Preparation and Property of Urease Immobilization with Cationic Poly(4-vinylpyridine) Functionalized Colloidal Particles

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We report here a novel immobilization matrix for high enzyme loading using cationic hairy latex particles. Specifically, poly(4-vinyl-*N*-ethylpyridine bromide)-functionalized polymeric colloidal particle was used to immobilize urease by its long charged chain structure. The successful immobilization of urease on the cationic hairy colloidal particles was confirmed by Field-emitting scanning electron microscopy (SEM) images, Fourier transform infrared (FT-IR) spectra and zeta potential measurements. Under the optimized conditions of pH = 8.0 and 1.4 mg mL^{-1} of urease concentration, the enzyme loading could reach 355 ± 22 mg g^{-1} support with the activity of $1.486 \times 10^4 \pm 169$ U g^{-1} support. The pH and thermal stability of urease was enhanced upon immobilization, and it could retain almost 100 % of its initial activity after repeating the catalysis reaction 10 times. The cationic hairy colloidal particles had not only high protein loading, but also enhanced the stability of enzyme. These properties prompt this type of cationic hairy colloidal particles to be used as a promising matrix for enzyme immobilization.

Key words:
Urease, poly(4-vinylpyridine), colloidal particles, immobilization

Introduction

Urease (urea amidohydrolase, EC 3.5.1.5), widely distributed in nature, catalyzes the hydrolysis of urea to ammonia and carbon dioxide at a rate 10^{14} times faster than the uncatalyzed reaction.[1–3] Among enzymes, urease is most extensively studied for immobilization due to the possible exploitation in practical applications, such as quantitative determination of blood urea in clinical examination and detection of heavy metal ions in environment.[4,5] It has been found that the immobilization of the enzyme enables the repeated use of the enzyme and thereby reduces the operating costs, as well as enhances stability and facilitates easy separation.[6] In the past decade, many immobilization approaches have been developed to immobilize the enzyme and a variety of matrixes have been used for urease immobilization.[7–16] Among them, polymeric microspheres have attracted much attention because they can be modified easily by functional groups for immobilization. For example, Elcin et al. immobilized urease within polyanionic carboxymethylcellulose/alginate microspheres coated with a cationic chitosan;[17] Kayastha et al. reported ureases from jack beans (Urs-JB) and pigeonpea (Urs-PP) had been immobilized onto poly(styrene-co-acrolein) microspheres.[18] However, for the various matrixes used as immobilization supports, the low loading capacity always resulted in low efficacy of the immobilized enzyme and limited their practical application.[19] Therefore, there is still constant exploration for new immobilization matrix until now.

The low loading capacity is always associated with the few sites for enzyme immobilization and low specific surface area. In our previous work, we developed a new microsphere support consisting of cationic poly(4-vinylpyridine) hairy colloidal particles for protein adsorption (such as bovine serum albumin). The capacity of protein adsorption could reach a high capacity of about 900 mg g^{-1}, because the chain length of charged hairy could be changed by the controlled synthesis, and there is a high specific area due to the nanosize (~50 nm).[20] Furthermore, the positive charges of the microsphere support remained at constant value in the entire pH range, and so it could be used to adsorb the protein with different isoelectric point (pI). We expect that this kind of nanoparticles as supports can provide more sites and high specific surface area for immobilizing enzymes, thereby leading to high loading capacity for enzymes and high catalytic efficiency.

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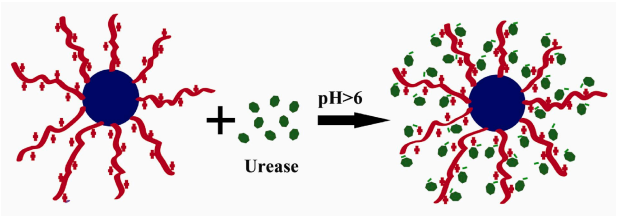
To the best of our knowledge, this is an area yet to be explored.

In this paper, the cationic poly(4-vinylpyridine) hairy colloidal particles was used as a novel immobilization matrix for significantly improving the enzyme loading capacity. Urease was selected as the enzyme model, which has an isoelectric point (pI = 4.9 – 5.5),[1] and above that urease should have a structure of negative charge and would be adsorbed on the positively charged support through the electrostatic interaction (Scheme 1). The immobilization of urease on the cationic hairy colloidal particles was characterized by Field-emitting scanning electron microscopy (SEM) imagines, Fourier transform infrared (FT-IR) spectra, and zeta potential measurements. The immobilization conditions were optimized, and the properties of the resultant immobilized urease were investigated. To the best of our knowledge, cationic hairy colloidal particles are used for the first time as the immobilization matrix for enzyme.



