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Covalent immobilization of trypsin onto thermo-sensitive poly(*N*-isopropylacrylamide-*co*-acrylic acid) microspheres with high activity and stability

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ABSTRACT: Poly(*N*-isopropylacrylamide-*co*-acrylic acid) (P(NIPAM-*co*-AA)) microspheres with a high copolymerized AA content were fabricated using rapid membrane emulsification technique. The uniform size, good hydrophilicity, and thermo sensitivity of the microspheres were favorable for trypsin immobilization. Trypsin molecules were immobilized onto the microspheres surfaces by covalent attachment. The effects of various parameters such as immobilization pH value, enzyme concentration, concentration of buffer solution, and immobilization time on protein loading amount and enzyme activity were systematically investigated. Under the optimum conditions, the protein loading was 493 ± 20 mg g^{-1} and the activity yield of immobilized trypsin was 155% ± 3%. The maximum activity (V_{max}) and Michaelis constant (K_m) of immobilized enzyme were found to be 0.74 μM s^{-1} and 0.54 m*M*, respectively. The immobilized trypsin showed better thermal and storage stability than the free trypsin. The enzyme-immobilized microspheres with high protein loading amount still can show a thermo reversible phase transition behavior. The research could provide a strategy to immobilize enzyme for application in proteomics. © 2016 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43343.

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INTRODUCTION

Trypsin is the most commonly used digestive enzyme for hydrolysis in proteomics studies, as well as for industrial production.[1] A major drawback of this enzyme is the low stability, which can lead to uncontrollable catalytic condition. The rapid autolysis of trypsin in solution will form undesirable and interfering fragments, causing the efficiency of the catalytic reaction decrease and the cost increase.[2,3] To solve these problems, immobilization of trypsin onto a heterogeneous support has been used to be an effective strategy, since it is associated with low degree of auto-digestion, reuse of enzyme and high digestion efficiency.[4] Different kinds of inorganic and organic materials, such as glass beads, silica nanoparticles and polymers have been chosen as the support matrices.[5–8]

Poly(*N*-isopropylacrylamide) (PNIPAM) is considered as a functional polymer material with thermo-responsive, and can undergo a phase transition from a hydrophilic state to a hydrophobic state reversible at temperature around lower critical solution temperature (LCST, about 32 °C) in its aqueous solution.[9] The phase transition of PNIPAM can significantly affect enzyme activity in response to temperature. PNIPAM-based polymers have been applied for the immobilization of enzyme through physical adsorption, covalent bonding, microencapsulation and matrix entrapment.[10] Among the various methods available for enzyme immobilization, covalent binding is particularly attractive because it provides stable immobilization.[11] The covalent attachment of enzymes to PNIPAM was realized by Hoffman's group.[12,13] They prepared reversible soluble-insoluble oligomer-enzyme conjugates by conjugating a thermally sensitive PNIPAM oligomer to trypsin. Chen *et al.* used a similar method to prepare α-chymotrypsin conjugated with oligo-NIPAM.[14] Covalent immobilization of enzymes on copolymer of *N*-isopropylacrylamide (NIPAM) and acrylamide (AAm) was reported by Kawaguchi *et al.*[15] Carboxyl groups and amino groups were introduced to the submicron hydrogel microspheres to immobilize trypsin. Hamerska–Dudra *et al.* immobilized glucoamylase and trypsin on crosslinked PNIPAM gels with ethylenediamine.[16]

Generally, in order to decrease diffusion limitations of carriers and retain a more native-like structure of enzyme, the size of matrix material is selected as nano-scale.[17] However, for protein identification,

immobilized enzyme is often filled into columns or packed-bed reactors. Micrometer-sized particles with narrow size distribution and controllable size are preferable. These particles can offer a clear and consistent response, easy separation from substrate and simply observation by optical microscopy. Therefore, it is essential to prepare size controllable PNIPAM microspheres with narrow size distribution for their biotechnological applications especially in enzyme immobilization. In previous studies, our group has developed a novel technique—membrane emulsification process to prepare different microspheres, and the results showed that the particle size was uniform and controllable.[18–21]

Recently, we have successfully prepared poly(*N*-isopropylacrylamide-*co*-acrylic acid) [P(NIPAM-*co*-AA)] microspheres with narrow size distribution by rapid membrane emulsification technique for the first time, and it was easy to covalently bond trypsin to the microspheres using a simple carbodiimide-based method.[22,23] Unfortunately, immobilization of trypsin on the microspheres resulted in a loss of the activity. Therefore, it is still necessary to further explore trypsin immobilization onto P(NIPAM-*co*-AA) microspheres for improving the activity and stability. In present study, P(NIPAM-*co*-AA) microspheres with a high value of AA content (10 wt %) were employed as carrier to immobilize trypsin. To improve the activity and stability, the effects of immobilization pH value, enzyme concentration, concentration of buffer solution and immobilization time on the activity of immobilized trypsin were investigated in detail. The kinetic parameters, thermal, and storage stabilities were assessed under free and immobilized conditions. Furthermore, the thermo-response behavior of trypsin-immobilized microspheres was investigated.

