Immobilization of Glucose Oxidase on a Novel Crosslinked Chitosan Support Grafted with L-Lysine Spacers

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A novel L-lysine modified semi-crosslinked chitosan resin (LMCCR) was synthesized and demonstrated to be a promising enzyme support by studying the enzymatic properties of glucose oxidases (GODs) immobilized on it. The prepared LMCCR beads have large specific surface and excellent chemical stability. The insertion of flexible L-lysine spacers between chitosan backbone and the immobilized GODs (I-GODs) increases the enzymes' activity and improves their affinity towards the substrate. Repetitive uses demonstrated that the LMCCR-immobilized GODs have excellent operational stability and reusability. Moreover, the enzyme activity at varied temperatures and pH indicated that the GODs immobilized on LMCCR have good thermostability and pH stability.

Key words:

Enzyme support, chitosan, L-lysine, enzyme immobilization, glucose oxidase, flexible spacers

Introduction

Chitosan (CT) is a polysaccharide that can be easily obtained by alkaline hydrolysis from chitin, the second most abundant polysaccharide after cellulose on Earth.[1] CT is considered to be an excellent support for enzyme immobilization since it is biocompatible, biodegradable, non-toxic, amenable to chemical modification, and highly affinitive to protein due to its hydrophilic nature.[2,3] During the past two decades, numerous studies have been carried out on the immobilization of many kinds of enzymes on chitosan. Meanwhile, in order to further improve the essential properties of chitosan for enzyme support such as chemical and mechanical stability, hydrophilicity, specific surface area, etc., various kinds of modified chitosan supports have been developed.[4–15] Chellapandian and Krishnan[4] studied the immobilization of ureases on Chitosan-poly(glycidyl methacrylate) copolymer prepared by grafting glycidylmethacrylates on the amino groups of chitosan. Diethylaminoethyl chitosan was synthesized by Spagna *et al.*[10] for a-L-rhamnopyranosidase immobilization. Yi *et al.*[11] prepared amino acid modified chitosan beads by activating chitosan backbone with epichlorohydrin followed by amino acid coupling. Lipase was then immobilized on the modified chitosan by using an adsorption-crosslinking method with glutaraldehyde as the crosslinking agent. Xu *et al.*[12] developed a water-soluble chitosan derivation, O-carboxymethyl chitosan, which was used for immobilizing horseradish peroxidase to construct a H_2O_2 biosensor. Ghica *et al.*[15] prepared a graphite-epoxy resin composite electrode modified with functionalized multi-wall carbon nanotubes immobilized in a chitosan matrix, which was used as a base to immobilize glucose oxidase. The resulting biosensor shows good capability of glucose determination without interferences.

Among the various methods available for enzyme immobilization, covalent linkage offers the advantage of stronger binding between support and enzymes, thus resulting in more stable and higher percentage of immobilization. However, if enzymes are connected directly with a support via covalent bindings, the interaction between the enzymes and support could denature enzymes. Moreover, the support can impede substrates to approach the active sites of the enzymes. Accordingly, arm molecules were always grafted on the support to serve as spacers for connecting the enzymes, which reduces the disturbance of the support on enzymes and increases enzyme mobility, thus resulting in higher enzyme activity and better affinity of enzymes toward the substrate. According to this theory, several research groups investigated the effect of different spacers on the activity of immobilized enzymes[16–19] and demonstrated that the activity of immobilized enzymes is significantly affected by the nature of the spacers. When flexible and hydrophilic spacers

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were introduced between the support backbone and the functional groups used for immobilization, the enzymes showed much higher activities over those immobilized *via* rigid spacers.