Scheme 1 – *Schematic for the immobilization of urease onto cationic hairy colloidal particles*

Material and methods

Material

Urease (Mn = 489 KDa) from jack bean and modified BCA protein assay kit were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Urea, mercury iodide and other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The functional PSt latex particles are prepared according to the literature method.[20]

Characterization

SEM images were taken by a HITACHI S-4700 microscope operated at an accelerating voltage of 15 kV. FT-IR spectra were recorded on a Varian-1000 spectrometer. The samples were ground with KBr crystals and the mixture was then pressed into a pellet for IR measurement. The zeta potential of the microspheres was measured using a Malvern Zetasizer with irradiation from a 632.8 nm He-Ne laser.

Immobilization of urease

The urease solution (1.4 mg mL^{-1}) was prepared in phosphate buffer (pH = 8.0, I = 0.008 mol L^{-1}). The urease solution (3 mL) and the latex particles (0.5 mL, 14.0 mg mL^{-1}) were mixed together and stirred gently for 1 h at room temperature. Then the immobilized enzyme was separated by centrifuging at 10,000 rpm at 4 °C for 30 minutes (Beckman Coulter, J-26xp, USA). The precipitate was then purified by re-suspending in 4 mL of the same buffer and centrifuging again under the same conditions, and once more until there was no protein detected. Finally, the immobilized enzyme was stored at 4 °C in 0.4 mL of phosphate buffer (pH = 8.0, I = 0.008 mol L^{-1}).

Protein concentrations were determined according to the procedure given in modified BCA protein assay kit (Sangon Biotech Co., Ltd. SK3051). The protein loading, i.e. the amount of protein immobilized on unit mass of the latex particles (mg g^{-1} support), was calculated as:

$$\text{Protein Loading (mg g}^{-1}\text{ support)} = (m_t - m_i)/W \quad (1)$$

where m_t, m_i is the amount of protein (mg) in the initial solution and the supernatant after immobilization, respectively, and W is the weight of the latex particles (g).

The retained activity of immobilized urease, i.e. the percentage of the activity of immobilized urease in the activity of free urease used for immobilization, was calculated as:

$$\text{Retained activity (\%)} = U_t/U_i \times 100\ \%. \quad (2)$$

where U_i and U_t is the activity (u) of the initial urease solution and the immobilized urease, respectively.

Activity assays of urease

0.55 mL of phosphate buffer (pH = 7.0, I = 0.008 mol L^{-1}) and urea solution (0.2 mol L^{-1}, 0.35 mL) were mixed together and pre-incubated at 37 °C for 5 minutes. The reaction was started by adding 0.10 mL of free enzyme solution (1 mg mL^{-1}) and carried out for 10 minutes with constant shaking at 37 °C (Water-bath Thermostatic Oscillator, SHY-2, Huanyu Co., Ltd, China). The enzymatic reaction was terminated by adding 0.5 mL of $ZnSO_4$ solution (10 %). The amount of generated NH_3 was determined at wavelength of 460 nm (SpectrumLab 22pC, Lengguang Co., Ltd, China) by measuring the intensity of the colored compound formed after the addition of Nessler's reagent. One unit of urease activity liberates 1μmol of NH_3 from urea per minute at 37 °C and pH = 7.0.

The immobilized urease was well dispersed in phosphate buffer (pH = 8.0, I = 0.008 mol L^{-1}) by ultrasonic cleaner before activity determination and the immobilized urease activity was determined as described above except that 0.1 mL immobilized urease was used instead of 0.1 mL free enzyme solution.

Properties of the immobilized urease

Properties of immobilized and free urease were measured using urea (0.2 mol L^{-1}) as substrate and carried out for 10 minutes with constant shaking. The effect of temperature on the activity of the free and immobilized enzyme was investigated at 37 – 62 °C in phosphate buffer (pH = 7.0, I = 0.008 mol L^{-1}). The effect of pH on the activity of the free and immobilized enzyme was investigated in citric acid buffer (pH = 3.0 – 5.0, I = 0.008 mol L^{-1}) and in phosphate buffer (pH = 6.0 – 8.0, I = 0.008 mol L^{-1}) at 37 °C. The results of relative activity about pH and temperature are presented in a normalized form, with the highest value of each group being assigned 100 % activity.