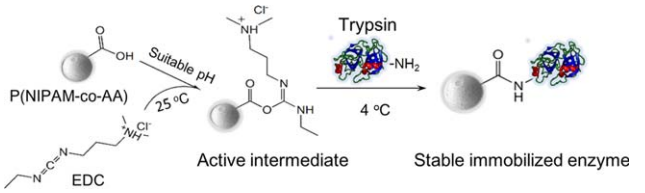
EXPERIMENTAL

Materials

N-isopropylacrylamide (NIPAM) was obtained from Tokyo Chemical Industrial. and was purified by recrystallization twice from n-hexane. Acrylic acid (AA), Span 80 and cyclohexane were supplied by Sinopharm Chemical Regent Beijing, China. Ammonium persulfate (APS) was provided from Shantou Xilong Chemical Factory, China. *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), *N*,*N*′-methylene-bisacrylamide (MBA), Rhodamine 123, fluorescein isothiocyanate (FITC), *N*-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE), Nα-benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA), trypsin from bovine pancreas (Type I), and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) was supplied from Sigma–Aldrich, USA. All reagents were of analytical grade and used as received.

Preparation of P(NIPAM-*co*-AA) and PNIPAM Microspheres

P(NIPAM-*co*-AA) microspheres were prepared according to our previous reported method.[22] Typically, 0.9 g NIPAM, 0.1 g AA, 0.15 g MBA, and 0.03 g APS were commingled together with 9.5 mL distilled water. Cyclohexane (100 mL) containing 1.9 wt % Span 80 in a beaker was used as oil phase. The water phase was dispersed into oil phase and this mixture was pressed through the SPG membrane. The obtain emulsion was stirred to remove dissolved oxygen with nitrogen bubbled about 30 min at 25 °C. Subsequently, 150 μL TEMED dissolving in 1.25 mL cyclohexane was added into the emulsion to initiate the polymerization reaction. Then, the obtained microspheres were centrifuged for 10 min and washed with acetone and water after 4 h reaction. The product was dispersed in deionized water and stored at 4 °C for further use. PNIPAM microspheres were obtained following the same method as used for the preparation of P(NIPAM-*co*-AA) microspheres, and the only difference was that AA monomers were absent in water phase. The PNIPAM microspheres were used as control sample during thermo-responsive behavior measurement.



Scheme 1. Schematic illustration of activation process of immobilization. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Trypsin Immobilization

Trypsin (EC 3.4.21.4) was immobilized onto P(NIPAM-*co*-AA) microspheres by the EDC coupling method and the schematic representation is shown in Scheme 1. A certain quantity trypsin solution of phosphate buffer saline (PBS, 50 m*M*, pH 7.0) was added to microspheres suspension (8 mL, 2.5 mg mL^{-1}) for 1 h incubation at 4 °C. A 1 mL fresh EDC solution (10 mg mL^{-1}) was added to activate carboxyl groups of microspheres at 25 °C. Then the immobilization process was performed at 4 °C for 10 h, afterward, the resultant microspheres were removed from the reaction solution by centrifuge, and washed with 10 mM phosphate buffer (pH 7.2) until no protein was detectable in the supernatant. Finally, the enzyme-immobilized microspheres were stored at 4 °C for further use. To obtain the highest activity of immobilized trypsin, several variables including pH (5.0–10.0) and concentration of trypsin solution (1–6 mg mL^{-1}), concentration of buffer solution (10–70 mM) and immobilization time (2–14 h) were optimized.

Characterization of Microspheres

Microspheres shape and surface morphology were observed by a JSM-6700F scanning electron microscopy (SEM) (JEOL, Japan). Before measurement, the microspheres were dried by CO_2 supercritical drying with K850 Critical Points Driers (Quorum/Emitech, England).[24] The resulted samples were coated with gold using a fine coater (JEOL JFC-1600, Japan) on a copper platform.

A TCS SP5 confocal laser scanning microscopy (CLSM) (Leica, Germany) was used to observe the morphology of wet microspheres. First, the microspheres were immersed in Rhodamine 123 solution and were incubated at 4 °C for 24 h. Then the unbound dye was separated by centrifugation. The suspension of microspheres was added into petri dish, and the CLSM images were captured using 488-nm excitation wavelength.

The volume-mean diameter of the microspheres was measured by laser diffraction using a Mastersizer 2000 (Malvern, UK).

Microspheres after being washed were added in a beaker with 500-mL water and were stirred by a screw propeller (1000 rpm).

The determination of transmittance change of microspheres solution at different temperatures or different pH values was carried out by an Ultrospec 2100 pro UV–visible spectrophotometer (Amersham Bioscience, America) with thermostatic control. The LCST value of microspheres was judged to be the initial break point of the transmittance verse temperature curve.

The zeta potential measurements were performed with a Zetasizer nano ZS instrument (Malvern, UK). Measurements were performed in distilled water and the average value of at least ten measurements was adopted on the zeta potential at different pH values.