In this paper, a novel chitosan derivative was synthesized by first semi-crosslinking chitosan using glutaraldehyde (GA) followed by grafting flexible L-lysine spacers on its active amino groups. The L-lysine modified semi-crosslinked chitosan resin (LMCCR) beads have excellent chemical stability and large specific surface. The potential application of LMCCR as an enzyme support was investigated by testing the enzymatic activity of glucose oxidases (GODs) immobilized on it. L-lysine molecules are very flexible and highly hydrophilic; their insertion between chitosan backbone and the active amino groups significantly increases the activity of immobilized GODs (I-GODs) and improves the affinity of I-GODs towards the substrates. Therefore, the grafting of L-lysine spacers makes LMCCR beads a more ideal enzyme support than the semi-crosslinked chitosan resin (CCR), whose potential application in enzymatic biosensors and reactions deserves further exploration.

Materials and methods

Materials and reagents

Glucose oxidase (GOD, EC: 1.1.3.4, Biochemical reagent grade, >100 U mg^{-1}, here one U is defined as the amount of GOD that catalyzes the conversion of 1 μmol D-glucose to product D-gluconic acid and H_2O_2 in 1 min at pH = 7.0 and temperature = 25 °C) was purchased from Great Wall Clinical Reagent Co. (Baoding, Hebei Province, China). L-lysine, alanine, 4-aminoantipyrine, N,N-dicyclohexylcarbodiimide (DCC) were purchased from Guoyuan Biotechnology Co. (Shanghai, China) and are all Biochemical Reagent Grade.

Chitosan with the deacetylation degree of 90.3 % was purchased from Yuhuan Biochemical Co. (Hangzhou, China). Aqueous solution of glutaraldehyde (50 %, mass concentration), liquid paraffin, Tween-80, phenol, glucose were purchased from Chongqing Chemical Head Factory (Chongqing, China), and all were of Analytical Reagent Grade.

Preparation of L-lysine modified semi-crosslinked chitosan resin (LMCCR) beads

When chitosan is used as enzyme support, it is usually semi-crosslinked *via* its active amine groups to improve its mechanical and chemical instability. Accordingly, in this study, semi-crosslinked chitosan resin (CCR) was firstly prepared by controlling the dose of the crosslinking agent. Then, CCR was modified by grafting L-lysines on its active amine groups to synthesize LMCCR. The reaction scheme of the synthesis procedure of LMCCR is illustrated in Fig. 1, the experimental procedure was briefly