The thermal stability of the free and immobilized urease was determined by the measurement of residual activity of the enzyme exposed to various temperatures (37 – 52 °C) at pH = 7.0 for 3 h. Activity of the samples was determined using urea (0.2 mol L^{-1}) as substrate at 37 °C, pH = 7.0 and carried out for 10 minutes with constant shaking. The thermal stability of urease was investigated by measurement of residual activity after incubation for 3h. Residual activity was calculated by using the initial activity of the free and immobilized urease as 100 %, respectively.

Operational stability of the immobilized urease was investigated by measurement of residual activity of the enzyme after each batch of reaction. After each reaction run, the reaction mixture was centrifuged at 10,000 rpm (4 °C) for 30 minutes. The precipitation was then re-suspended in buffer solution and reused to catalyze the next batch reaction as before to determine the activity again. Residual activity was calculated by using activity of the first time run as 100 %.

All of the experiments were carried out at least in triplicate.

Results and discussion

Characterization of immobilized urease

SEM image of the latex particles and immobilized urease

The morphology changes of the latex particles after urease immobilization were investigated by SEM images. The typical results are presented in Fig. 1. As can be seen from Fig. 1a, the latex particles have a spherical form with diameter of about 100 nm. After immobilization of urease, a significant change happened to the latex particles, i.e. the latex particles-urease complex formed cluster structures (Fig. 1b). This phenomenon may be attributed to the electrostatic interaction: the latex particles have many long hydrophilic chains with a large number of positive charges, resulting in electrostatic repulsion between the particles; whereas, after the urease with negative charges was adsorbed on the latex particles, positive charges on the latex particles were neutralized, resulting in the abatement of electrostatic repulsion between particles, and then the latex particles aggregated together. Actually, the cluster structure led to the easy separation of the immobilized enzyme by centrifugation after each reaction run, which enables the repeated use of the immobilized enzyme.

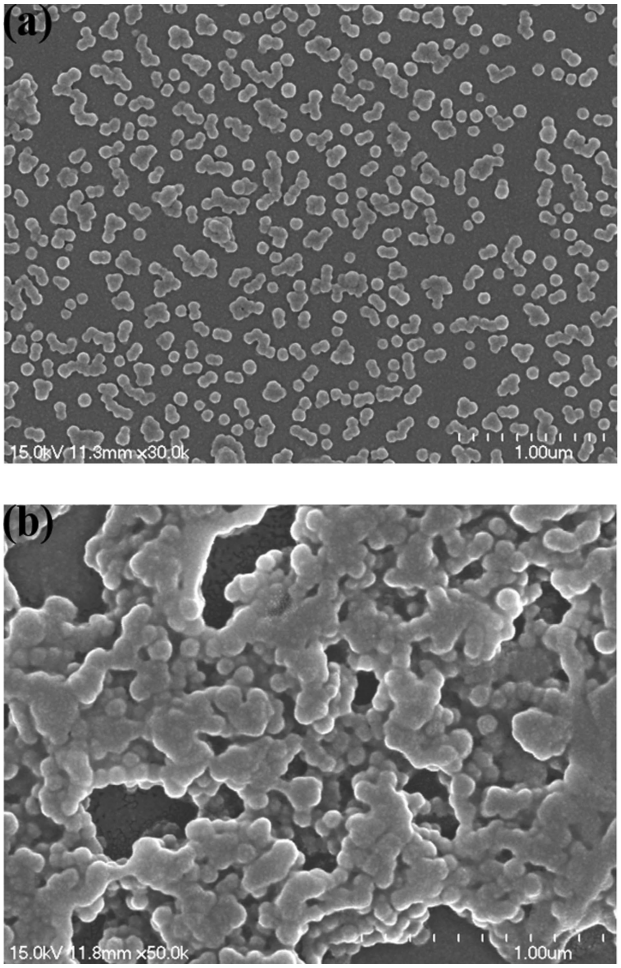


Fig. 1 – *SEM images of latex particles (a) and (b) latex particle-urease complex (Immobilization conditions: 1.4 mg mL^{-1} of urease concentration, pH = 8.0, I = 0.008 mol L^{-1})*

