Final Draft
of the original manuscript:

Dai, T.; Miletic, N.; Loos, K.; Elbahri, M.; Abetz, V.**:**
Electrospinning of Poly[acrylonitrile-co-(glycidyl methacrylate)] Nanofibrous Mats for the Immobilization of Candida Antarctica Lipase B
In: Macromolecular Chemistry and Physics (2010) Wiley

DOI: 10.1002/macp.201000536

DOI: (final version of manuscript: macp.201000536)
Article Type: (Full Paper)

Electrospinning of Poly(acrylonitrile-*co*-glycidyl methacrylate) Nanofibrous Mats for the Immobilization of *Candida antarctica* Lipase B

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A novel electrospun nanofibrous mat (nanomat) and its application for enzyme immobilization is reported in this article. Poly(acrylonitrile-*co*-glycidyl methacrylate) (PANGMA) nanofibers and nanomats with fiber diameters of 200 to 300 nanometers were fabricated by electrospinning. These nanomats contain reactive epoxy groups which can be used to covalently immobilize enzymes. *Candida antarctica* lipase B (Cal-B) was covalently immobilized onto the PANGMA nanomats via three different immobilization routes: (i) activated with hexamethylenediamine (HMDA) and glutaraldehyde (GA), (ii) activated with ammonia (Ammo) and GA, and (iii) direct immobilization. The properties of the Cal-B immobilized PANGMA nanomats were assayed and compared with the free Cal-B. Results indicate that, the observed Cal-B loading on these nanomats is up to approximately 50 mg/g and the hydrolytic activity of them is up to approximately 2500 nmol/min/mg, performing much higher hydrolytic activity than free enzyme powder, also a little bit higher than Novozyme 435. The optimum pH value of the buffer solution for the Cal-B immobilization is 6.8. When compared with free Cal-B, the Cal-B immobilized PANGMA nanomats have better

reusability, thermal stability and storage ability. The nanomats retain over 50% of their initial activity after 15 cycles, over 65% after 10 hours heat incubation, and over 75% after 30 days storage. This kind of enzyme immobilized nanomat could have good potential applications in the catalysis field.

Introduction

As biocatalysts, enzymes exhibit a number of excellent features like high activity, high specificity and high selectivity, and also can catalyze many organic reactions under mild and environmentally friendly conditions.[1,2] However, most of the natural enzymes will lose most of their powerful catalytic activity in organic solvents and will easily denature under industrial conditions (high temperature, mechanical shear, etc.). Moreover, the direct recovery of enzymes from reaction solutions and the separation of enzymes from substrates and products are generally difficult. Therefore, there have been many attempts aiming to stabilize enzyme activity and increase operational stability.[3-5]

Among them, enzyme immobilization is the most effective and popular strategy for most applications. Immobilization can, in general, improve the catalytic activity and selectivity of enzymes, in addition it can enhance the temperature and solvent stability, and the most important function is: immobilized enzymes can easily be recovered from the reaction medium and preferably be reused.[6-8] This is particularly important because of the high enzyme costs. In recent years, there is a trend to use nanostructured materials as the support for the immobilization of enzymes since the large surface area of nanomaterials can effectively improve the enzyme loading per unit mass of support. Both nanoparticles and nanofibrous mats (nanomats) were attempted for this purpose. Compared with nanoparticles, nanofibers and nanomats have big advantages on the easy recovery and reuse of the enzymes. They are suitable for the application on continuous operations.[9-12] Electrospinning is a simple and versatile method for fabricating nanofibers with fiber diameters ranging from several

micrometers down to several decades of nanometers.[13–15] Electrospun nanofibrous mats (nanomats) have extremely high specific area and porous structure, which makes them become excellent candidates for enzyme immobilization.[16–18] The immobilization of enzymes on both natural and synthetic polymeric electrospun nanomats has been widely reported.[19–26]

Candida antarctica lipase B (Cal-B) has proven to be one of the most recognized and versatile one among lots of lipases.[27–29] This enzyme has lots of outstanding advantages, including: good stability in acidic pH range, quality of end product, less side products and good performance at high temperatures. It can be used in a broad range of applications in chemical industry, such as: kinetic resolutions, aminolysis, esterification, and transesterification.[30–33]

Poly(acrylonitrile-*co*-glycidyl methacrylate) (PANGMA) is a new polymeric material which is the copolymer of acrylonitrile (AN) and glycidyl methacrylate (GMA). It has both the advantage of the chemical stability from the sturdy backbone of polyacrylonitrile and the advantage of the further reacting ability from the free and active epoxy group on GMA. The epoxy group offers the opportunity for the employment of PANGMA on a variety of activation/coupling chemistries for the covalent bonding of enzymes. An effectual covalent bonding of enzymes on the support not only can enhance the solvent and thermal abilities of the immobilized enzymes, but also can make the efficient reuse and recycling of the enzymes come to reality.[34,35] Godjevargova et al[36] cast PANGMA ultrafiltration membrane by phase-inversion method and used this membrane as a carrier for the immobilization of glucose oxidase. They proved that the immobilized glucose oxidase has similar or even better activity comparing to the free enzyme, and much better operational stability, reusability and storage stability. After that, they successively immobilized glucose oxidase onto ultrafiltration membranes made from different acrylonitrile copolymers and used them for the catalytic application. [37-39]