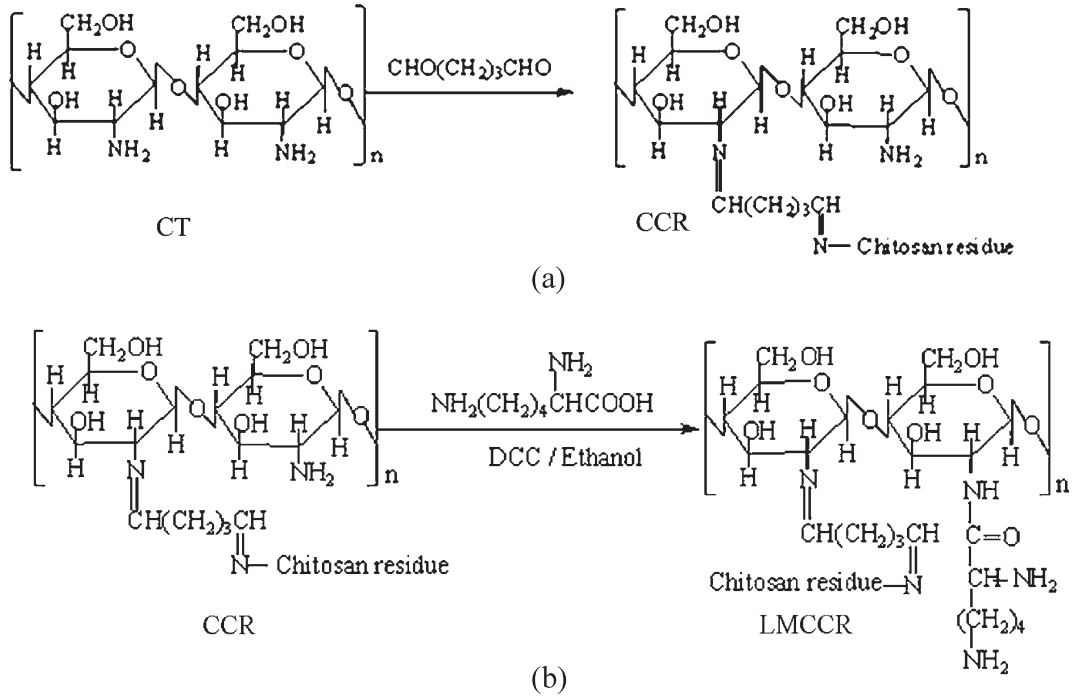


Fig. 1 – *Synthesis reactions of LMCCR. (a) Crosslinking reaction of CT to CCR via the formation of Schiff bonds (b) Grafting reaction of L-lysines on CCR to synthesize LMCCR. These figures have been reported previously by Xiao and Zhou.*[20]

introduced here and a more detailed description has been reported by Xiao and Zhou.[20]

In the first step to prepare CCR, 300 mL chitosan solution (2 %, w/v) in HCl (1 %, w/v) was firstly added into the mixed liquid of 300 mL paraffin oil and 1.0 mL Tween-80 under vigorous stirring by mechanical stirrer at room temperature. After 30 min, 30 mL glutaraldehyde solution (5 %, v/v) and 6.0 g $CaCO_3$ powder were slowly added into the liquid. Then, the reaction system was heated to 40 °C; after stirring for 1 h, the pH of the system was adjusted to 10.0 using a NaOH solution. Subsequently, the reaction system was heated to 60 °C, under which it was stirred for 3 h. Subsequently, the reaction mixtures were vacuum-filtered to obtain the crude product, which was washed with petroleum ether and ethanol successively, to remove the residual paraffin oil and Tween-80. After being added into HCl (0.2 mol L^{-1}) to dissolve $CaCO_3$ for forming pore networks, the crude product was washed using DI water to obtain the expected CCR product.

In the second step, after CCR was suspended in 300 mL L-lysine solution (0.1 mol L^{-1}) for 30 min to adsorb L-lysines, 0.03 mol DCC (dissolved in 20 mL of 95 % ethanol) was added to induce the acylation reaction. After the reaction was carried out at room temperature for 1 h under gentle agitation, the reaction mixtures were vacuum-filtered to obtain the crude LMCCR product, which was washed using ethanol (95 %) and DI water successively, to obtain the final product. Since DCC can also induce the self-reaction between L-lysines, the amount of L-lysine used in the process was overdosed.

Enzyme immobilization

In this study, GOD was chosen as a model enzyme to study the potential application of LMCCR as an enzyme support. The immobilization of GOD on LMCCR was implemented by using a support-activation method.[21,22] 10.0 g LMCCR was first dispersed in 100 mL phosphoric acid-citric acid buffer, into which 25 mL GA solution (1.0 %, mass concentration) was dripped. After 1 h reaction at 25 °C, the liquid was filtrated and washed to obtain the activated LMCCR support. Subsequently, the activated support was dispersed in the phosphoric acid-citric acid buffer solution again, and 25 mL GOD solution (80 U L^{-1}) in the same buffer was slowly added under mild stirring. After 1 h treatment at 25 °C, the reaction system was washed to obtain the immobilized enzymes, which were washed using DI water and stored at 4 °C before being used.

After the immobilization, the total mass of enzymes in the filtrate was measured using the Bradford method[23, 24] to calculate the mass of the immobilized GODs. The GOD coupling ratio was then determined according to eq. 1.

$$\text{GOD coupling ratio} = \frac{\text{mass of I-GOD}}{\text{total mass of GOD in solution before immobilization}} \cdot 100\ \% \quad (1)$$

Determination of enzyme activity

The enzyme activity of both free and immobilized GODs was assayed using a 4-Aminoantipyrine colorimetric method.[25] The principle of this method is that the GOD-catalyzed oxidation of glucose generates H_2O_2, which can rapidly react with 4-aminoantipyrine to generate red quinoneimine dye under the catalysis of horseradish peroxidase. Among these two sequential reactions, the oxidation of glucose is the limiting step, the reaction rate of which is much slower than the oxidation of 4-aminoantipyrine to red quinoneimine dye. For the concentration of red quinoneimine dye, which can be measured by using a colorimetric method, is proportional to H_2O_2 amount generated by the enzyme-catalyzed oxidation of glucose, one can determine GOD activity by measuring the red quinoneimine dye using a colorimetric method.

