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Immobilization of cellulase on polyamidoamine dendrimer-grafted silica

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ABSTRACT

Polyamidoamine dendrimer (PAMAM) is one of a number of dendritic polymers with precise molecular structure, highly geometric symmetry, and a large number of terminal groups, and is suitable to carry biomolecules due to its affinity and biocompatibility. In this study, PAMAM was grafted onto the surface of silica by microwave irradiation. A novel media was developed through immobilizing cellulase onto the prepared PAMAM-grafted silica by adsorption and crosslinking methods and applied in hydrolysis of carboxymethyl cellulose. The results demonstrate that the enzyme binding capacity and enzymolysis efficiency increased with generations of PAMAM. The properties of the immobilized cellulase-PAMAM-grafted silica were investigated, which possessed high enzymatic activity and exhibited better stability with respect to pH, temperature compared with free enzyme. The optimal immobilization conditions for adsorption and crosslinking method were respectively obtained at 5 and 4 mg ml^{-1} cellulase for 2 h of immobilization. A high enzymolysis efficiency was achieved by employing pH 4.8 and 5.8 substrate solution at 60 °C for adsorbed and crosslinked cellulase, respectively. After repeated three run cycles, the retained activities were found to be 75% and 82%. The results indicate that the PAMAM has a good performance as a carrier, and can be potentially adapted to support other biomacromolecules.

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1. Introduction

Immobilized enzymes have attracted widespread attention due to their reusability, easy separation from the reaction mixture and a possible increase in thermal and pH stability compared with free enzymes [1]. They have therefore potential applications in the field of biochemistry, food, clinical diagnosis and environmental engineering [2–5]. It is important for immobilized enzymes that the matrices should provide a biocompatible and inert environment and not interfere with the native structure of enzymes. Many researches and attempts have paid attention to developing new matrices or carrier to promote enzyme binding capacity, enzymolysis efficiency and stability [6].

Polyamidoamine dendrimer (PAMAM) is one of dendritic polymers that were introduced in the 1970s and 1980s and composed of a central core and branched monomers [7]. PAMAM is methodically constructed from an ethylenediamine or amino core through repetitive alkylation and amidation steps in which each iteration yields the next higher generation [8,9]. The cyclic manner of synthesis results in a hyperbranched, spherical and three-dimensional architecture for PAMAM, which is obviously different from that of typical linear polymers and leads to several remarkable advantages [10]. The globular shape and internal empty cavity are responsible for unique identifications and interactions with some reagents and solvents, and also can provide protection of the interior space. Furthermore, the terminal end groups and internal cavity structure are able to enther connect guest molecules or encapsulate them in the macromolecule interior [11]. These properties allow PAMAM suitable and attractive for biomolecules delivery [12–15]. Moreover, as the generation of PAMAM grows, the density of the surface amino groups exponentially increases, which can provide more sites for attaching special molecules such as enzymes, antibodies, nucleic acids and chiral selectors [16–19]. Due to these novel structure and unique functions, PAMAM has attracted widespread attention in the fields of biomaterials, drug delivery systems and catalysis [20–23]. PAMAM-grafted silica is a type of organic–inorganic hybrid material. The simple and easy preparation makes it possible to bond PAMAM dendrimers to silica. In previous work, we demonstrated successful grafting of PAMAM dendrimer onto the silica and a significant reduction in reaction time using microwave irradiation [24]. Our group also prepared a new type of off-line immobilized glucose oxidase capillary microreactors by introducing PAMAM with different generations onto the inner surface of fused-silica capillaries, which proved to be highly stable and efficient [25].

Cellulase is often used as a model enzyme in biochemistry, food, beverages and bioengineering, and hydrolyze β-1,4-glycosidic bonds of cellulose to produce oligomeric or soluble sugar, which is thus expected as a renewable source of fuels and chemicals [26–28]. However, free cellulase is often susceptible to denaturation and is

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difficult to reuse or recover. Some recent studies have focused on immobilized cellulase on silica-based matrices to improve immobilized enzymes properties [29–32].