IR spectra of immobilization urease

The surface structures of the immobilized urease were characterized by FT-IR spectra (Fig. 2). In comparison with the latex particles (Fig. 2, trace a), the characteristic peaks occur for the latex parti-

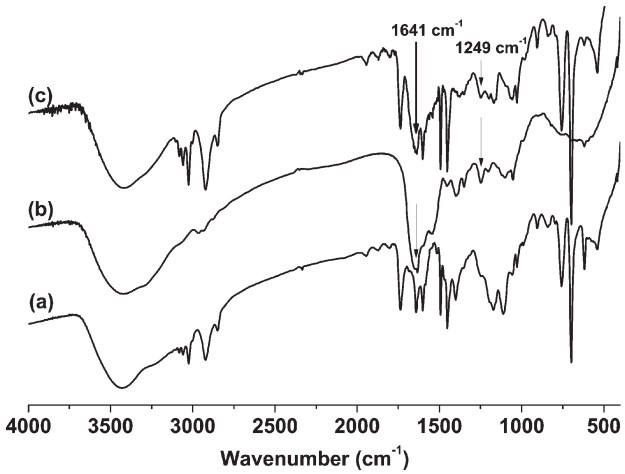


Fig. 2 – *FT-IR spectra of (a) latex particles, (b) urease, and (c) latex particles-urease complex (Immobilization conditions: 1.4 mg mL^{-1} of urease concentration, phosphate buffer pH = 8.0, I = 0.008 mol L^{-1})*

cle-urease complex (Fig. 2, trace c) at 1641 cm^{-1} and 1249 cm^{-1} corresponding to –CONH- (amide I) and C-N stretching (amide III) respectively, w, suggesting the successful immobilization of urease on the latex particles. Additionally, the similarity of the spectra of the free enzyme and the immobilized enzyme indicates that the immobilized enzyme retains the essential feature of its native structure on the support.[21]

Zeta potential of immobilized urease

The zeta potential is a measure of the number of charges per particle surface, and it can be used to assign the change of the surface charge created by anions and cations. The zeta potential measurement was also implemented for the surface characterization of the latex particles. Fig. 3 shows the zeta potentials of the latex particles, urease in buffer solution, and the latex particles-urease complex. The latex particle has a zeta potential of 22.6 mV (Fig. 3, trace a), and after immobilizing urease with a zeta

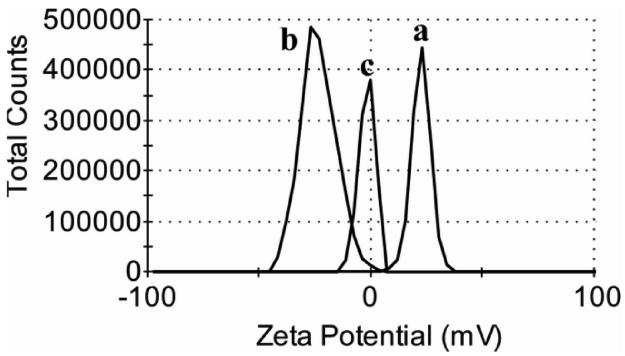


Fig. 3 – Zeta potential of (a) latex particles (b) urease in phosphate buffer (I = 0.008 mol L^{-1}, pH = 6.0), and (c) latex particles-urease complex (immobilization conditions: 1 mg mL^{-1} of urease solution, pH = 6.0, I = 0.008 mol L^{-1}, 1 h)

potential of –22.8 mV (Fig. 3, trace b), the zeta potentials are expectedly decreased to -0.27 mV for the latex particles-urease complex (Fig. 3, trace c). The results further indicated that urease was successfully immobilized on the support.

Optimal conditions for immobilizing urease

The effects of buffer pH and enzyme concentration on the immobilization of urease onto the latex particles were investigated. Urease was dissolved in buffer solution with different pH (phosphate buffer, pH = 6.0 – 8.0, I = 0.008 mol L^{-1}; or Tris-HCl buffer, pH = 8.5, I = 0.008 mol L^{-1}). The urease concentration in the range of 0.8 – 2.0 mg mL^{-1} (phosphate buffer, pH = 8.0, I = 0.008 mol L^{-1}) was investigated in the experiment.

