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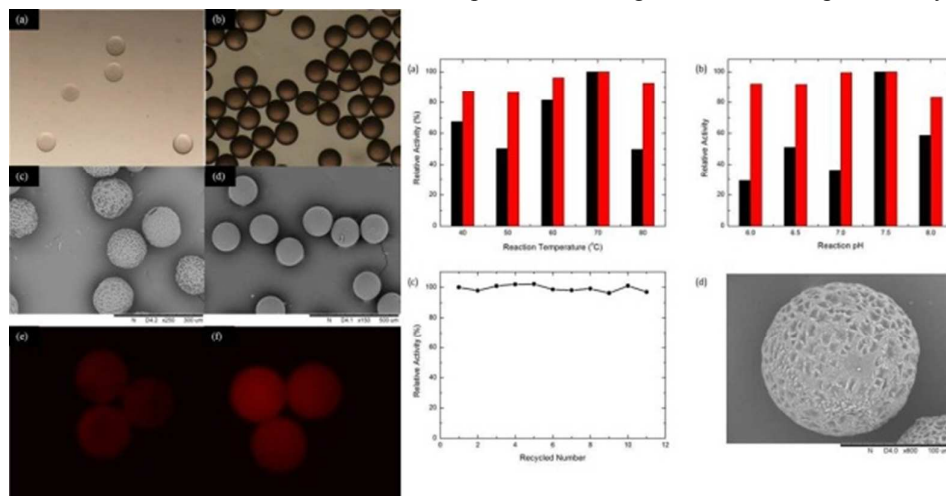
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Microfluidic Preparation of Chitosan-Poly(acrylic acid) Composite Microspheres with Porous Surface Structure[†]

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We prepared chitosan-poly(acrylic acid) composite microspheres with porous surface structure. Glucose isomerase immobilized on the microspheres showed great thermal and pH stability.



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This article presents a facile microfluidic method to prepare monodispersed chitosan-poly(acrylic acid) composite microspheres by combining chemical crosslinking and solvent evaporation. The prepared microspheres had porous surface and compact core, and the porous surface would become compact as the solidification time increased. Structures of the composite microspheres were successfully regulated by altering solidification time and dosage of crosslinker glutaraldehyde. The chitosan-poly(acrylic acid) composite microspheres with porous surface were used for the immobilization of glucose isomerase. Immobilized glucose isomerase showed good reusability and operational stability. High enzyme activity was maintained in the temperature range of 60 °C to 80 °C and in the pH range of 6.0 to 8.0. Increased thermal stability and pH stability were both realized by loading glucose isomerase on the composite microspheres.

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Introduction

Chitosan (CS) is the only alkaline cationic polysaccharide in nature, and it is the *N*-deacetylated derivative of chitin, the second most abundant polysaccharide on the earth. Chitosan is nontoxic, antibacterial, anticoagulant, biocompatible and biodegradable, which makes it a functional bio-material that can be applied in enzyme immobilization^{1,2}, drug controlled release³, adsorption of protein and heavy metal ions⁴⁻⁷, and so on. Abundant hydroxyl groups and amino groups exist on chitosan molecules. Accordingly, chemical reactions such as graft copolymerization and crosslinking are able to occur on the chitosan polymer chain^{8,9}.

Chitosan microspheres are widely applied in many fields on account of their special sizes and various structures. Chemical modification of chitosan microspheres has attracted more and more attention recently. Chemical modified chitosan microspheres have more novel and applicable structures, and many properties can be enhanced or enriched. Wei et al. successfully prepared four diverse structures of chitosan-based microspheres by adding two different crosslink reagents and a chitosan derivative^{10,11}. These prepared microspheres with different structures could provide the required protein drug release profiles for different clinical applications. Common methods of chemical modification are grafting new functional groups on chitosan polymers and blending chitosan with other polymers such as poly(vinyl alcohol), poly(ethylene oxide) and poly(acrylic acid).