In this paper, we describe the first results for a novel Cal-B immobilized on PANGMA electrospun nanofibrous mat (nanomat), which can be obtained by two steps, first electrospinning PANGMA/DMF solution and second a chemical immobilization of Cal-B via activation and covalent bonding. The enzyme loading, catalytic activity, optimum pH and temperature, reusability, thermal ability and storage ability of the immobilized enzymes are investigated, described and discussed in detail. These novel Cal-B immobilized nanomats have potential applications in the field of catalysis.

Experimental Part

Materials

Poly(acrylonitrile-*co*-glycidyl methacrylate) (PANGMA) was synthesized by GKSS-Forschungszentrum GmbH with a molecular weight (*M*n) of ca. 100,000 g/mol and GMA contents of 13 mol%.[34] *Candida antarctica* lipase B (Cal-B) in the form of a dried powder was purchased from BioCatalytics Co. (Grambach, Austria). Novozyme 435 was provided by Novozymes® (Bagsværd, Denmark). Hexamethylenediamine (HMDA), glutaraldehyde solution (25 wt.%) (GA), phosphate buffered saline (PBS) and *p*-nitrophenyl acetate (*p*NPA) were purchased from Sigma-Aldrich Co. Ammonium hydroxide solution (27 wt.%) (Ammo) was purchased from Fluka Co. *N,N*-dimethylformamide (DMF), methanol and 1,4-dioxane were purchased from Merck KGaA. All the chemicals were directly used without purification.

Preparation of PANGMA nanofibers via electrospinning

PANGMA was dissolved in dimethylformamide (DMF) at room temperature under moderate stirring for 48 h to form a homogeneous solution. The solution concentration was fixed to 20 wt.%. The solution was placed in a 5 mL glass syringe with a metal needle with the inner diameter of 0.8 mm. A high voltage generator was connected to the middle of needle. A

rectangular counter electrode covered with aluminium foil was used as the collector. Typically, electrospinning was performed at the applied voltage of 25 kV. The working distance (the distance between the needle tip and the collector) was 25 cm. The feed rate of the solution was controlled by a syringe pump (HARVARD PHD 4400, Harvard Apparatus Co.) to maintain at 1.2 mL/h and the electrospinning time was usually 5 h to obtain sufficient thickness of the mat. After electrospinning, the nanofibrous mat was detached and washed with distilled water and methanol to remove DMF and other impurities, and then was dried under vacuum at 50 ℃ for 24 h.

Enzyme immobilization

The immobilization of Cal-B on PANGMA nanofibrous mats was carried out by covalently binding Cal-B molecules to the epoxy groups on PANGMA via three different routes (Scheme 1).

Route 1: Indirect enzyme immobilization with HMDA and GA

PANGMA nanomats were first immersed in 10 wt.% HMDA aqua solution at room temperature for 1 h. Then the nanomats were washed with distilled water for several times. The washed nanomats were immersed in 10 wt.% GA aqua solution at 4 ℃ for another 1 h. After that, the activated PANGMA nanomats were extensively and carefully washed with distilled water and PBS buffer (PH 6.8) until total removal of unreacted HMDA and GA.

Enzyme immobilization was performed in lidded bottles loaded with Cal-B solution prepared with the concentration of 5 mg/mL in buffer solution with different pH values. The activated PANGMA nanomats were immersed into the Cal-B solution and the mixture was shaken at 4 ℃, room temperature (RT, 20 ℃) and 30 ℃ for up to 32 h. After reaction, the nanomats were taken out and washed several times with PBS buffer (pH 6.8) under shaking

condition until the complete removal of unbound enzymes. All the supernatants and washing solutions were collected carefully for the determination of enzyme loading capacity. The amount of residual protein after immobilization was determined by Bradford's method.[40] Bovine serum albumin was used as the standard to construct the calibration curve. The amount of immobilized protein onto the nanomats was estimated by deducting the amount of residual protein from the initial amount of protein used in the immobilization procedure (5 mg/mL). The enzyme loading capacity was defined as the amount of bound protein (mg) per gram of the enzyme immobilized PANGMA nanomat. Each reported value was the average of at least three experimental values.

Route 2: Indirect enzyme immobilization with Ammo and GA

Route 2 was carried out by the same technique as by route 1 but changing HMDA to ammonia aqua solution (27 wt.%) (Ammo). The ammonolysis was performed at 50 ℃ for 1 h.

Route 3: Direct enzyme immobilization

The direct immobilization was carried out by the same technique with route 1 except for the activation treatment with HMDA and GA.