In this study, cellulase was immobilized on PAMAM-grafted silica by adsorption and crosslinking methods and the resultant immobilized cellulase-PAMAM-grafted silica was applied in hydrolysis of carboxymethyl cellulose (CMC). The optimal immobilization and enzymolysis conditions were evaluated. Furthermore, the effect of PAMAM of different generations on the immobilization of cellulase was investigated. The activity, stability and reusability of free and immobilized cellulase were also compared.

2. Materials and methods

2.1. Chemicals and materials

Cellulase (EC 3.2.1.4 from *Trichoderma viride*) was purchased from Sinopharm Chemical Reagent (Shanghai, China). γ-Aminopropyltriethoxysilane (APES) and sodium cyanoborohydride were obtained from Acros Organics (Geel, Belgium). Methyl acrylate (MA), ethylenediamine (EDA), and glutaraldehyde were supplied by Beijing Yili Chemical (Beijing, China). Glucose was purchased from Beijing Biodee Biotechnology (Beijing, China). CMC and 3,5-dinitrosalicylic acid (DNS) was obtained from Tianjin Fuchen Chemical (Tianjin, China). Silica with an average particle size of 38–40 μm was obtained from Qingdao Haiyang Chemical & Special Silica Gel (Qingdao, Shandong, China). All other chemical reagents used were of analytical grade.

2.2. Instrumentation

Microwave-assisted synthesis was performed on an XH-100A microwave synthesis/extraction system (Beijing Xianghu Science and Technology Development Corporation, Beijing, China). The temperature of the reaction mixture was measured by an immersed platinum resistance thermometer. Elemental analysis was carried out using an Vario Micro Cube (Elementar corporation, Hanau, Germany). Scanning electron microscope (SEM) images were obtained using a Hitachi S4700 (Tokyo, Japan). Fourier-transform infrared spectra (FTIR; Thermo Scientific Nicolet 8700, Waltham, MA, USA) were determined to study the surface composition of the PAMAM-grafted silica.

2.3. Preparation of PAMAM-grafted silica by microwave-assisted technology

The 5 g silica was activated sequentially with 1 M NaOH for 4 h, deionized water for 15 min and then methanol for 10 min. Next, the silica was magnetically stirred with a 10% (v/v) solution of APES at room temperature for 10 min and put in microwave-assisted synthesis system. The silanization was carried out at 40 °C with 150 W microwave irradiation for 10 min under magnetic stirring. The prepared APES-modified silica (G0) was then washed with methanol and dried at 50 °C. Subsequently, grafting of PAMAM to the surface of G0 silica was achieved in two steps: (i) Michael addition of MA to amino groups; and (ii) amidation of the resulting esters with EDA. The grafting procedure is shown schematically in Fig. 1. The silanized silica was rinsed and treated with MA solution (20% [v/v] in methanol) for 10 min, and the reaction was carried out at 40 °C with 150 W microwave irradiation for 40 min under magnetic stirring. After cleaning with methanol, the silica was mixed with EDA solution (20% [v/v] in methanol) for 10 min and subjected to 150 W microwave irradiation with magnetic stirring at 40 °C for 60 min. The product was then filtered and washed repeatedly with methanol. In this way, G1 PAMAM-grafted silica was obtained. The Michael addition and amidation reactions were repeated to graft further generations of PAMAM to the surface of the silica.

2.4. Cellulase immobilization

Cellulase was immobilized on PAMAM-grafted silica respectively by adsorption and crosslinking methods. For the adsorption method, after washing with methanol, deionized water and acetate buffer, the PAMAM-grafted silica was mixed with 5 mg ml^{-1} cellulase acetate solution (pH 4.8) for 2 h. For the crosslinking method, the PAMAM-grafted silica was reacted with 0.4% (v/v) glutaraldehyde aqueous solution with stirring for 2 h. Thereafter, the silica was immersed in a 4 mg ml^{-1} cellulase acetate solution (pH 4.8) and incubated for 2 h. After that, 120 mg sodium cyanoborohydride was poured into the mixture. Thus, cellulase was attached to the surface of the PAMAM-grafted silica through adsorption and crosslinking methods, respectively. Finally, the immobilized cellulase-PAMAM-grafted silica, rinsed with acetate buffer, was stored at 4 °C until use. The amount of immobilized cellulase was calculated by determining the decrease of the cellulase solution concentration at 280 nm absorbance [22].