Poly(acrylic acid) (PAA) is an anionic polyelectrolyte and its pK_a value is 4.75. Poly(acrylic acid) is water-soluble, biocompatible and biodegradable. It shows broad application prospect in many fields, especially in biological area. Electrostatic attraction between cationic amino groups of chitosan and anionic carboxyl groups of poly(acrylic acid) can lead to the formation of chitosan-poly(acrylic acid) polyelectrolyte complex¹⁰. By using this method, researchers realized the composition of chitosan with poly(acrylic acid) and prepared the composite microspheres. Chitosan-poly(acrylic acid) microspheres have some properties improved by comparison with pure chitosan microspheres. Dai et al. and Zheng et al. used CS-PAA microspheres to adsorb copper ions and nickel ions separately, and the adsorption capacity was much higher than chitosan microspheres^{12,13}. CS-PAA microspheres also exhibit interesting swelling characteristics. Shi et al. studied the pH-responsibility of CS-PAA composite hydrogels and obtained different response patterns with different ratio of CS and PAA¹⁴. With diverse pH-response patterns, good mucoadhesion and biocompatibility, CS-PAA microspheres were also widely applied in drug delivery. Peniche et al. prepared porous CS-PAA microspheres with massive chitosan¹⁵. Release of meclufenamic acid from the microspheres was easier when pH was about 10, which showed an alkaline surrounding. In contrast, Cho et al. found CS-PAA microspheres with massive poly(acrylic acid) exhibited a burst release when pH was about 2, which was an acidic condition¹⁶.

CS-PAA microspheres are generally prepared with the method of inverse suspension polymerization. Traditional inverse suspension polymerization was reached by stirring. Most CS-PAA microspheres prepared with this method were

not monodispersed, and their sizes and structures were not easy to control. Preparing functional materials with microfluidic methods has been rapidly developed in the last decade. With a microfluidic method, it is facile to prepare microspheres with good monodispersity, controllable sizes and controllable morphology and structure. Monodispersed chitosan microspheres have been successfully prepared by a few researchers with microfluidic methods. By using chemical crosslinking, Xu et al. prepared chitosan microspheres with controllable sizes, and the microspheres showed good potential in biological application¹⁷. By combining chemical crosslinking and solvent extraction methods, they also prepared monodispersed chitosan microspheres and controlled the structures very well¹⁸. On this basis, Xu et al. successfully prepared monodispersed CS-PAA microspheres in a simple coaxial microchannel⁷. The prepared microsphere structures were controlled easily and the microspheres had higher adsorption capacity of copper ions compared with chitosan microspheres. In this method, chitosan was crosslinked and acrylic acid grafted onto the chitosan network first, then the monomers polymerized in situ. The shortage of this method was that the polymerization of monomers was difficult to control.

In this work, we use poly(acrylic acid) and chitosan solutions as raw materials in preparing CS-PAA composite microspheres to avoid the uncontrollability of polymerization. Firstly, we prepared CS-PAA microspheres in a coaxial microchannel. Structures of prepared CS-PAA microspheres were regulated by altering different solidification conditions and interesting structures with compact core and porous surface were obtained. Then we applied these microspheres in the immobilization of glucose isomerase (GI). Immobilized glucose isomerase was used in isomerization of glucose to fructose and the catalytic performance was studied. Glucose isomerase immobilized on the prepared CS-PAA microspheres exhibited good reusability and operational stability. Increased thermal stability and pH stability were also realized.

Materials and methods

Materials

Chitosan powders were purchased from Sinopharm Chemical Reagent Co., Ltd. The degree of deacetylation was 80.0% to 95.0%. 25.0 wt.% poly(acrylic acid) aqueous solution was purchased from Alfa Aesar. The average molecular weight was 24,000. Glucose isomerase from *Streptomyces rubiginosus* was purchased from Zhengzhou Zhongxin Chemical Reagent Co. Ltd. D-glucose purchased from Beijing Chemical Works was analytically pure. Other reagents were analytically pure or chemically pure.

Microfluidic Device

The microfluidic device used was fabricated with two polymethyl methacrylate (PMMA) plates. As shown in Fig. 1, the multiphase flow channel was made of a teflon (PTFE) tube with 0.5 mm inner diameter. A steel needle with inner diameter of 0.16 mm was inserted as the dispersed phase inlet, while two PTEE tubes were inserted as the continuous phase

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inlet. We used two microsyringe pumps and three gastight microsyringes to pump the fluids into the microfluidic device. Dispersed phase was sheared by continuous phase and then uniform droplets generated in the microchannel. The droplets were finally collected in Petri dishes filled with solidification bath.

