and the protein [36-37]. Thus, the binding performance is worst in Hepes buffer system. In addition, trypsin is positively charged in our studied buffer system, which may interact with the anion of the buffer system.

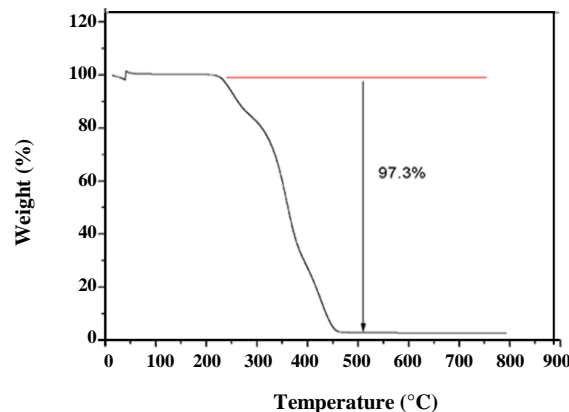


Fig. 5: TGA curves of the prepared polymer particles.

When compared PBS with Tris-HCl, we found that the buffer anions of Tris-HCl were the same as the anions of the surface of the material, thus, this may facilitate the protein adsorption. Thus, Tris-HCl buffer solution was chosen for the subsequent experiments. From Fig. 6b, the binding amount of the poly(ionic liquid)s materials to trypsin increased with the decrease of pH from 8.8 to 6.8. Taking into account of enzyme activity, we chose pH 6.8 as the optimum condition. Thus, the adsorption kinetics and adsorption capacity experiments were performed at pH 6.8 in Tris-HCl buffer solution. The results were

shown in Fig. 7. The adsorption capacity increased from 46 to 200 mg g^{-1} , when the concentration of trypsin changed from 0.1 to 0.6 mg/mL (Fig. 7a). When the concentration of trypsin was larger than 0.6 mg/mL , the adsorption capacity was kept unchanged. This capacity is superior to those of the previous report [38-39]. In the kinetic experiment, the adsorption capacity increased with the growth of the adsorption time. The adsorption capacity reached 126 mg/g at 60 minutes. When the adsorption time was extended to 360 minutes, 95% of adsorption capacity was achieved. The adsorption equilibrium was reached at 12 h (Fig. 7b). The adsorption capacity (199.3 mg/g) was approximately consistent with the result of the static adsorption experiment. So, the concentration of trypsin and the adsorption time were ascertained at 0.6 mg/mL and 12 h, respectively. In addition to these investigations, the binding stability of trypsin onto the poly(ionic liquid)s particles was studied by washing the particles loaded trypsin with different solutions. The results were shown in Table 1. It could be seen that only 53.75% of trypsin was removed from

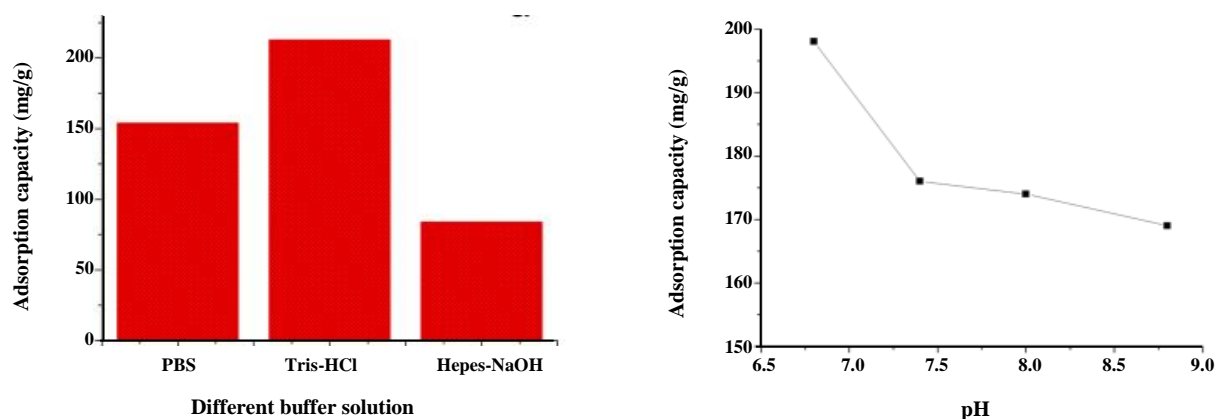


Fig.6: Effect of buffer and pH on the binding performance of the poly(ionic liquid)s to trypsin. (a) the effect of the different buffer; (b) the effect of pH in Tris-HCl buffer solution.