Effects of pH on the urease immobilization

Urease has an isoelectric point of pI = 4.9 – 5.5,[1] and above that urease should be negatively charged. Therefore, the immobilization of urease was performed in the buffer solution with pH = 6.0 – 8.5. Fig. 4 shows the activity of immobilized urease, retained activity, and protein loading as a function of buffer pH. The protein loading increased to a maximum value with the increase of buffer pH from 6.0 to 8.5. The result can be attributed to the fact that the immobilization of urease on the latex particles mainly depends on the electrostatic interaction between urease and support: the higher the pH, the more negative the urease, thereby leading to higher protein loading.

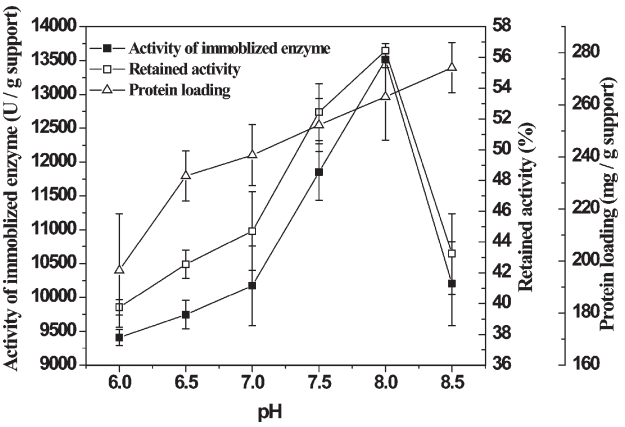


Fig. 4 – *Effect of pH on immobilization of urease onto latex particles (Immobilization conditions: 1 mg mL^{-1} urease solution, I = 0.008 mol L^{-1}, 1 h)*

In addition, the activity of the immobilized enzyme and the retained activity peaked at pH = 8.0 and then decreased. The significant decrease might be associated with the change of enzyme conformation at higher pH value.[5] Therefore, pH = 8.0 was

selected as the optimal pH condition for the immobilization of urease.

Effects of enzyme concentration on urease immobilization

As shown in Fig. 5, the protein loading increased with urease concentration, and reached 462 ± 10 mg g^{-1} support at the urease concentration of 2.0 mg mL^{-1}. It can be seen that the protein loading ability of the latex particles was far higher than that of other supports (Table 1).[6,22–24] The high protein loading should be attributed to the fact that the latex particles possess many long side chains with a large number of positive charges, which provides many electrostatic interaction sites for the enzyme, resulting in better enzyme immobilization in comparison with other supports.

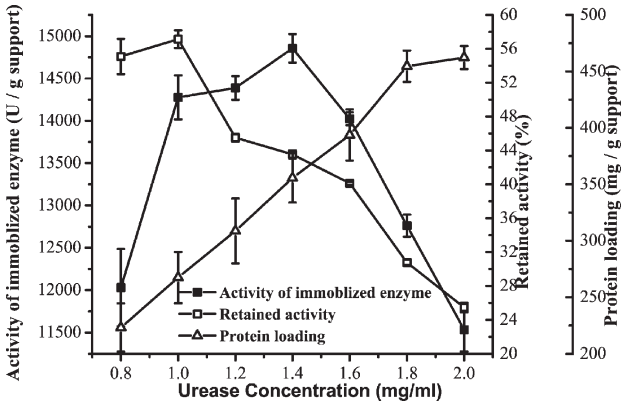


Fig. 5 – *Effect of urease concentration on immobilization of urease onto latex particles (Immobilization conditions: pH = 8.0, I = 0.008 mol L^{-1}, 1 h)*

Table 1 – *Amount of immobilized urease per unit of support on different supports*

Support type	Protein loading (mg g^{-1} support)	Reference
Polyamide hollow-fibre membrane	78	6
Poly(HEMA-MAH)	47.8	22
Poly(HEMA-MAH)–Ni(II)	66.1	22
Eupergit®C250L	171	23
Glass beads	13.71	24

It was also seen that the activity of immobilized urease reached $1.486 \times 10^4 \pm 169$ U g^{-1} support at urease concentration of 1.4 mg mL^{-1}, while the retained activity was 43.6±0.5 % (Fig. 5), corresponding to the protein loading 355±22 mg g^{-1} support. As the urease concentration further increased from 1.4 mg mL^{-1} to 2.0 mg mL^{-1}, the activity of the immobilized enzyme decreased rapidly to $1.157 \times 10^4 \pm 257$ U g^{-1} support though protein loading still increased. These results could be associated with the steric hindrance of the enzyme on the support: the more enzyme on the support, the more steric hindrance that resulted in lower activity yield. Thus, considering the best activity of immobilized enzyme and good retained activity, the urease concentration (1.4 mg mL^{-1}) could be used as the optimal concentration of urease for immobilization.