2.5. Optimization of immobilization and enzymolysis conditions

The optimal immobilization and enzymolysis conditions were obtained by measuring the amount of hydrolysis product glucose of CMC using the DNS method [33,34]. The 0.1 g of immobilized cellulase-PAMAM-grafted silica was added to 10 ml 2% (w/v) solution of CMC acetate buffer for 10 min enzymolysis. The reaction was stopped by putting the enzyme reaction tubes in a boiling water-bath for 5 min. After centrifugation, 0.5 ml of the supernatant was moved into a clear tube and colored by adding 2 ml of DNS. The concentration of the product glucose was measured by a Hitachi U3010 UV-Vis spectrophotometer (Tokyo, Japan) at 490 nm. A unit of the cellulase activity (U) was defined as producing 1 μg glucose per minute. The relative activity was defined as percentage of the maximum activity obtained in that series.

2.6. Thermal stability, reusability and storage stability of immobilized cellulase-PAMAM-grafted silica

The thermal stabilities of free and immobilized cellulase using adsorption and crosslinking methods were determined by measuring the residual activity of the enzyme exposed to 70 °C in acetate buffer for 3 h. After every 0.5 h time interval, 0.1 g immobilized cellulase were used and assayed for enzymatic activity. The reusability of immobilized cellulase was evaluated by repeated enzymatic hydrolysis six times. The storage stability was assessed by measuring activity after storage in buffer at 4 °C for 5 weeks and expressed as a percentage of the retained activity compared to the initial activity.

3. Results and discussion

3.1. Characterization of PAMAM-grafted silica

The PAMAM modified on silica were characterized by FTIR, SEM and elemental analysis. The FTIR result for the G3 PAMAM-grafted silica is shown in Fig. 2, which suggests that the bands at 1637 cm^{-1} (amide I) and 1556 cm^{-1} (amide II) are respectively assigned to the C═O stretch and N—H bending vibrations of amide groups. The absorption peaks around 2956 and 2858 cm^{-1} indicate the C—H stretching vibrations of methyl and methylene groups. The intense band between 3400 and 3500 cm^{-1} correspond to the stretching vibration of free N—H. The peak at 1743 cm^{-1} may result from