Determination of Protein Loading and Enzymatic Activity

The protein loading of immobilized trypsin was used to denote the amount of trypsin immobilized per gram of microsphere. The values of protein loading were determined by subtracting the residual amount of the enzyme from the original amount added for immobilization. The protein concentration was estimated by a typical micro bicinchoninic acid (Micro BCA) method with a sensitivity of 0.5–20 μg mL^{-1}, and bovine serum albumin was used as the standard protein.[25] The fluorescent microplate reader (Infinite M200, TECAN, Switzerland) was utilized to record the absorbance of different.

The activities of free and immobilized trypsin against BAEE were determined at 25 °C by the pH-stat method according to the literatures.[26] This pH-stat method was a method of Fédération International Pharmaceutique (F.I.P.).[27] A FE20 pH-meter (Mettler Toledo) was used to monitor the pH values of the system. Concisely, 1.0 mL BAEE (20 m*M*) in sodium phosphate buffer (0.1*M*, pH 7.0) was mixed with 10.0 mL disodium tetraborate (0.72 mg mL^{-1})/calcium chloride (2.78 mg mL^{-1}) buffer (pH 8.0) at 25 °C. A certain amount of enzyme solution was added into the substrate solution. About 50 m*M* NaOH was used to titrate with the solution. The pH of the reaction solution was maintained between 7.9 and 8.0 throughout the whole measurement. The total volume of NaOH was recorded after 5 min hydrolytic reaction, and the hydrolytic activity of enzyme was calculated from the following formulas:

$$\frac{V\times 0.05}{E_w\times 5}=\text{F.I.P.(u/mg)} \quad (1)$$

where V was the volume of NaOH consumed in 5 min (L), 0.05 was the concentration of NaOH, E_w was the amount of enzyme (mg), and 5 was the reaction time (minute). One unit of activity was expressed as the decomposition of 1 μmol BAEE per min in the assay conditions, and related terms were used in this study as follows:

$$\text{Activity yield (\%)}=\frac{\text{specific activity of immobilized trypsin}}{\text{specific activity of free trypsin}}\times 100\% \quad (2)$$

$$\text{Residual activity (\%)}=\frac{\text{activity of trypsin}}{\text{initial activity of trypsin}}\times 100\% \quad (3)$$

Measurement of the Kinetic Parameters

The kinetic constants, maximum enzyme velocity (V_{max}) and Michaelis constant (K_m) were determined using BAPNA as substrate in the concentrations range (0.05–0.5 m*M*). The apparent K_m and V_{max} values for the free and immobilized trypsin were calculated from Hanes–Woolf plot (a plot of $[S]$ versus $[S]/V$)[28]:

$$\frac{[S]}{V_0}=\frac{1}{V_{max}}\times[S]+\frac{K_m}{V_{max}} \quad (4)$$

where $[S]$ was the BAPNA concentration, V_0 was the initial enzyme velocity. Experimentally, the K_m from the plot was equal to $-[S]$, whereas the V_{max} was equal to 1/slope.

FITC-labeled Immobilized-trypsin Microspheres for CLSM Study

FITC dye was chosen to label trypsin using a method as reported.[29] Briefly, FITC (1 mg) in carbonate buffer (pH = 8) was added into trypsin solution. The reaction solution was stirred at 4 °C overnight. The unreacted labeling FITC was removed by centrifugal filter devices (10,000 MWCO, Millipore). Then, the FITC-labeled trypsin was immobilized onto microspheres under the same method as the unlabeled ones.

Thermal Stability and Storage Stability

The thermal stability of the immobilized and free enzyme was assessed by determining the activity under 45 °C in phosphate buffer (50 m*M*, pH 7.2) for 180 min. Every 30-min interval, a part of sample was taken for its activity measurement. Similarly, the storage stability was monitored by measuring the activity of enzyme stored at 4 °C, and the measuring interval was 5 days.

RESULTS AND DISCUSSION

Characterization of P(NIPAM-*co*-AA) Microspheres

As reported previously, it has been proven that the hydrophilicity of the materials will be beneficial to the immobilization of trypsin, and the pore size can affect the activity of immobilized enzyme.[30] Prior work has also shown that the hydrophilicity and LCST of the P(NIPAM-*co*-AA) hydrogel can be affected by the amount of copolymerized AA, and a high acrylic acid amount in the hydrogel can reduce or even eliminate its temperature sensitivity in some cases.[31,32] Our previous research showed that the increase of AA amount would increase the content of carboxyl group in the surface of the microspheres and decrease the percentage of macropores(>50 nm) in the microspheres.[24,33] Likewise, the AA content higher than 10 wt % in the microspheres would lead to a complete suppression of thermo sensitivity at pH 7.0.