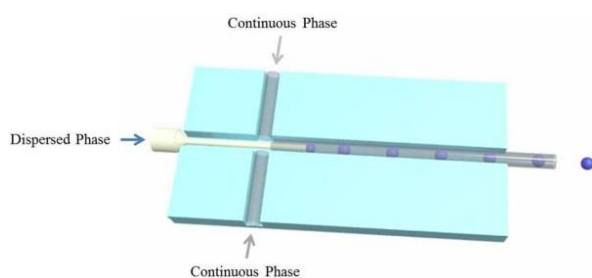


Fig. 1 The microfluidic device

Preparation of CS-PAA microspheres and CS microspheres

The chitosan and poly(acrylic acid) were mixed in a neutral condition, CS-PAA copolymer formed quickly because of strong electrostatic interaction between two polymers. Precipitate of the composite copolymer dissolved in strong acid environment as excessive acetic acid was added. In this work, the dispersed phase was aqueous solution of 1.5 wt.% chitosan, 0.1 wt.% poly(acrylic acid) and 40 wt.% acetic acid. The continuous phase was liquid paraffin. The solidification bath contained 98 wt.% n-octane, 2 wt.% Span as a surfactant and trace of glutaraldehyde as a crosslinker. The droplets were solidified for three to five hours at room temperature. Prepared CS-PAA composite microspheres were rinsed three times with n-octane and dried with a freeze-drying method for one day.

The dispersed phase of preparing CS microspheres was aqueous solution of 1.5 wt.% chitosan and 2.0 wt.% acetic acid. The continuous phase and solidification method were the same as those in preparing CS-PAA microspheres.

Characterization of CS-PAA microspheres

CS-PAA droplets were observed using an optical microscope (Type BX-61, Olympus, Japan) and an on-line CCD (Pixelink, Canada). To understand detailed morphology and structures of microspheres, a scanning electron microscopy (Type TM3000, Hitachi, Japan) was also used. Fourier transform infrared spectroscopy (Type Tensor27, Bruker, Germany) was used to analyze composition of composite microspheres. The fluorescence micrographs were taken by laser confocal fluorescence microscopy (Type BX61, Olympus, Japan).

Immobilization of glucose isomerase

2.0 g of glucose isomerase powders were dissolved in 100 mL of deionized water. The solution was centrifuged and supernatant was separated as GI solution. GI solution was first diluted by phosphate buffer (0.2M, pH=7.0). CS-PAA microspheres were incubated in the diluted solution at 25 °C for 24 hours at the rotational speed of 120 rpm. Then diluted GI solution was displaced with GLA solution diluted by phosphate buffer (0.2M, pH=7.0). CS-PAA microspheres with

adsorbed GI were incubated at 25°C for 30 min standing to crosslink chitosan with GI for further immobilization.

Enzyme activity assay

Enzyme activity of GI was determined by catalyzing isomerization of glucose to fructose for 30 min by stirring in a three-neck flask. The substrate were 5 mL glucose and 4 mL phosphate buffer. Enzyme used here was 1 mL GI solution or CS-PAA microspheres loaded with GI. 0.5 mL MgSO₄ and 0.5 mL CoCl₂ were also added into the substrate to stabilize the enzyme and increase the enzyme activity. Reaction temperature was 70 °C and pH value of phosphate buffer was 7.5 if there was no specific illustration. Fructose concentration was analyzed with cysteine-carbazole method. By adding cysteine hydrochloride, H₂SO₄ and carbazole into the diluted reaction liquid after isomerization, color-developing reaction occurred at 60 °C and lasted for 10 min before cooled down. We used UV spectrophotometry to determine fructose concentration by measuring its absorbance at 560 nm. One unit of GI activity was defined as the amount of GI used to produce 1 μmol of fructose per minute.

Relative activity was defined as the ratio of measured enzyme activity and initial enzyme activity. Initial enzyme activity was uniformly determined at the reaction temperature of 70 °C and the reaction pH of 7.5.