Hydrolytic activity assay of free and immobilized Cal-B

A 1,4-dioxane solution (5 ml) containing *p*NPA (40 mM) and methanol (80 mM) was added to 20 ml vials containing 0.500 mg of enzyme. The assay reactions were carried out for 50 min at 35 ℃ (250 rpm) and were terminated by removal of the enzyme by filtration. The concentration of the reaction product *p*-nitrophenol (*p*NP) was determined by UV/VIS spectrophotometry (HITACHI U-3000, HITACHI) at λ_{max} (304 nm) of *p*NP. Enzyme

hydrolytic activities for immobilized Cal-B are defined herein as the nanomoles of *p*NPA hydrolyzed in 1,4-dioxane per unit of weight of enzyme per time (nmol of *p*NP/min mg).

Reusability, thermal stability and storage stability

The reusability of the Cal-B immobilized nanomats was examined by a recycling hydrolytic activity assay. After each recycling reaction run, the immobilized nanomats were washed several times with PBS buffer (pH 6.8) to remove any residual substrates on the nanomats. The recycled nanomats were subjected to the new hydrolytic activity assay for the next cycle and so on. Activity retention was given as percentage of activity taken as 100% for the initial hydrolytic activity before recycling.

Thermal stabilities of Cal-B immobilized nanomats and free Cal-B were determined by incubating them in PBS buffer (pH 6.8) at 60 ℃ for 10 h. Periodically, the samples were withdrawn and their residual hydrolytic activities were determined. Activity retention was given as percentage of activity taken as 100% for the initial hydrolytic activity before incubation.

Storage stabilities of Cal-B immobilized nanomats and free Cal-B were determined by storing them in PBS buffer (pH 6.8) at 4 ℃ for 30 days. The residual hydrolytic activities of the samples were measured at intervals of 1 to 5 days within the 30 days. Activity retention was given as percentage of activity taken as 100% for the initial hydrolytic activity before storage.

Morphology characterizations of PANGMA nanofibers

The morphology of the pure and Cal-B immobilized PANGMA nanofibers and nanomats was observed with a scanning electron microscope (LEO Gemini 1550 VP, Zeiss) at 10 kV

accelerating voltage after sputter-coating with Au/Pd. The average diameters of the nanofibers were calculated from 10 different randomly chosen single values.

Structure characterizations of PANGMA nanofibers

The structure of both neat and Cal-B immobilized PANGMA nanofibers and nanomats was characterized by Attenuated Total Reflection Fourier transform infrared spectroscopy (ATR-FTIR) with a FTIR spectrophotometer (Bruker Equinox 55, Bruker Optics) in the mid-infrared range from 4000 to 500 cm^{-1}.

Results and Discussion

Fabrication PANGMA nanomats by electrospinning

It has been proven that electrospinning is an effective way for fabricating polymeric nanofibers and nanomats. In this work, PANGMA with GMA content of 13 mol.% was electrospun into nanofibers. The morphology of the PANGMA nanomats before and after Cal-B immobilization was observed by using the scanning electron microscope. As shown in **Fig.1(a)**, an almost homogeneous network of the electrospun nanofibers with diameters in the range of 200 to 300 nanometers was obtained when the electrospinning solution concentration was 20 wt.% and the applied voltage was 25 kV. The nanofibers show a smooth surface and a uniform body with a narrow distribution of fiber diameter.

From the SEM photos it can be also found that the morphology of the nanofibers remains uniform and the structure of nanomat remains complete after Cal-B immobilization reaction and washing with PBS buffer. As mentioned in the introduction, PANGMA has a good chemical stability and mechanical strength. It is quite stable during the Cal-B immobilization reaction and the following hydrolytic activity assay. Moreover, it can be seen clearly from **Fig.1(b)** that there are some small agglomerations of Cal-B on the surface of the nanofibers

after Cal-B was immobilized. This result also verifies the successful binding of the Cal-B on the PANGMA nanofibers.

FTIR results also show the successful immobilization of Cal-B on PANGMA nanofiber. **Figure 2** shows the FTIR spectrum of neat (curve (a)) and Cal-B immobilized (curve (b)) PANGMA nanofibers. It could be observed that first on curve (a) there is a clear peak near 908 cm^{-1} (characteristic peak of epoxy group), which shows the existence of epoxy groups in neat PANGMA nanofibers. Meanwhile in curve (b), the epoxy peak is sharply diminished, which proves that epoxy groups in PANGMA have been reacted with enzymes. In addition, a single peak near 1650 cm^{-1} represents the existence of secondary aldimine (RCH＝NR′), which is the result of the imine formation reaction between the amine groups in modified PANGMA nanofibers and Cal-B and the aldehyde groups in glutaraldehyde. A double peak near 3300 cm^{-1} and a single peak near 1550 cm^{-1} both show the evidence of secondary amine, which is generated during the opening of the epoxy group by the immobilization of Cal-B. A peptide peak at 2180 cm^{-1} and two peaks near 2000 cm^{-1} give the direct information of the Cal-B structure. Finally, a peak near 3600 cm^{-1} is the characteristic peak of the hydroxyl group conversed from the epoxy group and the primary amine during the immobilization reaction (Scheme 2).