In the process, a specific amount of soluble GOD or suspended I-GOD was added to a reaction solution containing 1.5 mL of mixed chromogenic reagents and 1.5 mL of 6.5 % (w/v) glucose solution. The mixed chromogenic reagents were freshly prepared, containing 3.5 mg horseradish peroxidase, 3.5 mg 4-aminoantipyrine, 1 mL of 3.0 % (w/v) phenol solution and 20 mL of 0.2 mol L^{-1} sodium phosphate buffer. About 10 minutes after the reaction, the absorbance of the solution at 500 nm was measured and the GOD or I-GOD activity (U mg^{-1}) was determined according to a standard calibration curve relating the enzyme activity with the absorbance. The activity retention of I-GOD (immobilization percent) was determined according to eq. 2. Here, one unit of enzyme activity (1 U) is defined as the amount of GOD that catalyzes the conversion of glucose to product 1 μmol H_2O_2 in 1 min at the above operating condition.

$$\text{I-GOD activity retention} = \frac{\text{specific activity of I-GOD}}{\text{specific activity of free GOD}} \cdot 100\ \% \quad (2)$$

Determination of Michaelis-Menten kinetic parameters

The Michaelis-Menten kinetic parameters, K_m and V_{max} were determined according to the Lineweaver-Burk plots, in which the experimental reaction rates and the corresponding substrate con-

centrations were fitted into the adjusted Michaelis-Menten equation (eq. 3) by using a linear regression method in Excel. In eq. 3, V_0 and V_{max} are, respectively, the initial reaction rate and the maximum reaction rate achieved by the enzyme-catalyzed reaction; [*S*] is the substrate concentration; Michaelis constant, K_m is the substrate concentration at which the reaction rate is half of V_{max}.

$$\frac{1}{V_0} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \quad (3)$$

Results and discussion

LMCCR synthesis and characterization

The primary amino groups in CT are very active and can serve as the reactive groups for both the crosslinking and spacer grafting reactions.[26] The synthesis procedure of LMCCR illustrated in Fig. 1 was confirmed in a previous publication.[20] The chemical structure of the synthesized CCR and LMCCR was characterized by using FTIR, ^{13}C-NMR and XRD, which demonstrated that the amino groups in chitosan had participated in the formation of both the Schiff bonds (N=C) during the crosslinking reaction and the amide bonds during the grafting of L-lysines. The grafting of L-lysines inserts a flexible spacer between chitosan backbone and the free amino groups and makes LMCCR possess some excellent properties as an ideal enzyme support.

SEM images show that the prepared LMCCR beads are porous microspheres, whose diameter is around 800–1000 μm and pore size is around 50–100 μm in diameter. The BET specific surface of LMCCR is 403 $m^2\ g^{-1}$ and that of CCR is 769 $m^2\ g^{-1}$, which is much larger than that of chitosan (285 $m^2\ g^{-1}$).[20] Chitosan is dissoluble in acidic media due to the protonation of the primary amino groups at low pH.[27] Both CCR and LMCCR swell in acidic media instead of dissolving because the crosslinking reaction creates covalent bindings between chitosan backbones, leading to the formation of a network structure. Thus, the crosslinking treatment endows CCR and LMCCR with better chemical stability, which makes them suitable enzyme supports at various pH.[20]

GOD immobilization on LMCCR

Glucose oxidase (GOD) (EC: 1.1.3.4) is an enzyme that naturally catalyses the oxidation of β-D-glucose to D-gluconic acid. In this study, the properties of GODs immobilized on LMCCR were investigated with the aim to verify the potential application of LMCCR as an enzyme support. Because the properties of I-GODs are affected by the operating conditions of the immobilization process, the immobilization of GODs on LMCCR was performed at the optimum conditions obtained via a group of orthogonal experiments: GOD : GA : Amino groups in LMCCR = $15 \cdot 10^{-5} : 0.55 : 1$ (mol/mol/mol); pH = 8.0; immobilization time = 3.5 h.