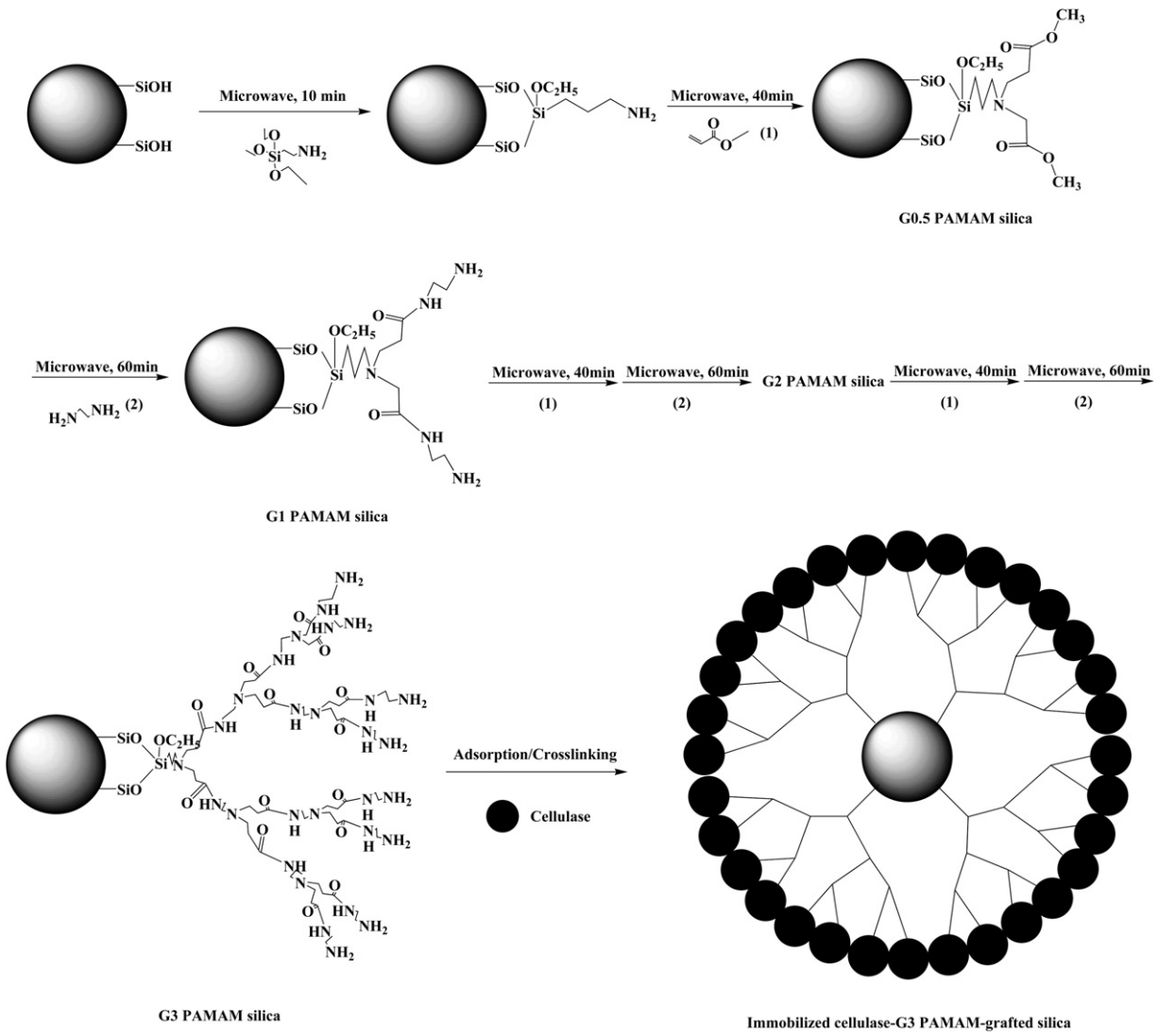

Fig. 1. Schematic of the process for grafting PAMAM to the surface of the silica by microwave irradiation and cellulase immobilization.

the absorption of C=O in residual ester groups during the amidation reaction. The FTIR results are favorably in agreement with our previous results and literatures [17,24,35]. The morphologies of PAMAM-grafted silica of different generations were shown in Fig. 3, and the comparison of these SEM images clearly exhibits that the supports were of homogeneous and degree of branching of PAMAM rised with its generation. The results of elemental analysis further supported an increase in the proportion of C, H and N with increasing the generations of the PAMAM, as shown in Table 1. These results demonstrate that PAMAM of different generations was successfully grafted onto the silica surface by repeated Michael addition and amidation reactions.

3.2. Effect of generations of PAMAM on cellulase binding capacity

After immobilization, the characteristic bands of cellulase at 1656, 1552 and 1453 cm^{-1} could be observed in the FTIR spectrum of immobilized enzyme, which indicates that the cellulase was successfully immobilized on the PAMAM-grafted silica. The effect of different PAMAM generations on cellulase binding capacity is shown in Table 1. All enzyme capacities and activities of both adsorption and crosslinking immobilization methods improved with generations of PAMAM because as the generation of PAMAM increased, an increasing density of terminal amino groups on PAMAM-grafted silica can form more hydrogen bonds and enhance

Table 1
Elementary analysis of PAMAM-grafted silica and effect of generations of PAMAM on cellulase binding capacity.