Covalent immobilization of Cal-B on PANGMA nanomats

It is well known that the amount of immobilized enzyme and its activity depends on the physical and chemical structure of the carriers. PANGMA nanomats contain epoxy groups which are very suitable for direct and indirect immobilization of Cal-B. For indirect immobilization, PANGMA nanomats were first modified with HMDA/Ammo (spacers) in order to introduce amino groups into their structure. The presence of amino groups is needed for the further GA activation. For direct immobilization, PANGMA nanomats were directly coupled with Cal-B without any modification (**Scheme 2**). The properties (enzyme loading

and leaching, hydrolytic activity, reusability, thermal stability, storage stability, etc.) of the Cal-B immobilized nanomats prepared by different routes are compared and discussed.

Cal-B loading

Figure 3 shows the Cal-B loading of samples prepared via different immobilization routes at different immobilization temperatures. For each sample studied, immobilization saturation is achieved within 10 h. It is obvious from **Fig. 3(a)** that GA activation can enhance the Cal-B loading of the PANGMA nanomats (detailed data seen in **Table 1**). Nanomats with GA activation have much higher amount of bound Cal-B than those prepared by direct immobilization. As modifiers, HMDA and ammonia do not show significant differences in the amount of bound Cal-B. Samples modified with HMDA only give a little bit higher Cal-B loading than those modified with Ammo. It is reported that GA-activated epoxy resins attach more enzymes than epoxy resins themselves. For instance, López-Gallego *et al.* immobilized Glutaryl-7-aminocephalosporanic acid acylase (GAAA) onto both GA-activated and standard epoxy Sepabeads and found that 100% immobilization yield could be obtained for GA-activated epoxy Sepabeads whereas only 75% immobilization yield was got for standard one.[41] Their reported results are consistent with the results we have obtained.

The immobilization temperature seems to have limited influence on the Cal-B loading (**Fig. 3(b)**). This behavior is due to the high reactivity between Cal-B and the GA molecules introduced onto PANGMA nanofibers, and therefore the effect of the temperature does not play an important role on the loading. Nevertheless, small difference between immobilization temperatures is noticeable.

The hydrolytic activity of Cal-B immobilized PANGMA nanomats

All these Cal-B immobilized nanomats were studied to assess how these parameters (pH value, temperature, recycling times, storage environment, etc.) affect their hydrolytic activity (hydrolysis of *p*-nitrophenol acetate).

Tab.1 shows the immobilization conditions and working parameters of all the nanomats studied in this article. For comparison purpose, hydrolytic activity results of free Cal-B powder and commercially available Cal-B preparation, Novozyme 435, are also included. As the Cal-B loading, also the hydrolytic activity is highly influenced by the immobilization route. Indirect immobilization is found to give higher hydrolytic activity to the Cal-B immobilized nanomats than the direct immobilization. Nanomats modified with HMDA show advantages on the hydrolytic activity than those modified with Ammo. Hydrolytic activity also increases a little bit with the increase of immobilization temperature. Results in Tab. 1 indicate that Cal-B immobilized on all nanomats studied perform much higher hydrolytic activity than free enzyme powder. Furthermore, Cal-B immobilized at 30 ℃ on nanomats modified with HMDA has higher activity than Novozyme 435.[42]

Determination of proper pH value of the buffer solution for the optimum immobilization

It is well known that the pH value of the buffer solution for immobilization will affect the activity of the immobilized enzymes. Cal-B immobilization was carried out at different pH values and the optimum value was determined. The variation of the activity retention for the Cal-B immobilized nanomats with the pH value is given in **Fig.4**. Here, the activity retention was defined as the percentage ratio of initial activity to the maximum initial activity achieved in this set. It can be seen that Cal-B immobilization in the pH range of 6.0–7.5 provided relatively high activity values. This is because the enzyme conformation, which is essential for the enzymatic activity, changes with different pH values. It is well known that the

behavior of an enzyme molecule, such as conformation, is susceptible to its vicinal microenvironment. An alteration of the pH value results in a possible influence on the immediate vicinity of the enzyme environment. By this way, the pH value of the buffer solution finally affects the activity of the enzymes.[43-46] Therefore, there was an optimum pH value for enzyme activity in the enzyme immobilization process. In this case, the highest activity was observed at a pH 6.8, for all the samples prepared from the three different routes. This result is consistent with our previous work on the immobilization of Cal-B on polystyrene nanoparticles by physical adsorption.[43] Since the highest hydrolytic activity was found under the pH = 6.8, all other steps of this work were carried out at this pH value.