After the completion of immobilization, the activity of GODs immobilized on both CCR and LMCCR was determined at 45 °C and pH =5.5. The results, as well as the activity retention and coupling ratio, are shown in Table 1. Rauf *et al.*[25] studied the immobilization of GODs on a novel cellulose acetate–polymethylmethacrylate membrane, the maximum activity retention that they reported was 75 %, which is higher than the activity retention for the CCR-immobilized GODs but lower than that for the LMCCR-immobilized GODs. However, they acquired a much higher coupling ratio of around 94 %. The maximum activity retention reported by Bautista *et al.*[28] when investigating the immobilization of GOD on amorphous $AlPO_4$ support is 79.17 % with a corresponding coupling ratio of 54.95 %.

Table 1 – *Results of GOD immobilization*

Items		Activity (U mg^{-1} support)	Activity retention (%)	Coupling ratio (%)
Support	CCR	3.31	64.87	27.85
	LMCCR	7.86	82.65	50.12

Since an L-lysine molecule has two amino groups, LMCCR possesses more free amino groups due to the grafting of L-lysines and is able to immobilize more GODs. Consequently, the coupling ratio of GODs immobilized on LMCCR is about 1.8 times that on CCR, indicating that the amount of GODs immobilized on unit LMCCR is 1.8 times that on unit CCR. Meanwhile, the activity retention of I-GODs on LMCCR is 1.28 times that on CCR, demonstrating that the activity of unit GOD immobilized on LMCCR is 28 % higher than the activity of unit GOD immobilized on CCR due to the presence of flexible L-lysine spacers. Summarily, LMCCR is not only able to immobilize more GODs than CCR, but also the GODs immobilized on LMCCR are more active. The combination of these two factors leads to the result that I-GODs on unit LMCCR exhibit much higher activity than those on unit CCR, 7.86 versus 3.31.

I-GOD properties

Michaelis-Menten kinetic parameters

Fig. 2 shows the Linweaver-Burk plots for free GOD, GOD immobilized on both CCR and LMCCR in catalyzing the oxidation of glucose,

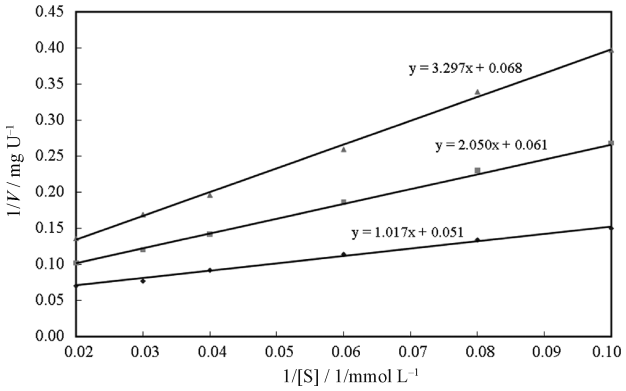


Fig. 2 – *Lineweaver-Burk plots and the fitting equations of free GOD and immobilized GOD. ◆: free GOD, ■: GOD immobilized on LMCCR, ▲: GOD immobilized on CCR.*

and Table 2 lists the corresponding values of K_m and V_{max}. The Michaelis constant for GOD was calculated by various researchers and they found differences in their values depending on GOD nature and the testing conditions. The K_m value for the free GOD from *T. favus* is calculated to be 10.9 mmol L^{-1},[29] and 33 mmol L^{-1} from *A. niger*,[30] The calculated K_m value for free GOD in this study is 20.01 mmol L^{-1}, which is a reasonable result based on the previous reports.[31]