Generation	Elemental proportion in PAMAM-grafted silica			Enzyme capacity (mg g^{-1})	
	N (%)	C (%)	H (%)	Adsorption	Crosslinking
G0	1.91	6.16	1.89	32.47	27.86
G1	3.35	10.83	2.59	53.47	58.10
G2	3.96	13.29	2.97	62.55	83.71
G3	5.02	15.69	3.37	86.85	98.19

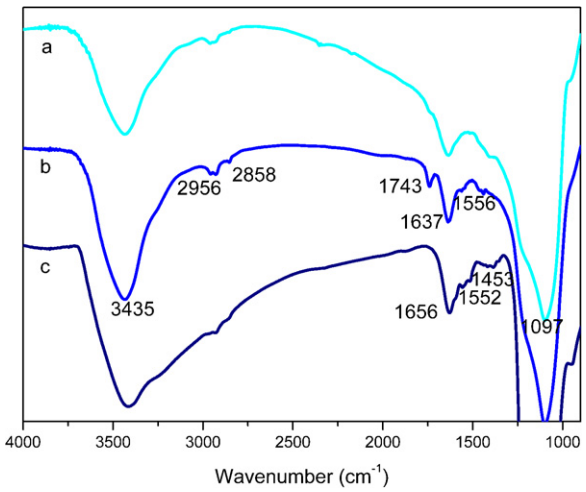

Fig. 2. FTIR spectra of G3 PAMAM-grafted silica and immobilized cellulase. (a) Bare silica; (b) G3 PAMAM-grafted silica; (c) immobilized cellulase-G3 PAMAM-grafted silica.

electrostatic forces that contributed to adsorption of cellulase. On the other hand, large number of the surface functional amino groups provided more reactive sites for the attachment of cellulase through glutaraldehyde crosslinking. In addition, the external spatial structure of silica was gradually changed and biocompatibility of silica was improved when generations of PAMAM grew, resulting in an increase of immobilization efficiency. However, the cellulase molecule is large and causes severe steric hindrance, preventing the cellulase binding capacity from attaining their theoretical values. Moreover, high-generation PAMAM-grafted silica needs longer synthesis time and more steps. G3 PAMAM-grafted silica was therefore adopted for immobilizing cellulase in subsequent experiments.

3.3. Optimization of immobilization and enzymolysis conditions

Immobilization time is a important factor in enzyme immobilization process. Fig. 4A presents the effect of immobilization time on the activities of immobilized cellulase-G3 PAMAM-grafted silica using adsorption and crosslinking methods. The results showed that the enzymatic activities for two methods were improved with immobilization time up to 2 h. As time continued to increase, the relative activity changed only insignificantly because the immobilization capacity of carrier saturated. A longer immobilization time allows cellulase to attach more completely with the PAMAM-grafted silica and therefore a higher activity and hydrolysis efficiency of immobilized cellulase will exhibit, but longer immobilization time reduces the speed of experiments. An optimal immobilization time of 2 h for both adsorption and crosslinking methods was chosen to give an acceptable enzymolysis efficiency and time.

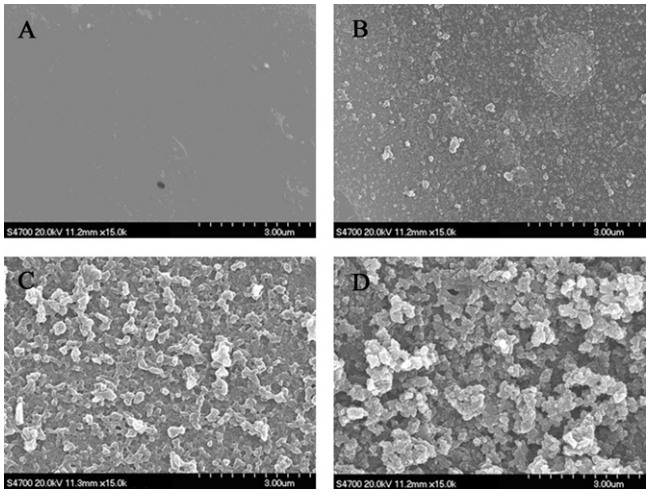

Fig. 3. SEM images of PAMAM-grafted silica of different generations. (a) Bare silica; (b) G1; (c) G2; (d) G3.