Reusability of Cal-B immobilized PANGMA nanomats

The reusability of the immobilized enzymes is of importance for their practical application because of the sustained costliness of the enzymes. The effect of repeated use on the activity of Cal-B immobilized nanomats is presented in **Fig.5**. The activity retention of the sample prepared with HMDA and GA at 4 ℃ was chosen as a representative since all the samples prepared under different conditions showed almost the same behavior and activity retention in reusability measurements. It can be found that the activity of the Cal-B immobilized nanomat decays when being recycled. The nanomat maintained about 72% of its initial activity after 5 cycles, about 60% after 10 cycles, and about 51% after 15 cycles, respectively. Such reusability is advantageous for the continuous use in industrial applications. The loss of activity could be explained by the inactivation of the enzyme due to the denaturation of protein and the less leakage of enzyme from the nanomats supports upon the repeated use. The elution of minor residual surface-adsorbed enzymes perhaps could also decrease the activity during the first few cycles.[47]

Thermal stability of Cal-B immobilized PANGMA nanomats

The drawback on the thermal stability of native enzymes is one of the most important limitations for their application in continuous reactors. It has been reported in several articles that the activation and the covalent binding between the enzyme and support can enhance the thermal stability.[44,48,49] Therefore, the thermal stability at 60 ℃ of Cal-B immobilized nanomats and free Cal-B were studied in this work. **Fig. 6** shows the activity retentions of the Cal-B immobilized nanomats and the free Cal-B under the incubation at 60 ℃. It can be seen that the free Cal-B almost loses all of its initial activity after 2 h, while the Cal-B immobilized nanomats prepared from three different routes retain their initial activities of about 96% for HMDA-GA, 95% for Ammo-GA and 88% for directly immobilization after 2h heat incubation, respectively. Even after 10 h incubation, all three Cal-B immobilized nanomats still maintain over 65% of their initial activities. These results indicate that the Cal-B immobilized nanomats are much more stable at high temperature than the free Cal-B. This also could be explained by the multipoint attachment of the Cal-B molecules with the epoxy groups on the nanomats. The formation of multiple covalent bonds between the enzyme and the nanomats supports restricts the conformation transition of the enzyme, resulting in the reduction of mobility and the protection of distortion of the enzymes at high temperature. This protects the enzymes from thermal deactivation.[44,45]

Storage stability of Cal-B immobilized PANGMA nanomats

Storage stability of the immobilized enzymes is one of the significant parameters to evaluate the properties of enzyme, which can greatly benefit the transportation and storage of the immobilized enzymes. **Fig. 7** shows the activity retentions of the Cal-B immobilized nanomat and the free Cal-B. Since there is no obvious difference of retentions among the Cal-B immobilized nanomats prepared under different conditions, the retention of the sample prepared with HMDA and GA at 4 ℃ is shown as a representative. It is obvious that as the

time of storage increases, there is a remarkable difference in the activity retentions between the Cal-B immobilized nanomat and the free one. The immobilized nanomat still retained about 77% of its initial activity after being stored in PBS buffer (pH 6.8) at 4 ℃ for 30 days, while the free Cal-B lost most of its initial activity after the same period of time. This indicates that immobilization can prolong the storage period and consequently increase the times of reusability of enzyme. The good storage stability of Cal-B immobilized nanomats could be attributed to two reasons: first the covalent bonds between Cal-B and PANGMA nanomat support prevent the structural denaturation of the enzymes; and second the leaching of Cal-B is effectively held back by the firm covalent bonds.[44,50,51]

Conclusion

Poly(acrylonitrile-*co*-glycidyl methacrylate) (PANGMA) nanofibers and nanomats with the fiber diameter of 200 to 300 nanometers were fabricated by electrospinning a 20 wt.% PANGMA/DMF solution at the applied voltage of 25 kV. *Candida antarctica* lipase B (Cal-B) was covalently immobilized onto the PANGMA nanomats via three different immobilization routes: (i) activated with hexamethylenediamine (HMDA) and glutaraldehyde (GA), (ii) activated with ammonia (Ammo) and GA, and (iii) direct immobilization. Results indicate that the Cal-B has been successfully immobilized onto PANGMA nanomats (FTIR). The observed Cal-B loading on these nanomats is up to approximately 50 mg/g and the hydrolytic activity of them is up to approximately 2500 nmol/min/mg. All the enzyme immobilized nanomats perform much higher hydrolytic activity than free enzyme powder, Cal-B immobilized at 30 ℃ on nanomats modified with HMDA even have higher activity than Novozyme 435. The optimum pH value of the buffer solution for the Cal-B immobilization is 6.8. Cal-B immobilized nanomats retain over 50% of their initial activity after 15 cycles of reuse. They also have good thermal and storage stabilities, retaining over

65% of their initial hydrolytic activities after 10 hours heat incubation, and over 75% after 30 days storage, which are obviously higher than those of the free Cal-B. These improvements show that such types of enzymes immobilized on nanomats could have good potential applications in the field of catalysis.

Acknowledgements: This work was financially supported by a DAAD-HGF fellowship for Tianhe Dai. The authors thank Karen-Marita Prause for recording the SEM micrographs.