Table 2 – *Michaelis-Menten kinetic parameters for free GOD and immobilized GOD*

Enzyme	K_m (mmol L^{-1})	V_{max} (U mg^{-1})
Free GOD	20.01	19.68
I-GOD on LMCCR	33.71	16.45
I-GOD on CCR	48.27	14.64

Once an enzyme was immobilized, its affinity toward the substrate decreased due to the steric hindrance of the active sites by the support, the loss of enzyme flexibility necessary for substrate binding, and the possible change of enzyme structure resulting from the multi-point covalent attachment with the support. Accordingly, K_m of GODs immobilized on both CCR and LMCCR becomes higher than that of free GODs, and V_{max} becomes lower, demonstrating that the affinity of I-GODs toward glucose is decreased, which is a typical result for the transfer of free enzymes to immobilized state.[32] However, compared with CCR-immobilized GOD, K_m of GODs immobilized on LMCCR is lower and V_{max} is higher, demonstrating that LMCCR-immobilized I-GODs have better affinity towards the substrate since the grafted L-lysine spacers reduce the disturbance of chitosan backbone on I-GODs and increase enzyme mobility. Therefore, it can be observed that LMCCR is a more ideal enzyme support than CCR.

In the report by Rauf *et al.,*[25] once GOD was immobilized on the novel cellulose acetate-polymethylmethacrylate membrane, its K_m value increased 2.4 times from 17.42 mmol L^{-1} to 41.65 mmol L^{-1}. In comparison, the K_m value for the LMCCR-immobilized GOD is only 1.68 times that of the free GOD, indicating that LMCCR is a better enzyme support from one aspect.

The effect of temperature on I-GOD activity

After immobilization, enzymes become more "rigid", which impairs their activity; consequently, the activity of immobilized enzymes is always lower than that of free enzymes. However, immobilized enzymes, especially in a covalently bound system, become more stable against heat and denaturing agents, they can function in a broader range of pH and temperatures.[33] Fig. 3 compares the enzyme activities of GODs immobilized on LMCCR with that of free GODs at different temperatures, where the relative enzyme activity is the ratio between the enzyme activity at different temperature with the maximum enzyme activity at the optimum temperature. The result shows that the optimum temperature of free GODs is 35 °C, the deviation from the optimum temperature leads to a significant decrease in their activity. The optimum temperature for I-GOD was found to be 45 °C and it can keep relatively high activity in a broad range from 25 to 60 °C. This result agrees with the previous reports that optimum temperature is increased during enzyme immobilization and the immobilized enzymes have better thermostability.[34–37]

To further study the thermostability of I-GOD, its activity under a high temperature (60 °C) was in-