The effect of initial cellulase concentration on the activity of immobilized enzyme was investigated. The enzymatic activities for samples incubated with cellulase of different concentrations are shown in Fig. 4B. The activity of immobilized enzyme increased with rising initial cellulase concentrations until it reached 4 mg ml^{-1} for crosslinking method and 5 mg ml^{-1} for adsorption method. However, further increases in concentration did not improve the relative activity significantly. In addition, the concentration of crosslinking agent, glutaraldehyde, is another major parameter for crosslinking method and was estimated. The experimental results were obtained for a range of 0.1–1% (v/v) glutaraldehyde. The relative activity initially increased with rising glutaraldehyde concentration, whereas higher concentration may led to enzyme denaturation and side reactions, which severely lowered the activity of immobilized cellulase-G3 PAMAM-grafted silica. An optimal glutaraldehyde concentration of 0.4% (v/v) was selected for the subsequent experiments.

It is known that enzyme activity is dependent on the ionization state of the amino acids in the active site, so pH plays a significant role in maintaining the proper conformation of an enzyme. The effect of pH on the hydrolysis activities of free and immobilized cellulase-G3 PAMAM-grafted silica using adsorption and crosslinking methods was determined for 10 ml 2% (w/v) CMC acetate buffer (pH 2.8–7.8) enzymolysis for 10 min at 20 °C and the results are presented in Fig. 4C. Both free and adsorpted cellulase activities are strongly pH-dependent, and the maximum for free and adsorbed cellulase was achieved at pH 4.8. Compared with free and adsorpted cellulase, the immobilized cellulase using crosslinking method exhibited higher stability over a wider range of pH values, showing a maximum at pH 5.8. The difference in the optimal pH for free and the immobilized cellulase using crosslinking method was caused by the change of the pendent groups and the enzyme microenvironment. The aldehyde group of crosslinking agent and the residual amino of enzymes could result in the Schiff base and further form the stable secondary amine formation, which would affect the enzyme active center. In addition, the strong covalent bond may also lead to the spatial rigid structure that could affect the intra-molecular forces, and influence the conformation of the enzymes. Consequently, the change of optimal pH was observed.

Temperature is one of the most important factors in enzymatic hydrolysis. The effect of temperature on the activities of free and immobilized cellulase was examined. The results of the experiments performed on 10 ml 2% (w/v) CMC acetate buffer (pH 4.8 for free and adsorbed cellulase; pH 5.8 for crosslinked cellulase) enzymolysis for 10 min between 20 and 70 °C are presented in Fig. 4D, which shows that free enzyme activity initially increased with increasing temperature up to 50 °C and that cellulase was deactivated as the temperature continued to rise, leading to a dramatic decline in enzymolysis efficiency. In contrast, the immobilized cellulase-G3 PAMAM-grafted silica using both adsorption and crosslinking methods showed a maximum at 60 °C and retained approximately 90% activity when temperature reached 70 °C, which demonstrated that the cellulase immobilized on G3 PAMAM-grafted silica possessed more stable three-dimensional structure and thermal stability than those of the free enzyme.

Based on the results obtained above, the actual enzyme activity was measured by based on the amount of released glucose during