Properties of immobilized urease

Effect of pH on the activity of urease

The effect of pH on the activities of the free and immobilized urease was examined in the pH range 3.0 – 8.0. As can be seen in Fig. 6, both free and immobilized enzymes were sensitive to pH value of solution. Upon immobilization, the optimal pH for the activity of urease shifted down from 7.0 to 6.0 (Fig. 6). The phenomenon should be ascribed to the diffusional constraint of the support: A higher concentration of the enzyme product, ammonia, in the vicinity of the enzyme.[14] Furthermore, the cationic structure of the support will repel H^+ at pH = 6. Thus, the microenvironment around the enzyme immobilized on support was more basic than the pH of the bulk solution. Similar results were obtained by Monier and co-workers.[14]

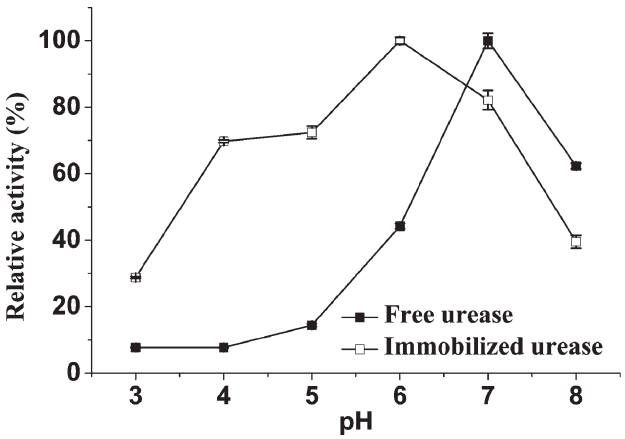


Fig. 6 – *Effect of pH on the activities of free and immobilized urease. Urease was immobilized under conditions: 1.4 mg mL^{-1} of urease concentration, pH = 8.0, I = 0.008 mol L^{-1}.*

Effect of temperature on the activities of urease

The effect of temperature on the enzyme activity was studied in the temperature range of 37 – 62 °C. The temperature optimal curves for both free and immobilized urease are shown in Fig. 7. The maximum relative activity was observed at 52 °C for both free and immobilized enzymes, and the immobilized enzyme exhibited higher relative activity than that of free urease. It was noticed that broader temperature

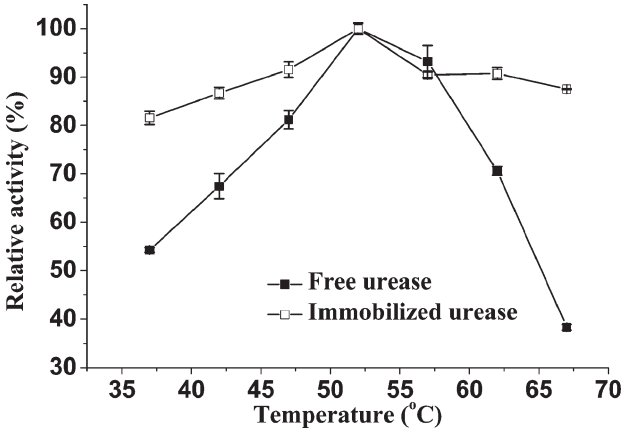


Fig. 7 – *Effect of temperature on the activities of free and immobilized urease. Urease was immobilized under conditions: 1.4 mg mL^{-1} of urease concentration, pH = 8.0, I = 0.008 mol L^{-1}.*

profile shows less sensitivity to the temperature for immobilized urease. The resistance of immobilized urease to high temperature may be attributed to the protection afforded by the support and the increase of the rigidity of enzyme structure after immobilization.