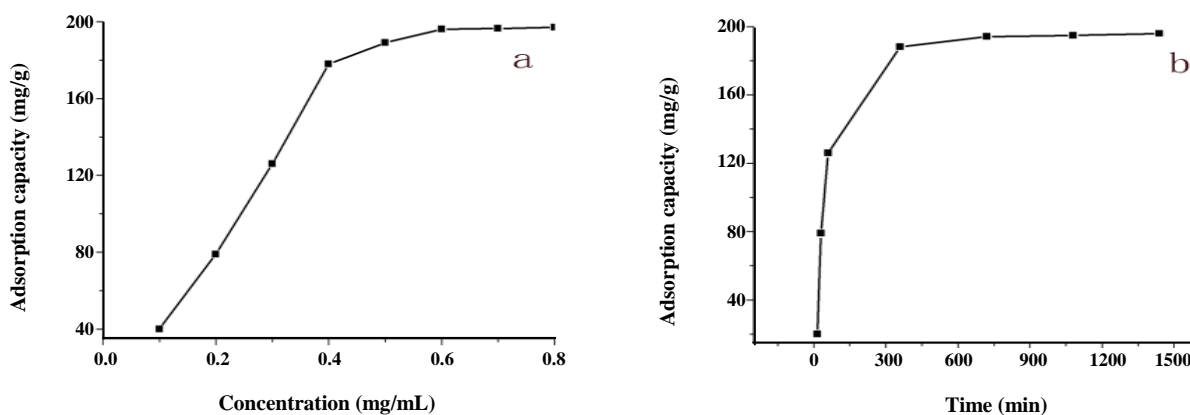


Fig.7: Binding performance of the poly(ionic liquid)s to trypsin. (a) Static adsorption; (b) kinetic adsorption

the materials with the mixtures of 70% acetonitrile + 30% water + 2% trifluoroacetate as the eluent. When trifluoroacetate was changed to acetate acid, the elution ratio decreased significantly. In addition, the elution ratio was decreased with the decrease of the acetonitrile content in the eluent. It was worth to note that when 50 mM ammonium bicarbonate, in which the enzyme reactor would be utilized for the protein digestion, was chosen as the eluent, only 1.5% of trypsin was removed from the poly(ionic liquid)s particles. These results demonstrated that the stability of trypsin onto the poly(ionic liquid)s particles was very excellent, and the loss of trypsin during the digestion period could be avoided.

Digestion ability of the enzyme reactor to β -casein

For traditional enzyme digestion method, time-consuming is one of the drawbacks. In order to

investigate the digestion ability of the enzyme reactor, digestion of β -casein was carried out at different intervals. For comparison, the traditional solution digestion was also performed under the same amount of enzyme. All results were shown in Table 2 and Fig.S1. When digestion time was 4h, only 8 matched peptides with 6% sequence coverage were obtained for the traditional digestion, however, 32 matched peptides with 38% sequence coverage were identified for the enzyme reactor digestion. When digestion time was extended to 16h, the traditional solution digestion exhibited similar results that obtained via the enzyme reactor within 4h in view of matched peptides and sequence coverage. Thus, the enzyme reactor could shorten digestion time from 16h to 4h. These results indicated that the present enzyme reactor was superior to the traditional solution digestion.

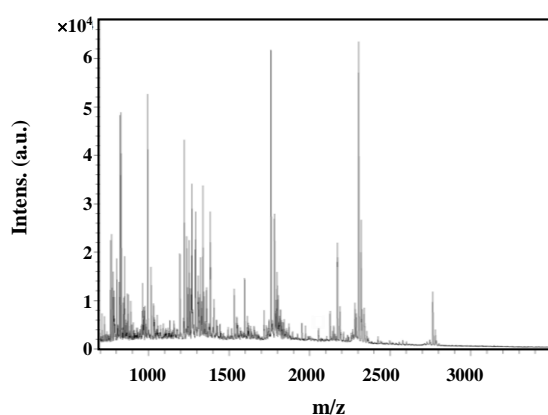
In order to investigate the potential, the digestion time

Table 1: Binding stability of trypsin onto the poly(ionic liquid)s.

Eluent	Elution ratio
70% acetonitrile+30% water+2% trifluoroacetate	53.75%
70% acetonitrile+30% water+2% acetic acid	19.56%
50% acetonitrile+50% water+2% trifluoroacetate	25.44%
50% acetonitrile+50% water+2% acetic acid	13.94%
50Mm ammonium bicarbonate	1.5%

Table 2: Results of sequence coverage and the matched peptides by both the enzyme reactor and the traditional in-solution digestion.

		4h	8h	12h	16h
Enzyme reactor digestion	sequence coverage	38%	32%	33%	56%
	matched peptides	32	33	31	33
Traditional digestion	sequence coverage	6%	17%	28%	29%
	matched peptides	8	22	27	31

**Fig. 8: MALDI-TOF mass spectra of β -casein solution within 5 min digestion by the enzyme reactor.**

was curtailed to 5 min (Fig.8). It could be seen that 18 matched peptides with the sequence coverage 14% were affirmed, indicating the relatively high performance of the new digestion method by the prepared enzyme material. The present digestion time was shorter than that of the previous report [30, 39].