Received: ((will be filled in by the editorial staff)); Revised: ((will be filled in by the editorial staff)); Published online: ((will be filled in by the editorial staff));
DOI: ((will be filled in by the editorial staff))

Keywords: *candida antarctica* lipase B (Cal-B); electrospinning; enzyme immobilization; nanomats; poly(acrylonitrile-*co*-glycidyl methacrylate) (PANGMA)

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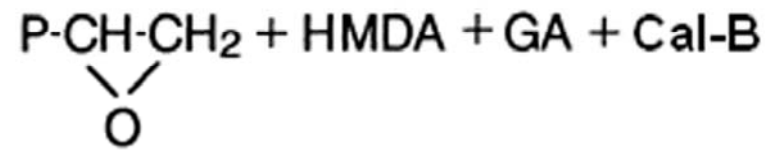
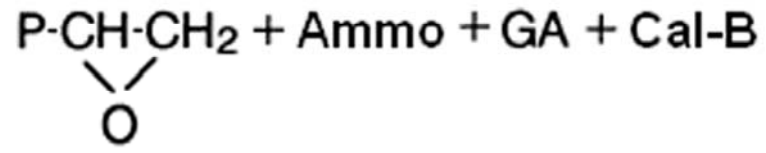
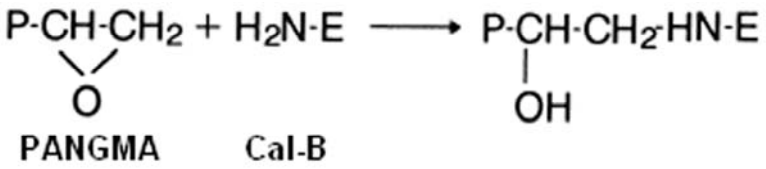


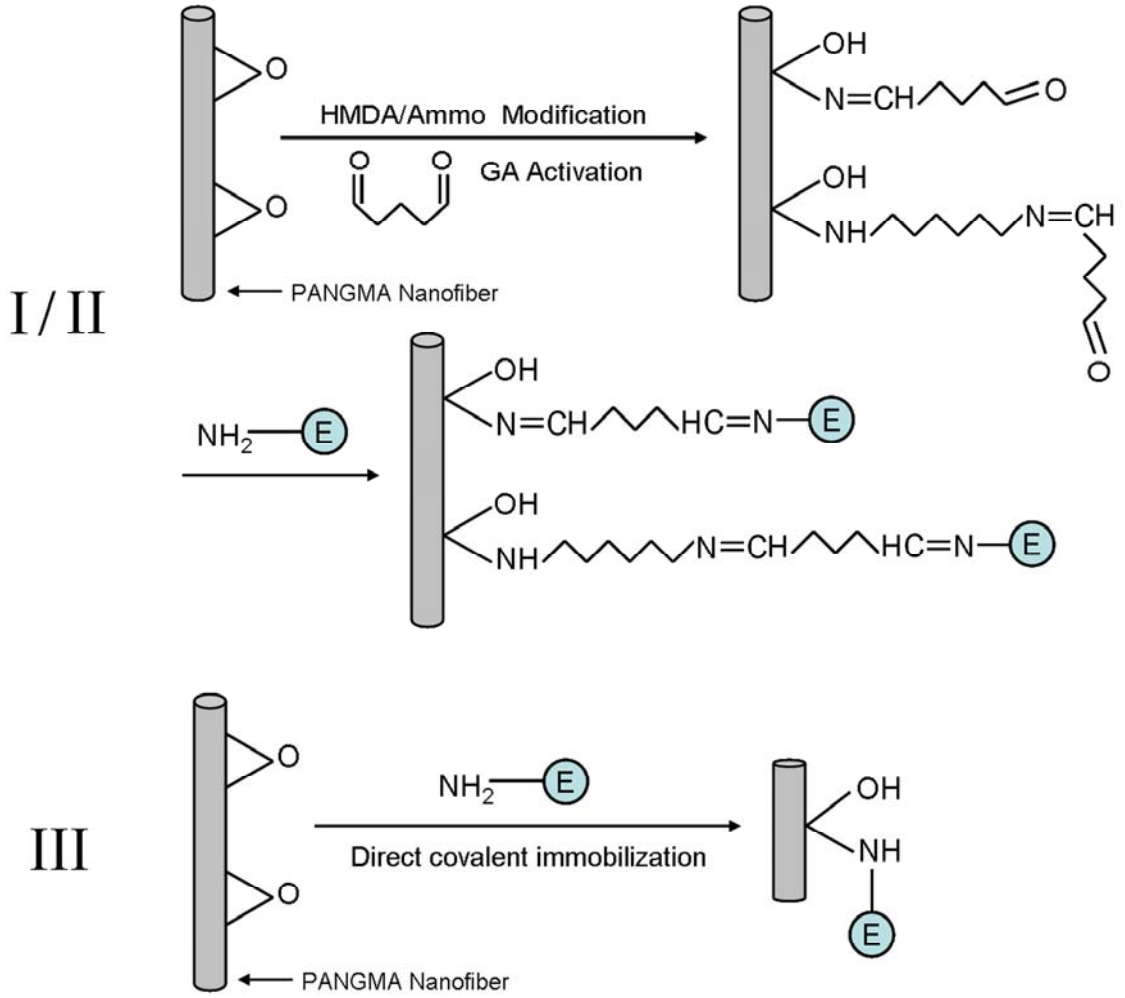


(a)