Results and Discussion

Characterization of CS-PAA microspheres

Fig. 2(a) and (b) show the micrographs of CS-PAA droplets and microspheres solidified for 1 day. The size of CS-PAA droplets could be easily controlled in a wider range from 500 μm to 260 μm by varying the flow rate of continuous phase, as is shown in Fig S1. After solidification, the size of microspheres is about half of the droplet because of the extraction of water from the droplet. The prepared CS-PAA microspheres appeared light yellow and the color became darker as the solidification time extended. SEM micrographs of CS-PAA microspheres and CS microspheres solidified for 4h were shown in Fig. 2(c) and (d), respectively. The smaller SEM micrograph inserted in the left corner exhibited the inner structure of one CS-PAA microsphere in Fig. 2(c). CS microspheres and CS-PAA microspheres prepared were in good monodispersity and high degree of sphericity. The CS microspheres were compact and possessed smooth surface. Different from this, the CS-PAA microspheres had porous surface and compact core when solidified for 4h. This interesting structure could be regulated controllably and also had good potential applications. In Fig. 2(e) and (f), we observed LCFM micrographs of CS-PAA microspheres and CS microspheres solidified for 4h under the same excitation light source. Two types of microspheres both exhibited fluorescence, while fluorescence intensity of CS-PAA microspheres appeared weaker than CS microspheres. As is known, fluorescence lies in crosslinking degree of CS and GLA. Some of the CS polymers were complexed with PAA polymers, resulting in less CS polymers crosslinked with GLA. Consequently, CS-PAA microspheres exhibited weaker

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fluorescence intensity than CS microspheres prepared in the similar method. Moreover, a few dark spots were observed in LCFM micrographs of CS-PAA microspheres in the places where holes existed on the surface. The polydispersity index of the chitosan-poly(acrylic acid) composite microspheres was less than 4% as shown in Fig. 2(g). The monodispersed microspheres had uniform sizes and structures. Active substances like enzymes could be loaded on every microsphere in the same way to ensure uniformity of the catalytic properties. This is important for subsequent fundamental research and modeling.

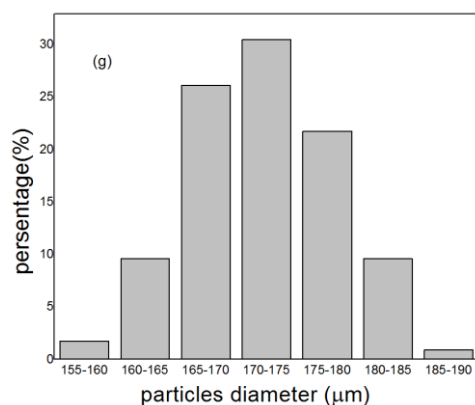
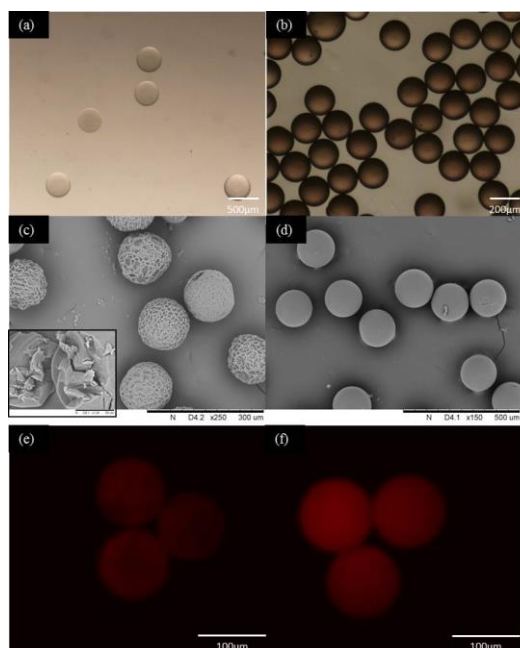


Fig. 2 Micrographs of CS-PAA droplets and microspheres. (a)Optical micrograph of CS-PAA droplets. (b)Optical micrograph of CS-PAA microspheres solidified for 1d. (c)SEM micrograph of CS-PAA microspheres solidified for 4h. (d)SEM micrograph of CS microspheres solidified for 4h. (e)LCFM micrograph of CS-PAA microspheres. (f)LCFM micrograph of CS microspheres. (g)The size distribution of microspheres and the polydispersity index of the diameters is 3.2 %.