In order to affirm the feasibility and efficiency of the enzyme reactor to semi-complex samples, two different proportions of mixed solutions with the ratio of BSA to β -casein at 100:1 and 500:1 (v/v) were chosen as the samples, which were digested by the enzyme reactor for 30min. The results were shown in Fig. 9. When the ratio

of BSA/ β -casein was set at 100:1, 53 matched peptides with 14% sequence coverage and 19 matched peptides with 23% sequence coverage were observed for BSA and β -casein, respectively. The sequence coverage was better than that of the previous report [40]. Under the ratio of 500:1 (BSA/ β -casein), 21 matched peptides with 20% sequence coverage and 54 matched peptides with 18% sequence coverage were obtained for β -casein and BSA, respectively. These results indicated that the enzyme reactor showed excellent enzymatic activity in the case of semi-complex samples.

In order to further check the performance of the enzyme reactor, skim milk was selected as an actual sample for the large-scale proteome research. When the skim milk solution was digested by the enzyme reactor for 30 min, the result of 29 matched peptides with sequence coverage as high as 84% were obtained for β -casein (Fig. 10a). However, 5 matched peptides with sequence coverage of 14% were observed for traditional free enzyme digestion method (Fig. 10b). The detail results were shown in Table S1. The results indicated that the enzyme reactor showed potential applications for complex samples.

In order to investigate reusability, the present enzyme reactor was utilized to digest

skim milk. From Table 3 and Fig.S2, we could infer that the residual activity of enzyme reactor toward skim milk decreased slightly as the cycle number increased;

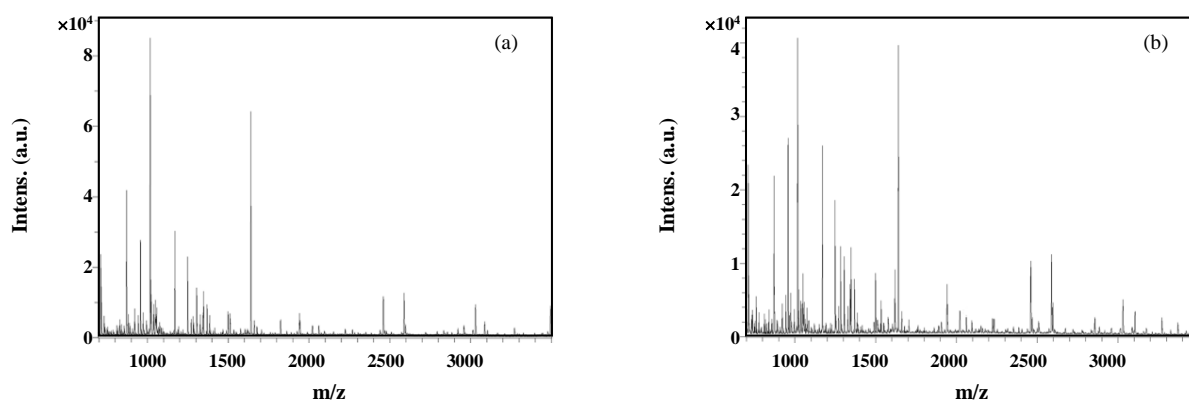


Fig. 9: MALDI-TOF mass spectra of BSA and β -casein mixture within 30 min digestion by the enzyme reactor. (a) 100:1 (BSA/ β -casein); (b) 500:1 (BSA/ β -casein).

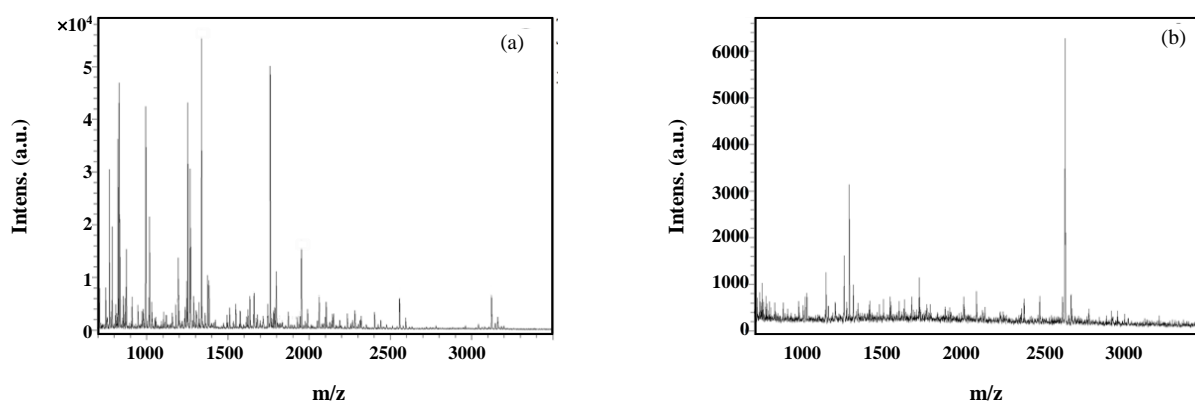


Fig. 10: MALDI-TOF mass spectra of the result of skim milk solution within digestion of 30 min used enzyme reactor. (a) Enzyme reactor digestion method. (b) The traditional solution digestion method.