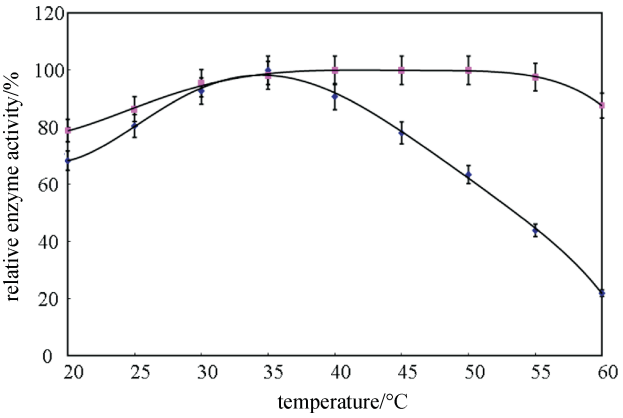
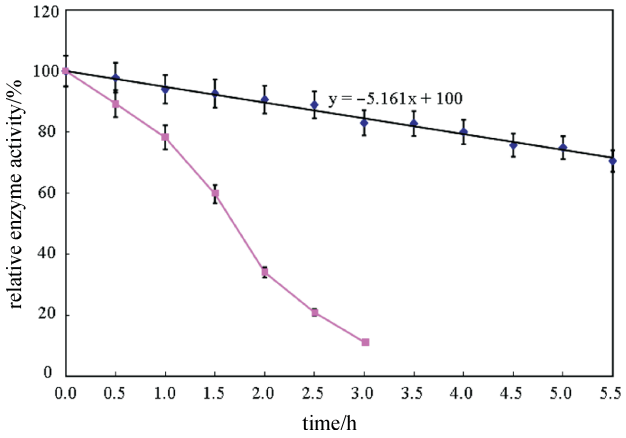
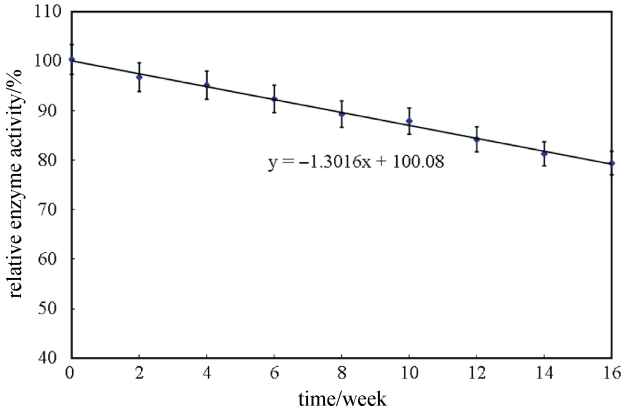


Fig. 3 – *Relative enzyme activity of free GOD and LMCCR-immobilized GOD at different temperatures at pH = 6. ◆: free GOD, ■: GOD immobilized on LMCCR.*



F i g . 4 – *Relative enzyme activity of free GOD and LMCCR-immobilized GOD versus time at 60 °C. ◆: free GOD, ■: GOD immobilized on LMCCR.*

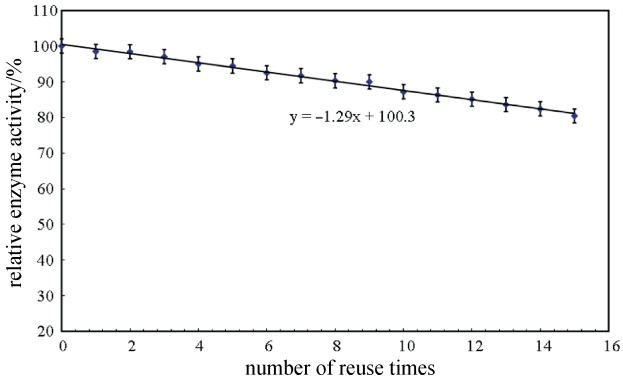
vestigated. Fig. 4 compares the time course of the activity of I-GODs under 60 °C in a phosphate buffer (pH = 6) with that of free GODs, where the relative enzyme activity is the ratio between the enzyme activity at different time with its initial activity. After being heated, the samples were quickly cooled and assayed for enzymatic activity at 35 °C. After 3 h treatment, the activity of free GODs was only 15 % of its initial activity, while I-GODs could keep 85 % of their initial activity. By fitting the activity data to a linear equation, the half-life of the I-GOD activity at 60 °C is estimated to be 10 h, 5 times higher than that of the free GOD. By using the same procedure, the half-life of I-GOD activity stored at 4 °C is calculated to be 270 days (Fig. 5).