The FTIR spectrogram was shown in Fig. 3. The overlapped peaks around 3444cm^{-1} represented stretching vibrations of $-\text{OH}$, $-\text{NH}$ and intermolecular hydrogen bonding. 1715cm^{-1} corresponded to stretching vibration of $-\text{COOH}$. 1635cm^{-1} was assigned to bending vibration of $-\text{NH}_3^+$, while 1558cm^{-1} and 1409cm^{-1} corresponded to asymmetry vibration and symmetry vibration of $-\text{COO}^-$, respectively.

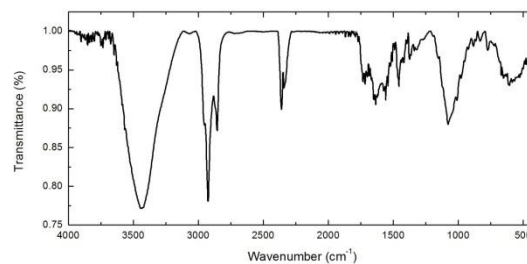


Fig. 3 FTIR spectrogram of CS-PAA microspheres

Structure controlling of CS-PAA microspheres

We found prepared CS-PAA microspheres solidified for 4h were with porous surface and compact core in the SEM micrographs. But CS-PAA microspheres solidified for 1d were compact both in core and on surface. In further research, it was found out that by controlling structure evolution process, we could control structures of CS-PAA microspheres effectively. The size of the microspheres will not affect the porous morphology obviously referring to Fig S2. CS-PAA microspheres we used to regulate structures here were with a diameter of $120\text{ }\mu\text{m}$.

Structures of CS-PAA microspheres evolved in the solidification process. By controlling solidification time, structures of CS-PAA microspheres could be controlled successfully. Some of the amino groups on chitosan polymer chains were complexed with carboxyl groups, and residual amino groups crosslinked with aldehyde groups in GLA. We defined the residual amino groups as extra $-\text{NH}_2$. Fig. 4(a-d) showed the evolution process of CS-PAA microspheres when solidification time extended, molar ratio of $-\text{CHO}$ and extra $-\text{NH}_2$ was 4:1. Inner structure of the microspheres was compact all through. With the increase of solidification time, the structure varied from porous to compact on the surface.

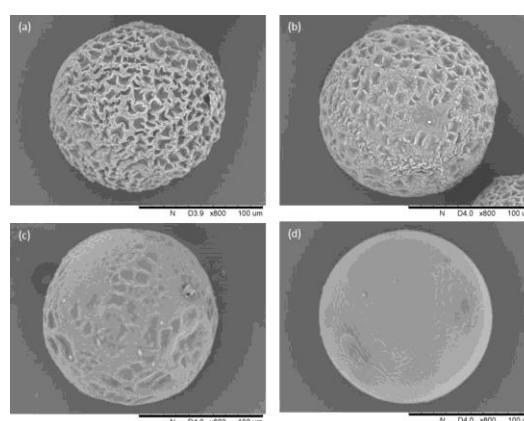


Fig. 4 Surface structure of CS-PAA microspheres solidified for (a) 2.5h; (b)3h; (c)3.5h; (d)4h. n(CS):n(PAA)=8:1. n(-CHO):n(extra $-\text{NH}_2$)=4:1.

Microsphere structure was also influenced by the amount of the crosslinker, GLA. Structures of CS-PAA microspheres solidified for 4.5h varied as the amount of crosslinker increased, as shown in Fig. 5(a-d). We used molar ratio of $-\text{CHO}$ and extra $-\text{NH}_2$ to express amount of GLA. With the dosage increase of GLA, pore sizes and depth decreased on

the surface and the microsphere surface became compact eventually.