however, the present enzyme reactor could be repeated usage for more than 4 times according to the results of the sequence coverage matched peptides and enzyme activity.

The stability was also evaluated by maintaining the enzyme reactor at 4 °C for 12 d. When it was used to digest skim milk, the obtained MALDI-TOF score could reach 88 with 43 matched peptides (Fig.11.), which was equivalent to those obtained by freshly made enzyme reactor. The same conditions were utilized for the traditional digestion, only one matched peptide was obtained. The obtained results indicated such high stability of the enzyme reactor. The batch-to-batch reproducibility of the present enzyme reactor for β -casein digestion was also evaluated, and the RSD of the sequence coverage

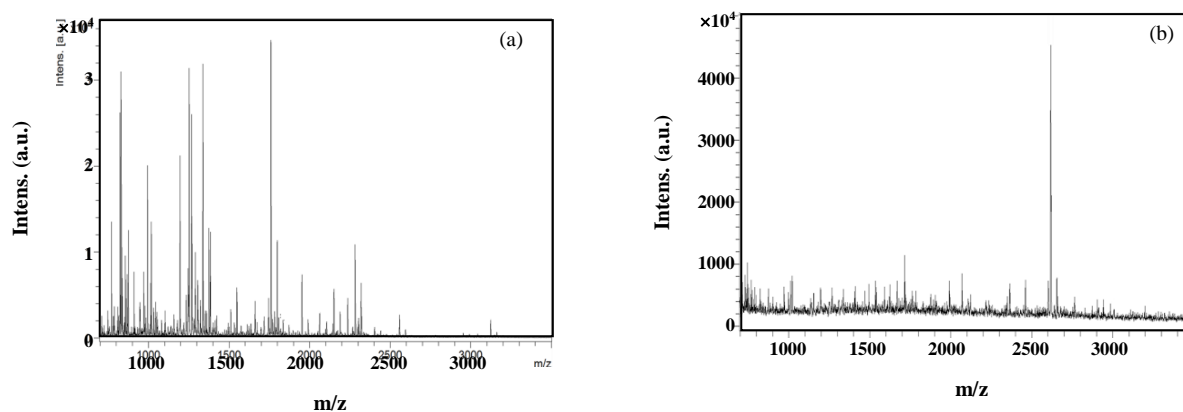
was 1.68% ($n = 5$). These results demonstrated the excellent reproducibility of the present enzyme reactor.

CONCLUSIONS

A recyclable poly(quaternary ammonium ionic liquid)s enzyme reactor for highly efficient protein digestion was first demonstrated. The monodispersed spherical poly(quaternary ammonium ionic liquid)s particles were prepared through a simple one-step way. Although, the enzyme was immobilized onto the poly(quaternary ammonium ionic liquid)s particles based on non-covalent interaction, only 1.5% enzyme was lost in 50 mM NH_4HCO_3 solution. The digestion time could be reduced from 16 h to only 5 min. Compared

Table 3: Result of the obtained matched peptides after each process of continuous enzymatic used the enzyme reactor.

Recycle times	1	2	3	4	5	6	7	8
Sequence coverage	43%	48%	46%	41%	38%	35%	42%	28%
Matched Peptides	38	37	38	36	29	25	18	16

**Fig. 11: MALDI-TOF mass spectra of the result of skim milk solution within digestion of 30 min (a) enzyme reactor (the material was stored at the condition of 4 °C for 12 d); (b) the traditional solution digestion method.**

with traditional solution digestion, the present enzyme reactor showed excellent biocompatibility, good enzyme loading capacity, and reusability. Our work offered a novel enzyme loaded material with great potential for application in proteomic analysis.

Supplementary Materials

Supplementary material published online alongside the manuscript (Figure S1 : MALDI-TOF mass spectra of the result of β -casein solution, (a,b) 4h, (c, d) 8 h, (e, f) 12 h, (g, h) 16 h. The spectra of a,c,e and g obtained by the enzyme reactor and the spectra of b, d, f, h obtained by in-solution traditional digestion, Figure S2 : Effect of recycling on the activity of the enzyme reactor, Table S1 : Difference of peptides obtained by enzyme reactor digestion and traditional solution digestion.)

Acknowledgments

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