To improve the activity of immobilized trypsin and keep the thermo sensitivity of the microspheres, P(NIPAM-*co*-AA) microspheres with 10 wt % AA content were successfully prepared by rapid membrane emulsion technology. As shown in Figure 1(a), the microspheres with narrow size distribution were obtained, and the average size of resultant microspheres was about 7.0 μm. It also can be seen that the P(NIPAM-*co*-AA) microspheres were shaped well with smooth surface in Figure 1(b). Interestingly, the particle size shown in SEM images was smaller than those measured by laser particle size distribution. Similar results have been reported by other researchers.[34,35] This phenomenon could be attributed to the swelling behavior of the microspheres in solution during the laser diffraction measurement and the dry state in the SEM observation. From Figure 1(c), fluorescence was observed to be uniformly distributed in the microspheres. Rhodamin 123 had amino groups and

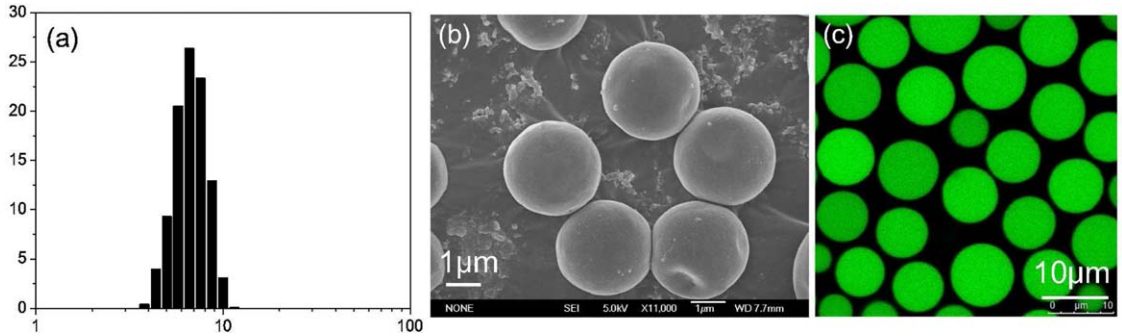


Figure 1. (a) Size distribution of P(NIPAM-*co*-AA) microspheres dispersed in water at 20 °C. (b) SEM image of P(NIPAM-*co*-AA) microspheres. (c) CLSM photograph of P(NIPAM-*co*-AA) microspheres. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

would attract with carboxyl groups, thus the CLSM image demonstrated that carboxyl groups were existed throughout the whole microspheres. The hydrophilic polymer chains and narrow size distribution could afford microspheres an ideal support for enzyme immobilization.[16,36]

The thermo-responsive behavior of P(NIPAM-*co*-AA) microspheres was very important for performance of enzyme. The activities of immobilized enzymes within thermo-sensitive hydrogels could be modulated by thermal cycling through the lower critical solution temperature (LCST).[13] From Figure 2, it could be seen clearly that the transmittance of the microspheres suspension was changed as temperature increased. The LCST of the P(NIPAM-*co*-AA) microspheres was about 38 °C, while PNIPAM microspheres had a LCST at about 33 °C. This was attributed to that an incorporation of hydrophilic monomer into PNIPAM would increase the LCST of the microgel, as a result of hinder the phase transition.[37,38] These results showed that the microspheres had a LCST behavior at temperature closed to the optimum temperature of trypsin (37–40 °C[39–41]). At temperature above LCST, the hydrophobic microspheres created a crowded and hydrophobic local microenvironment surrounding the enzymes.[42] The distinctive microenvironment was expected to have influences on preservation of trypsin at elevated temperature.

Effect of pH Value on Immobilization

The immobilization pH was an essential parameter for enzyme immobilization, which could affect the state of the carrier's surface functional groups and the charge distribution of enzyme.[41] Figure 3(a) showed the transmittance changes of the microsphere suspensions at pH ranging from 5.0 to 10.0. It was found that the transmittances of microsphere suspensions were changed slightly, implying a small changing of microspheres size. Thus, the influence of particle size on immobilization process could be ignored. As seen in Figure 3(b), the zeta potentials of the microspheres without enzyme increased with pH value until a plateau region was reached around pH 7.0. It was known that the value of pK_a for AA was at 4–5,[43] so most of carboxyl groups on the microspheres were dissociated at pH 7.0. The isoelectric point (pI) value of trypsin was 10.8,[44] and the trypsin molecules were positively charged when pH was lower than its pI value.

The effect of pH on enzyme activity and protein loading of immobilized trypsin was examined, and the results were shown in Figure 4. It was clear that protein loading and trypsin activity increased with pH value increasing from 5.0 to 7.0. With further increase in pH value, the protein loading was decreased, and the activity of immobilized trypsin reached its maximum values at pH value 7.0–8.0 and then declined. The change of protein loading could be attributed to several possible reasons. First, the optimum pH value for EDC to activate functional groups was 4.0–6.0. When pH value was higher than 7.0, the activation capacity of EDC was decrease with increasing pH, which lead to a decrease in protein loading. Second, the electrostatic adsorption between negative microspheres and positive trypsin would promote the immobilization of trypsin at pH 7.0.