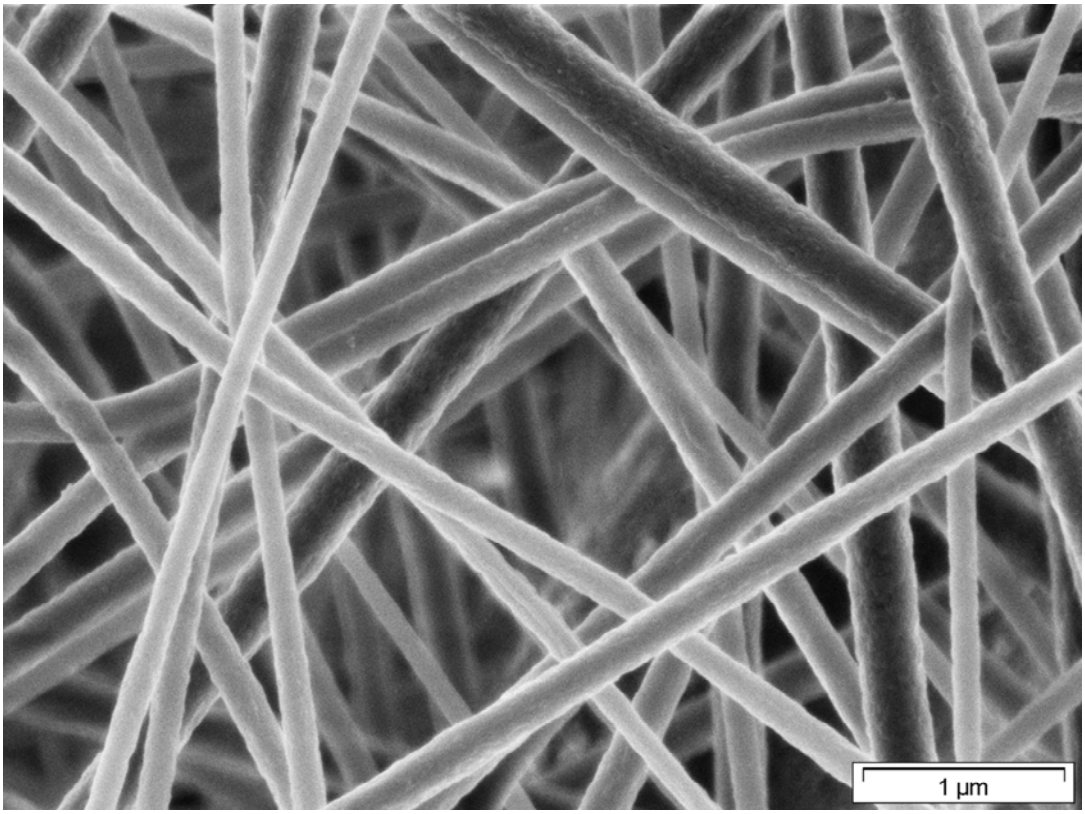


(b)

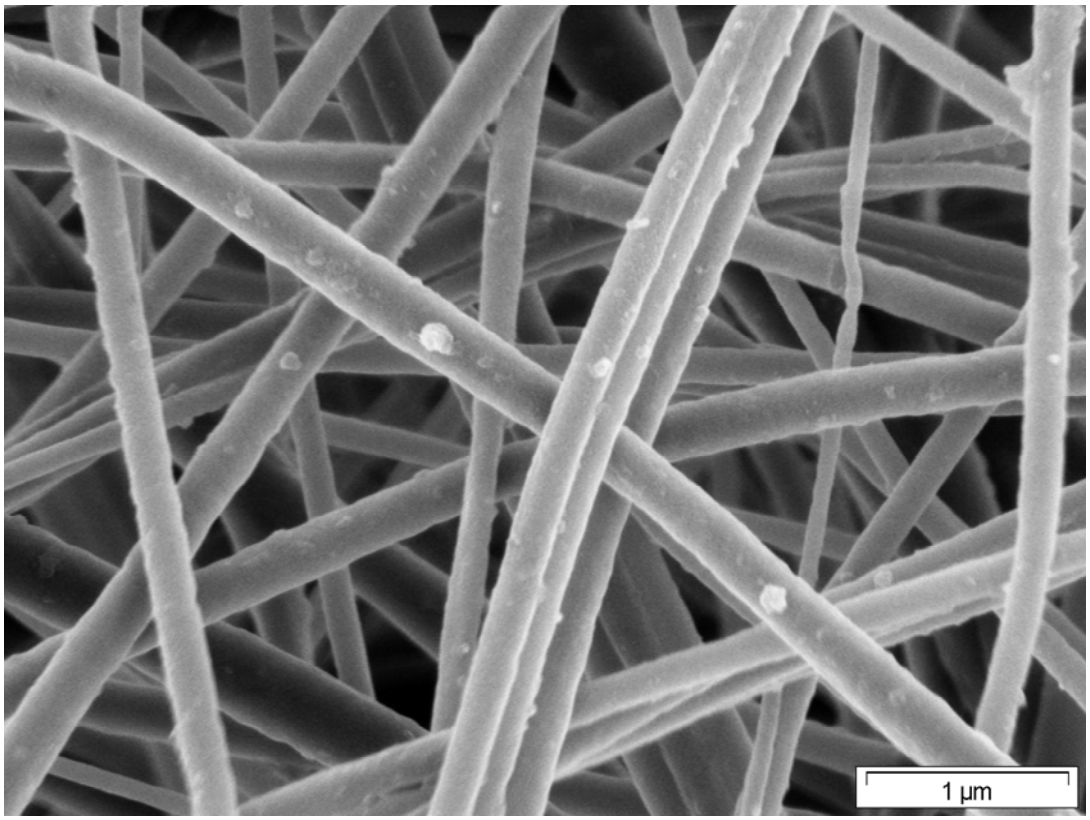
Scheme 1. Immobilization routes of Cal-B on PANGMA nanomats: (a) indirect immobilization; (b) direct immobilization