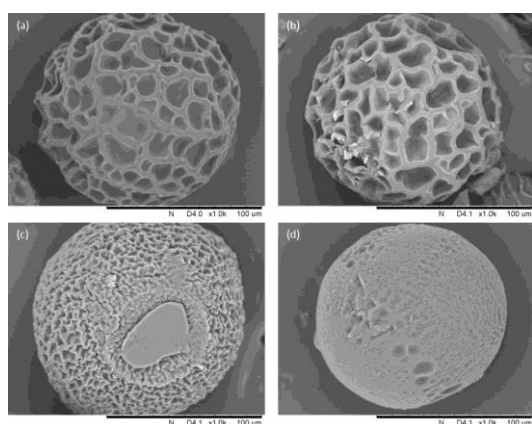


Fig. 5 Surface structure of CS-PAA microspheres when $n(-\text{CHO}):n(\text{extra } -\text{NH}_2)$ was (a)1:1; (b)2:1; (c)3:1; (d)4:1. $n(\text{CS}):n(\text{PAA})=8:1$ and solidified for 4.5h.

Solidification of CS-PAA droplets experienced composite and crosslinking steps, as shown in Fig. 6. In a strong acidic surrounding, amino groups were protonated while carboxyl groups were not ionized, and then electrostatic interaction between CS and PAA was shielded. CS and PAA were first mixed homogeneously when the pH value was about 2 inside of the droplets. As the pH value of internal medium increased with evaporation of acetic acid, electrostatic interaction between amino groups and carboxyl groups recovered and CS was composited with PAA whose pKa was 4.75. Schiff base reaction occurred and CS was crosslinked with GLA at the same time. Evaporation of acetic acid had not finished for CS-PAA microspheres solidified for less than 5h, which resulted in concentration distribution inside. Acetic acid distribution brought out pH decrease from the interior to the surface, so compositing and crosslinking reaction took place in the interior first and expanded to the surface gradually. Pores observed on the surface once acted as channels of molecules of acetic acid, GLA and water. With solidification proceeding, CS-PAA microspheres with compact core and porous surface were obtained.

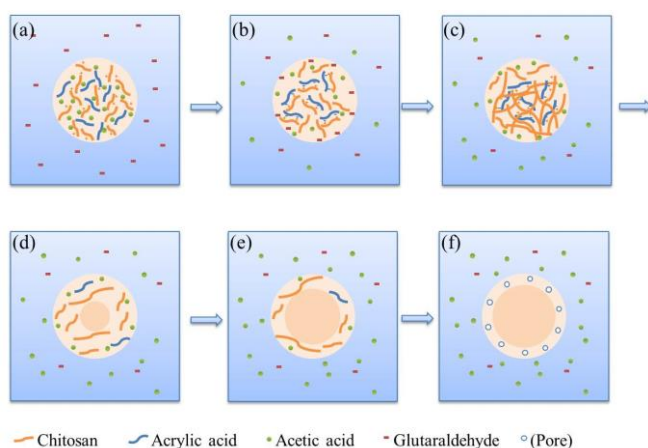


Fig. 6 The mechanism of CS-PAA microspheres generation process

The prepared CS-PAA composite microspheres had high mechanical strength. There were not any acrylic acid monomers inside of prepared CS-PAA composite

microspheres. Chitosan and poly(acrylic acid) were blended with strong electrostatic interaction. The CS-PAA composite microspheres also had a specific structure. They were with compact core and porous surface. This novel structure can be applied in many fields, such as loading enzymes on the surface and ensuring the mechanical strength.

Immobilization of glucose isomerase

CS-PAA microspheres with porous surface and compact core could be applied in loading active substances such as metal catalyst and enzymes. Good active-substance carriers need sound mechanical strength and large specific surface area. As the compact core could act as a firm support and the porous surface was to load active substances, the prepared CS-PAA composite microspheres possessed of both two properties.

Glucose isomerase (EC 5.3.1.5), also known as D-xylose isomerase, is a water soluble enzyme widely applied in food industry. GI can catalyze substrates like D-glucose, D-xylose, D-ribose and D-rhamnose. This enzyme is mainly applied in isomerization of glucose to fructose, which is the key step of producing high fructose corn syrup (HFCS). HFCS is an important sweetener since it is sweeter and has better fructose-solubility than sucrose¹⁹. Moreover, fructose can be absorbed without metabolic regulation of insulin, which makes it possible for diabetics to intake directly. GI is also used to catalyze isomerization of xylan to xylulose, and that is important in production of ethanol from hemicellulose. GI for industrial application should be with increased thermal stability, lower pH optimum and effective reuse property. Immobilization of GI on proper carriers

Here, CS-PAA composite microspheres with porous surface structure were utilized to load GI and catalyze isomerization of glucose to fructose. The molar ratio of CS and PAA was 8:1, molar ratio of $-\text{CHO}$ and *extra* $-\text{NH}_2$ was 4:1 and solidification time was 3h. As shown in Fig. 7(d), the prepared CS-PAA microspheres were with compact core and porous surface.