As a serine protease, trypsin could express a high activity in the pH range 7.0–9.0.[45] Under strong acid or alkaline condition, trypsin would be denatured, decreasing the catalytic activity of immobilized enzyme. Meanwhile, a change in pH would affect the intermolecular hydrogen bonding resulting in a distorted conformation, and this would reduce the activity of the enzyme consequently.[46,47]

Effect of Enzyme Concentration on Immobilization

The effect of trypsin concentration on the protein loading and catalytic activity of immobilized trypsin was investigated in a

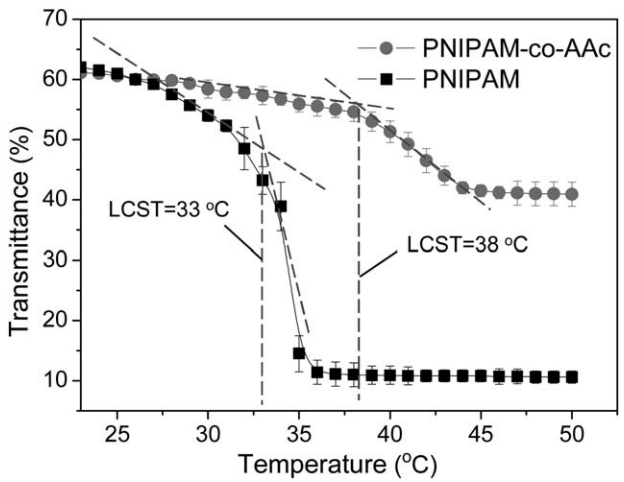


Figure 2. Phase transition behavior of PNIPAM and P(NIPAM-*co*-AA) microspheres. (pH 7.0, 10 m*M* phosphate buffer).

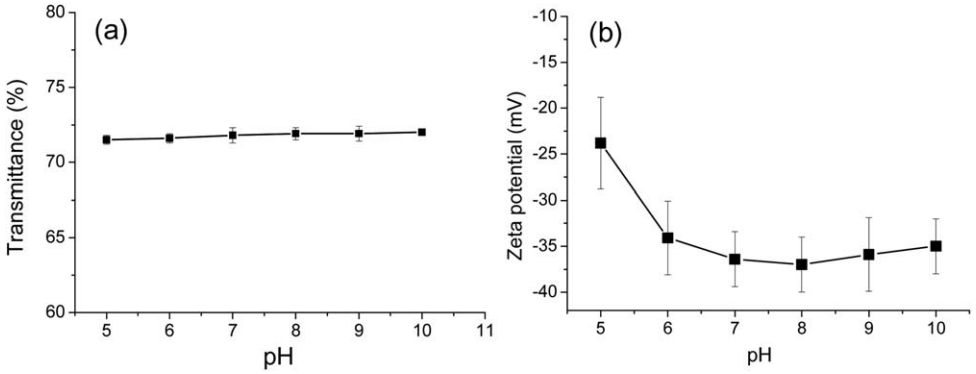


Figure 3. (a) Transmittance of P(NIPAM-*co*-AA) microsphere suspension at different pH value. (b) Zeta potential of P(NIPAM-*co*-AA) microspheres at different pH value.

range from 1 to 6 mg mL^{-1}. As shown in Figure 5, the protein loading for immobilized trypsin was increased constantly, while activity of the enzyme showed a trend of decline with enzyme concentration increasing. The decreased activity of enzyme could be considered as the result of the interaction between enzyme molecules. At a suitable enzyme concentration, trypsin could be immobilized onto microspheres in single layer status, which restrained the intermolecular reaction of immobilized trypsin molecules, and the immobilized trypsin could exhibit its maximal activity.[48] However, a high enzyme concentration led to the increase of protein loading. In this situation, trypsin molecules would entangle together easily which limited reaction substrates access to the active center of enzyme, and then the activity was decreased. This finding was in agreement with the previous literature.[49]

Effect of Concentration of Buffer Solution on Immobilization

Figure 6 illustrated the effect of PBS concentration on the protein loading and catalytic activity of immobilized trypsin. It can be seen that the protein loading amount increased a little at the concentration ranging from 10 to 40 m*M*. A decrease was observed after the PBS concentration exceeded 50 m*M*. Presumably, the main reason was that the concentration of buffer solution would affect the electrostatic interactions between the carriers and enzyme.[50,51] The high ionic strength in the higher buffer concentration solution could shield the charge effects on the support, and thus the protein loading amount was decrease.[46,52]

The profile of the activity of immobilized trypsin also showed an "up and down" tendency. It is known that metal ions can maintain the enzyme in active and stable state.[53] The increase of activity of trypsin was because that a small amount of Na^+ ion in buffer solution would combine with enzyme molecules to form activated complex, and then elevated the activity of enzyme.[54] However, an excessive level of PBS concentration would accelerate protein denaturation and the generation of salting-out phenomenon. Similar observations for immobilized lipase have also been reported.[55]

Effect of Immobilization Time on Immobilization

The effect of immobilization time on the enzyme activity was studied in the range of 2–14 h, and the results were presented in Figure 7. It was found that the activity increased significantly with increasing immobilization time up to 10 h, in which the maximum value was obtained. With the extended immobilization time, the latter binding trypsin molecules and the former ones would interact with each other intensely, which led to conformation change of trypsin molecules. At the same time, the over-loading of trypsin could lead to a multilayered stacking of enzyme, which would block the enzyme molecules from accessing to the microspheres and thus