To clarify this point, thermal stability of the free and immobilized urease was also investigated by incubation at 37 – 52 °C for 3 h before measurements, the results are listed in Table 2. It can be seen that at relatively higher temperature (such as 47 and 52 °C), the residual activity of the immobilized urease was maintained above 90 % of the initial maximal activity, while the free urease lost ~35 % of its activity. The result indicated that the immobilization of urease on the latex particles could indeed lead to enhanced thermal stability. Similar results were reported previously.[6,12,25]

Table 2 – *Thermal stabilities of free and immobilized enzyme*

	Temperature / °C			
	37	42	47	52
Residual activity of free urease*	106.6±0.7 %	104.5±1.5 %	63.4±5.6 %	64.2±0.7 %
Residual activity of immobilized urease*	104.0±4.3 %	100.9±2.7 %	107.9±1.8 %	94.6±2.8 %

*mean±SD

Reusability of the immobilized urease

It is well known that the immobilization of enzyme enables repeated use and thus significantly reduce operating costs. Therefore, operational stability is one of the most important advantages of immobilized enzyme. In order to investigate this property, the immobilized urease was reused 10 times and the residual activity was measured. As indicated in Fig. 8, the immobilized urease retained ~100 % of its original activity after 10 reaction runs. This result implies that the immobilized urease has good operational stability and can successfully be used for continuous decomposition or repeated detection of urea. It should be noted that the residual activity slightly increased to above 100 % during the repeating uses, which might be attributed to the detachment of some urease from the support, resulting in less steric hindrance of the immobilized urease and thus high activity.

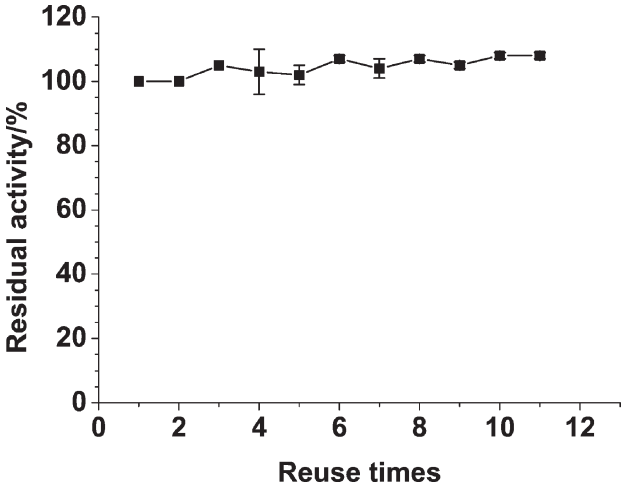


Fig. 8 – *Operational stability of the immobilized urease. (Urease was immobilized under conditions: 1.4 mg mL^{-1} of urease concentration, pH = 8.0, I = 0.008 mol L$^{-1;}$ operational condition: 0.55 mL of phosphate buffer pH = 7.0, I = 0.008 mol L^{-1}, and 0.2 mol L^{-1} of urea solution, 0.35 mL).*

Conclusion

Nanosized cationic hairy latex particles with long charged chains displayed high protein loading ability, and successfully applied as immobilization support. The successful immobilization of urease on cationic poly(4-vinylpyridine) functionalized colloidal particles was confirmed by SEM images, FT-IR spectra, and zeta potential measurements. The immobilization conditions were optimized, and the maximum activity ($1.486\times10^4\pm169$ U g^{-1} support) of immobilized urease was obtained at pH = 8.0 and urease concentration of 1.4 mg mL^{-1}, while the protein loading reached 355±22 mg g^{-1} support. This method offered a higher enzyme loading (~460 mg g^{-1} support) than other literature's support. The effects of pH and temperature were studied on the activity and stability of immobilized urease, and the results showed the stability of urease was enhanced upon immobilization. Moreover, the

immobilized urease retained ~100 % of its initial activity after repeating the catalysis reaction 10 times. All the results indicate that this support is a promising matrix for urease immobilization. We expect that a similar concept should be generically applicable to the immobilization of other enzymes. To the best of our knowledge, this is the first time that cationic hairy colloidal particles have been used as the immobilization matrix for enzyme.

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Abbreviations

SEM – Field-emitting scanning electron microscopy
FT-IR – Fourier transform infrared
pI – Isoelectric point
PSt – Polystyrene
BCA – Bicinchoninic acid

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