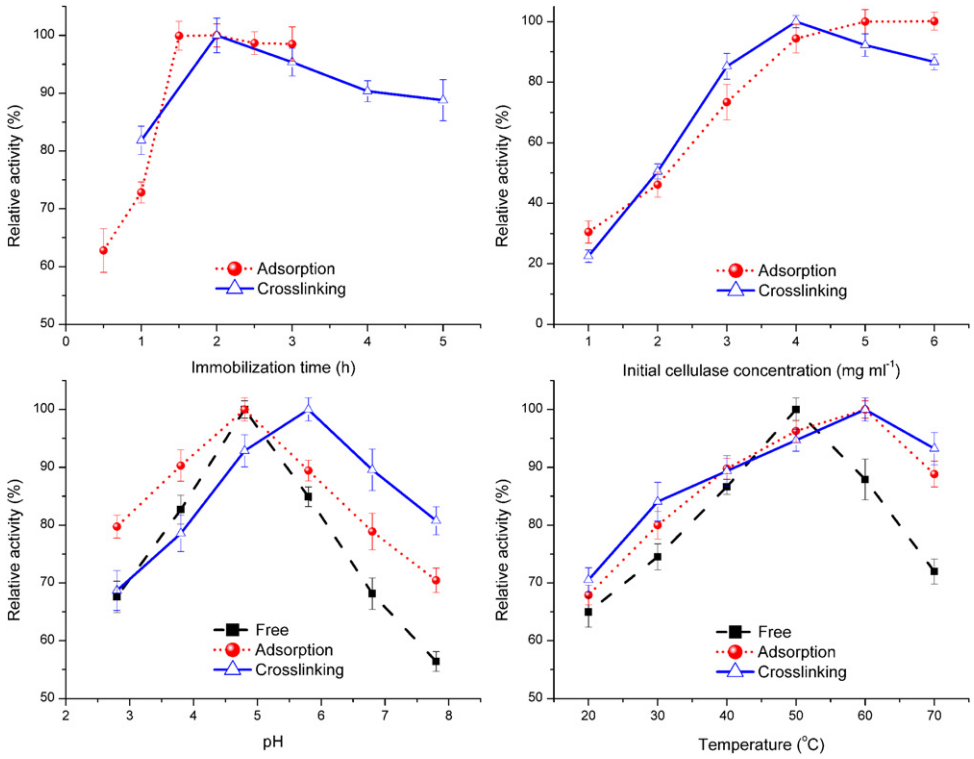

Fig. 4. (A) Effect of immobilization time on the activities of immobilized cellulase-G3 PAMAM-grafted silica using adsorption and crosslinking methods. (B) Effect of initial cellulase concentration on the activities of immobilized cellulase-G3 PAMAM-grafted silica using adsorption and crosslinking methods. (C) Effect of pH on the activities of free and immobilized cellulase-G3 PAMAM-grafted silica using adsorption and crosslinking methods. (D) Effect of temperature on the activities of free and immobilized cellulase-G3 PAMAM-grafted silica using adsorption and crosslinking methods.

the hydrolysis of CMC solution. The results show that the actual activities of free and immobilized cellulase-G3 PAMAM-grafted silica using adsorption and crosslinking methods were respectively 1513.9, 1335.3 and 984.6 U. Compared with free enzyme, the activities of adsorbed and crosslinked cellulase retained 88.20% and 65.04%. The results indicate that the immobilized cellulase on G3 PAMAM-grafted silica could retain a major activity for performing the enzymatic reaction.

The most important kinetic constant of the enzyme reaction, the Michaelis constant, can be determined by the Lineweaver–Burk method [36]. The Lineweaver–Burk plot was linear (Fig. 5) and gave a Michaelis constant of 0.26 mg ml^{-1} for free enzyme, 0.33 mg ml^{-1} for adsorbed cellulase and 0.41 mg ml^{-1} for crosslinked cellulase.

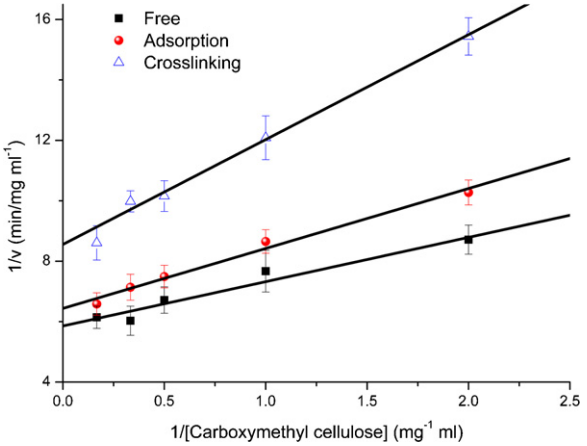

Fig. 5. Lineweaver–Burk plot for free and immobilized cellulase-G3 PAMAM-grafted silica using adsorption and crosslinking methods.

Compared with free enzyme, an increase of Michaelis constant for immobilized cellulase implied the slight decline of biological affinity to substrates. The smaller change of Michaelis constant of the adsorbed cellulase indicated that immobilized cellulase using adsorption retained more affinity and activity.