F i g . 5 – *Relative enzyme activity of LMCCR-immobilized GOD stored at 4 °C*

Reusability

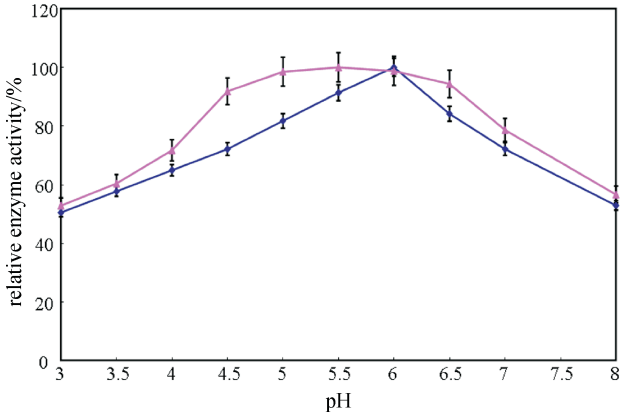
Fig. 6 shows the enzyme activity of GOD immobilized on LMCCR after repetitive uses, where the relative enzyme activity is the ratio between the residual activity of I-GOD after being used for specific times with its initial activity. After 16 uses, I-GOD can still retain 80.40 % of its initial activity. According to the fitting curve, the half-life of I-GOD is estimated to be 39 times, demonstrating that the I-GOD has excellent operational stability. For comparison, the half-life of GOD immobilized on the novel cellulose acetate–polymethylmethacrylate membrane is 30 min,[25] indicating that LMCCR-immobilized GOD has a better operational stability.



F i g . 6 – *Repetitive use of LMCCR-immobilized GOD*

pH

Every enzyme has an optimum pH range, in which the enzyme has the highest activity. Deviation from the optimum pH leads to a significant decrease in enzyme activity because pH affects the dissociation states of both enzyme and substrates. Immobilization varies enzyme's micro-environment and conformation; therefore, the optimum pH of the immobilized enzymes is usually different from that of free enzymes in general cases.[38–41] Fig. 7 shows that the optimum pH for I-GOD (pH = 5.5) is shifted to a more acidic range compared to that for free GOD (pH = 6). One reason for this observation is due to the deviation of the local pH around I-GODs from the bulk pH, which resulted from the



F i g . 7 – *Relative enzyme activity of free GOD and LMCCR-immobilized GOD at different pH. ◆: free GOD, ▲: GOD immobilized on LMCCR. Temperature = 35 °C.*

attraction of cations by the support.[42] LMCCR is an anionic support; its amino groups are positively charged in water, which attract cations in the solution to aggregate in the vicinity of LMCCR. Thus, pH in the local microenvironment around I-GODs is higher than the bulk pH. In order to make the local pH equal to the optimum functional pH, the bulk pH has to be shifted downward. Another reason is due to the steric hindrance of the crosslinked LMCCR, which obstructs the diffusion of protons to the bulk. Fig. 6 also demonstrates that I-GOD is less sensitive to pH variation; it maintains relatively high activity at a broader pH range, which confirms the increased pH stability of I-GODs compared with free GODs.

Conclusions

A novel chitosan derivative was synthesized by first semi-crosslinking CT using GA followed by grafting flexible L-lysine spacers via the active amino groups. The prepared LMCCR has excellent physical and chemical properties; the investigations on the enzymatic properties of immobilized GODs demonstrated that the prepared LMCCR beads are a promising enzyme support, whose potential application in enzymatic biosensors and reactions deserves further exploration. Compared with GOD immobilized on CCR, the insertion of flexible L-lysine spacers between chitosan backbone and the active amino groups increases the activity of the immobilized GOD and improves its affinity towards the substrate. Repetitive uses demonstrated that the LMCCR-immobilized GODs have excellent operational stability. Moreover, the enzyme activity at varied temperatures and pH indicated that the LMCCR-immobilized GODs have good thermostability and pH stability.

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List of symbols

BET – Brunauer-Emmett-Teller
CT – chitosan
CCR – crosslinked chitosan
DCC – N,N-dicyclohexylcarbodiimide
GA – glutaraldehyde
GOD – glucose oxidase
I-GOD – immobilized GOD
K_m – Michaelis constant
LMCCR – L-lysine modified semi-crosslinked chitosan
S_w – the swelling percentage
$[S]$ – substrate concentration, mol L^{-1}
V_0 – the initial reaction rate, mol L^{-1} S^{-1}
V_{max} – maximum reaction rate, mol L^{-1} S^{-1}
SEM – Scanning electron microscopy

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