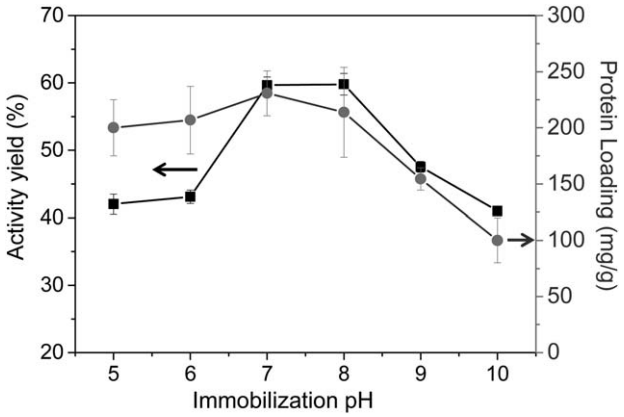


Figure 4. Effect of immobilization pH on protein loading and activity yield.

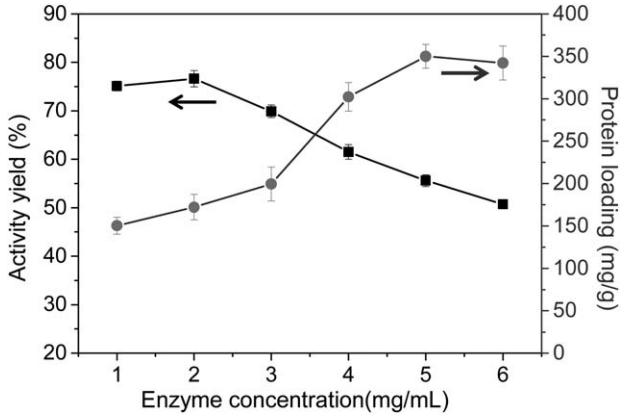


Figure 5. Effect of trypsin concentration on protein loading and activity yield.

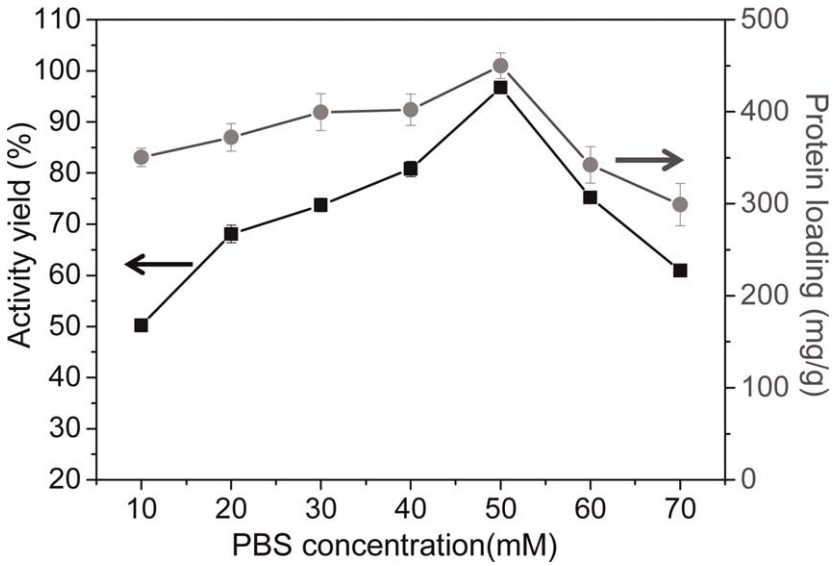


Figure 6. Effect of PBS concentration on protein loading and activity yield.

decreased the protein loading.[56] In addition, the susceptible autolysis of trypsin would be serious. Similar results have been reported elsewhere.[57] The maximum value of protein loading was at 12 h, suggesting that the immobilization of trypsin reached its saturation point.

From the above optimization results, the optimal preparation conditions for immobilization of trypsin onto P(NIPAM-*co*-AA) microspheres by covalent binding were determined as follows: the pH value was 7.0, trypsin concentration was 2 mg mL^{-1}, PBS concentration was 50 m*M* and immobilization time was 10 h. Under these optimized conditions, the protein loading of immobilized trypsin was 493 ± 20 mg g^{-1} (protein/support), and the activity yield of the immobilized trypsin was $115\% \pm 3\%$. These results indicated that the abundant functional groups of the P(NIPAM-*co*-AA) microspheres were excellent reaction sites for trypsin immobilization.

Kinetic Parameters

The kinetic parameters of free trypsin and trypsin immobilized on the microspheres were shown in Figure 8, and the apparent V_{max} and K_m values calculated from Hanes–Woolf plots were given in Table I. K_m values that indicated the effective diffusion characteristics of the enzyme were found as 0.34 and 0.54 m*M* for free and immobilized trypsin respectively, suggesting that the affinity of trypsin to its substrate was slightly decreased by immobilization. This increased K_m might be attributed to that immobilization induced conformational changes of the enzyme and an increase of diffusion limitations, resulting in a lower possibility to form substrate–enzyme complex.[58] V_{max} values that reflected the enzymatic reactions were estimated as 0.51 and 0.74 μM s^{-1} for the free and immobilized trypsin, respectively. This result suggested that the microenvironment created by the soft microspheres was compatible with enzyme molecules, which was beneficial to expression of activity of trypsin.[59]