3.4. Thermal stability, reusability and storage stability of immobilized cellulase-G3 PAMAM-grafted silica

Thermal stability experiments were carried out with free and immobilized cellulase-G3 PAMAM-grafted silica using adsorption and crosslinking methods, which were incubated in the absence of substrate at 70 °C from 0.5 h to 3 h, and then the enzymatic activities were measured respectively. As shown in Fig. 6A, the free cellulase lost more than half of its initial activity within 2 h. After a 3 h treatment at 70 °C, adsorbed and crosslinked cellulase retained about 53 and 66% of their initial activities and decreased more slowly than those of the free counterpart. The results shows that immobilization of cellulase can enhance the thermal stability, especially at a higher temperature. The increase in the thermal stability of the immobilized cellulase may arise from the conformational integrity of the immobilized enzyme structure after multi-point adsorption immobilization or covalent binding to the G3 PAMAM-grafted silica.

The reusabilities of the adsorbed and crosslinked cellulase were studied throughout six successive runs. Fig. 6B presents that the retained activities of immobilized cellulase-G3 PAMAM-grafted silica using adsorption and crosslinking methods were found to be 75 and 82% after 3 run, respectively. At the end of six run cycles, these values were found to be 41 and 67%. The higher values were obtained for crosslinked cellulase due to the strong covalent bonds between the enzyme molecules and the G3 PAMAM-grafted silica. The storage stability was assessed by measuring activity

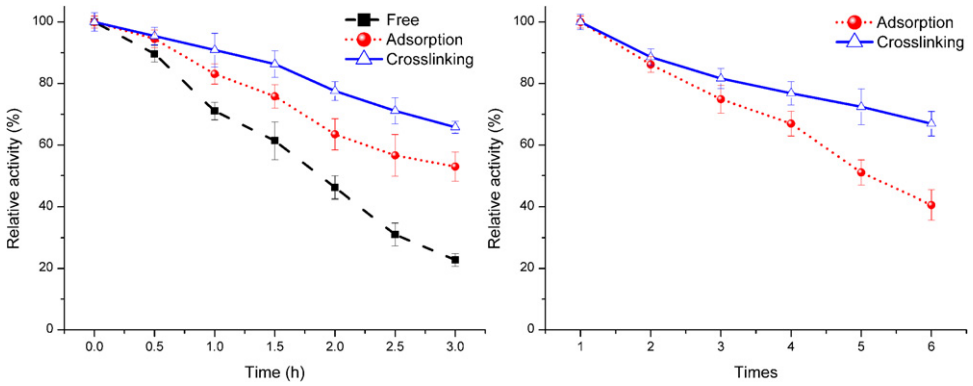

Fig. 6. (A) Thermal stabilities of free and immobilized cellulase-G3 PAMAM-grafted silica using adsorption and crosslinking methods. (B) Reusability of the adsorbed and crosslinked cellulase.

after storage in buffer at 4 °C for 35 days and the activities of the adsorbed and crosslinked cellulase respectively remained above 70 and 80%.

4. Conclusions

PAMAM was grafted onto the surface of silica with microwave-assisted synthesis, which was used as a new type of carrier for immobilization of cellulase with adsorption and crosslinking. These two methods have different immobiliztion effects and advantages. The adsorption tends to retain the enzyme activity well, while the crosslinking method is appropriate for clearly promoting the stability and reusability. The results indicate that the introduction of PAMAM enables higher amounts of cellulase to be immobilized, markedly improving the enzymolysis efficiency with increasing PAMAM generations. The presented immobilized cellulase-PAMAM-grafted silica could be applied in a wider range of temperature and pH and possess the enhanced reusability, thermal stability and storage stability in comparison to free enzyme, which demonstrates that a stable three-dimensional structure of enzyme was established after immobilization. The PAMAM-grafted silica provides a favorable microenvironment to not only retain the high enzymatic activity and specificity, but also markedly improve the immobilized enzymes properties such as capacity, enzymolysis efficiency and stability. This protocol can be potentially adapted to support other biomacromolecules in future biochemical and biotechnological applications.

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