The effects of reaction temperature and pH on enzyme activity were studied by changing temperature and pH value of substrate. We studied enzyme activity of free and immobilized GI at the temperature range of 40 °C to 80 °C, and found that the optimum reaction temperature was 70 °C from Fig. 7(a). Thermal stability of GI was reinforced when immobilized on CS-PAA microspheres. Enzyme activity of free GI at lower temperatures decreased a lot in comparison with that at 70 °C, but relative activity of immobilized GI maintained above 85% as reaction temperature changed. Reinforced thermal stability owed to protection of GI from CS-PAA microspheres since GI was loaded on the microspheres with covalent interaction. Effects of reaction pH values on enzyme activity was shown in Fig. 7(b). The optimum reaction pH for free GI was 7.5, while free enzyme activity decreased significantly at lower pH. In contrast, relative activity of immobilized GI maintained above 80% in the pH value range of 6.0 to 8.0. Enzyme activity of GI at lower pH could be successfully increased with the immobilizing method. It was deduced that microenvironment on the surface of CS-PAA microspheres had an effect on catalytic properties of GI, making it more stable and efficient

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at lower pH. The reason may be that CS-PAA microspheres were composed of cationic CS and anionic PAA, and influenced electrochemical property of GI. Immobilized GI also had good operating stability since its relative activity kept around 98% after recycled for 11 times, as shown in Fig. 7(c).

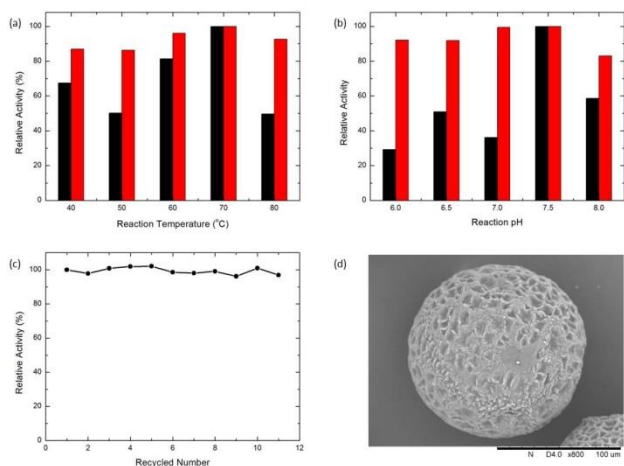


Fig. 7 (a)Effect of reaction temperature on enzyme activity of free (black column) and immobilized (red column) GI; (b)Effect of reaction pH on enzyme activity of free (black column) and immobilized (red column) GI; (c)Operating stability of immobilized GI; (d)SEM micrograph of used CS-PAA microsphere.

Therefore, the CS-PAA composite microspheres with porous surface structure prepared in this work was a good carrier for GI immobilization. Catalytic activity under lower temperature and lower pH could be increased significantly. Moreover, CS-PAA microspheres with both compact core and porous surface could also load other active substances and had good potential in biological applications.

Conclusions

Monodispersed CS-PAA composite microspheres with controlled structures were successfully prepared by using a facile microfluidic method. The CS-PAA microspheres were with compact core and porous surface under defined conditions. The composite microspheres would all become compact, provided that solidification time was longer than 5h. By varying solidification time, amount of crosslinker and molar ratio of CS and PAA, surface structure could be regulated effectively. CS-PAA composite microspheres with porous surface structures were applied in immobilization of GI. The optimum reaction temperature of free GI was 70 °C and the optimum pH was 7.5, while enzyme activity decreased a lot at lower temperature and pH. Immobilized GI maintained high activity at the temperature range of 40 °C to 80 °C and at the pH range of 6.0 to 8.0. The relative activity of immobilized GI kept around 98% after recycled for 11 times. So the immobilized GI had good thermal, pH and operating stability. The CS-PAA composite microspheres with porous surface structure have good potential applications in food industry, wastewater treatment and biological areas.

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Notes and references

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