Thermo-Responsive Behavior of Enzyme-immobilized Microspheres

The thermo-responsive behavior of enzyme-immobilized microspheres was also examined, and the result was shown in Figure 9. The transition temperature of microspheres with trypsin binding was around 39–42 °C, indicating that immobilization of trypsin caused a shift of LCST when compared with pristine

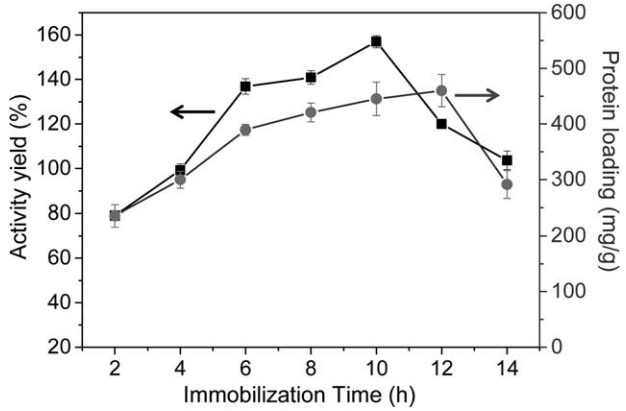


Figure 7. Effect of immobilization time on protein loading and activity yield.

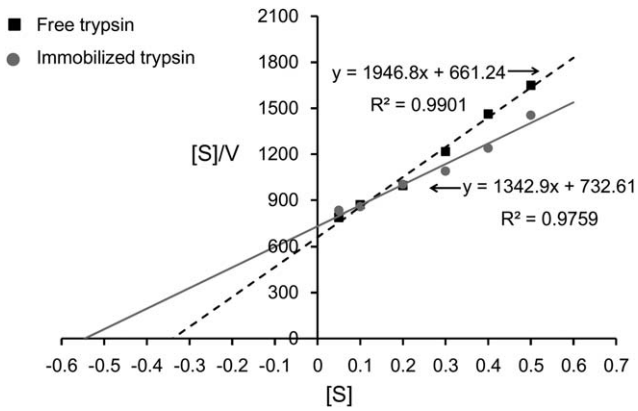


Figure 8. Hanes–Woolf plot curve for free and immobilized trypsin.

Table I. Yields of Protein Loading, Activity, K_m and V_{max} Values of Free and Immobilized Trypsin

Sample	Protein loading (mg g^{-1}-support)	Activity (F.I.P.u mg^{-1})	Activity yield (100%)	K_m (m*M*)	V_{max} (μM s^{-1})
Free trypsin	-	10,000	100%	0.34	0.51
Immobilized trypsin	493 ± 20	15,500	155%	0.54	0.74

microspheres. The result was consistent with previously reported result.[15] The reasonable explanation was that after immobilization, enzyme molecules tightened the PNIPAM-*co*-PAA network,[50] which increased the rigidity of microspheres and hindered the phase transition of microspheres.

Figure 9 showed the CLSM images of FITC-labeled trypsin. It can be seen that the enzyme-immobilized microspheres showed stronger fluorescence on the surface of the microspheres, suggesting the trypsin molecules was situated on the surface of the microspheres. Additionally, the average particle size of the enzyme-immobilized microspheres at 25 °C was bigger than that of the microspheres at 45 °C, which indicated that the volume transition of the microspheres was reversible by changing temperature above and below the LCST. It was noteworthy that the leaching phenomenon of fluorescence in the microspheres was hardly observed during the heating and cooling cycles. The results confirmed that trypsin molecules were stably bound onto the microspheres by covalent attachment.

Thermal Stability and Storage Stability

The stability of the immobilized enzyme is significant for industrial uses. The capability of the immobilized trypsin to remain active was investigated by incubating trypsin at 45 °C for 180 min and compared with the initial activity. Figure 10 showed the resultant thermal stability of free and immobilized trypsin for various periods. It could be seen that after 60 min of incubation, the free trypsin retained only 60% of its original activity, whereas the immobilized trypsin maintained 91% of its activity. Prolongation of the incubation time, both the residual enzymatic activities of free and immobilized trypsin decreased. The result was similar with that reported by other literature.[60] However, it was obvious that the immobilized trypsin exhibited a residual activity of 70% after 180 min. The enhanced stability can be due to multi-point fixation between the trypsin and the support, which restricted the conformational flexibility of the enzyme.[54] In addition, an amount of trypsin molecules were immobilized on and in the vicinity of the microsphere surface. At temperature over the LCST of microspheres, the microspheres would collapse and thus prevented protein denaturation processes.[61]

Storage stability of free and immobilized trypsin for longer periods at 4 °C was further investigated, and the results were presented in Figure 11. After 60 days of storage, the immobilized trypsin still kept about 80% of its activity, while the activity of the native trypsin decreased to <55% of the initial value. This phenomenon was in good agreement with other literature.[62] It was because the covalent bond between trypsin and microspheres restricted the movement of trypsin molecules, and the auto-proteolysis was weaken in immobilized trypsin.[40] These results demonstrated that the immobilized trypsin displayed more resistant to thermal inactivation than its free form, and had excellent storage stability throughout the whole time range.

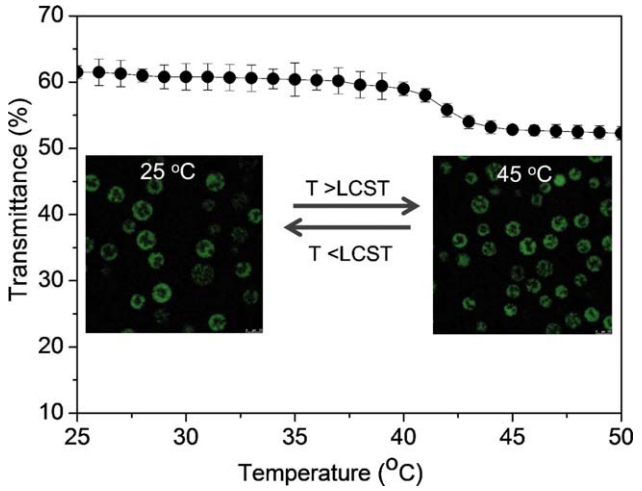


Figure 9. Phase transition behavior of enzyme-immobilized P(NIPAM-*co*-AA) microspheres. (pH 7.0, 10 m*M* phosphate buffer). The CLSM images of FITC-labed enzyme-immobilized P(NIPAM-*co*-AA) microspheres at 25 and 45 °C are shown inset. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

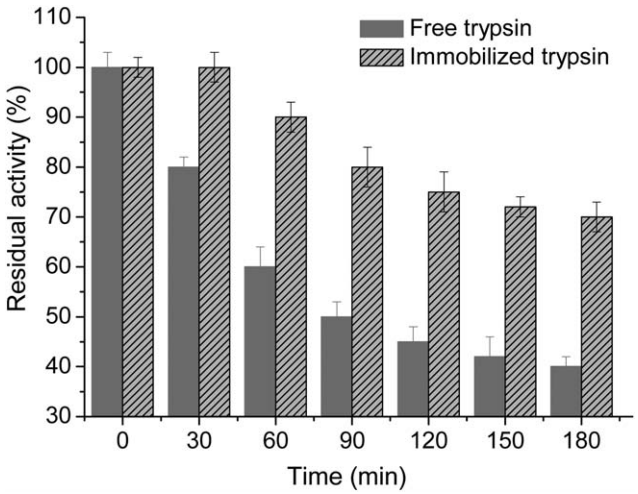


Figure 10. Thermal stability of free and immobilized trypsin at 45 °C for 180 min.

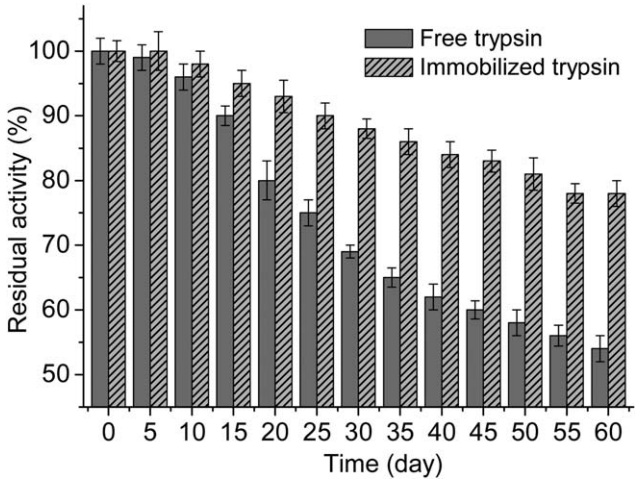


Figure 11. Storage stability of free and immobilized trypsin at 4 °C for 60 days.

CONCLUSIONS

In summary, trypsin was successfully covalently bound to the thermo-sensitive P(NIPAM-*co*-AA) microspheres with high activity and stability. The uniform microspheres with a high value of AA content (10 wt %) had an improved hydrophilicity and well thermo response behavior, which enhanced the activity of enzyme. The optimal parameters in the immobilization process were as follows: pH value was 7.0, trypsin concentration was 2 mg mL^{-1}, PBS concentration was 50 m*M* and immobilization time was 10 h. Under the optimum conditions, the protein loading amount and the activity yield of the immobilized trypsin were 493 ± 20 mg g^{-1} and $155\% \pm 3\%$, respectively. The V_{max} and K_m value of the immobilized trypsin were found to be 0.74 μM s^{-1} and 0.54 m*M*, respectively. The enzyme-immobilized microspheres exhibited an inverse temperature response behavior at around 39–42 °C higher than LCST of P(NIPAM-*co*-AA). The immobilized trypsin maintained 91% of its initial activity after 60 min at 45 °C comparing with only 60% for the free form. After storage for 60 days at 4 °C, the immobilized trypsin retained about 80% of its original activity. These results demonstrates that the thermo-sensitive P(NIPAM-*co*-AA) microspheres can be employed as enzyme carrier to improve the enzyme activity and stability. The immobilized system will have a great potential application in proteomics research.

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