Scheme 2. Schematic diagram for the preparation of covalently attached enzymes on nanofibers



(a)



(b)

Figure 1. SEM micrographs of the PANGMA nanofibers: (a) neat nanofibers; (b) Cal-B immobilized nanofibers

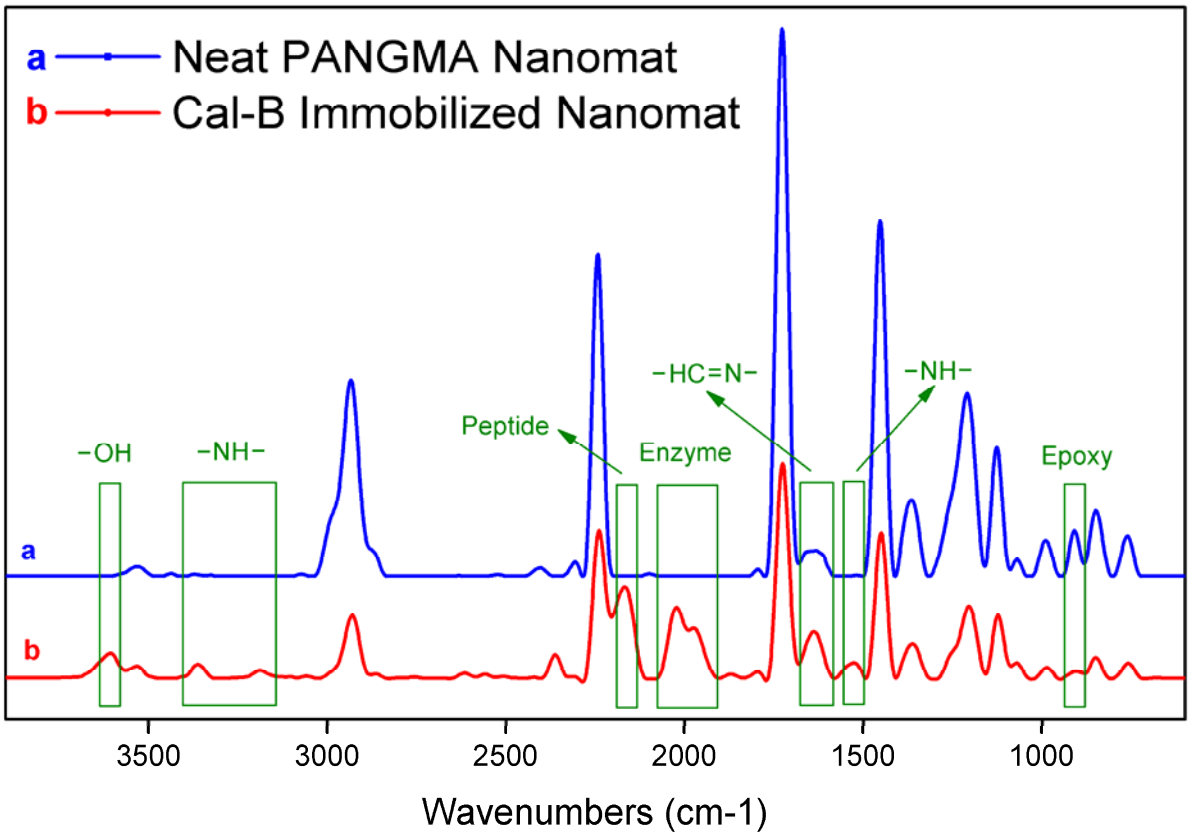
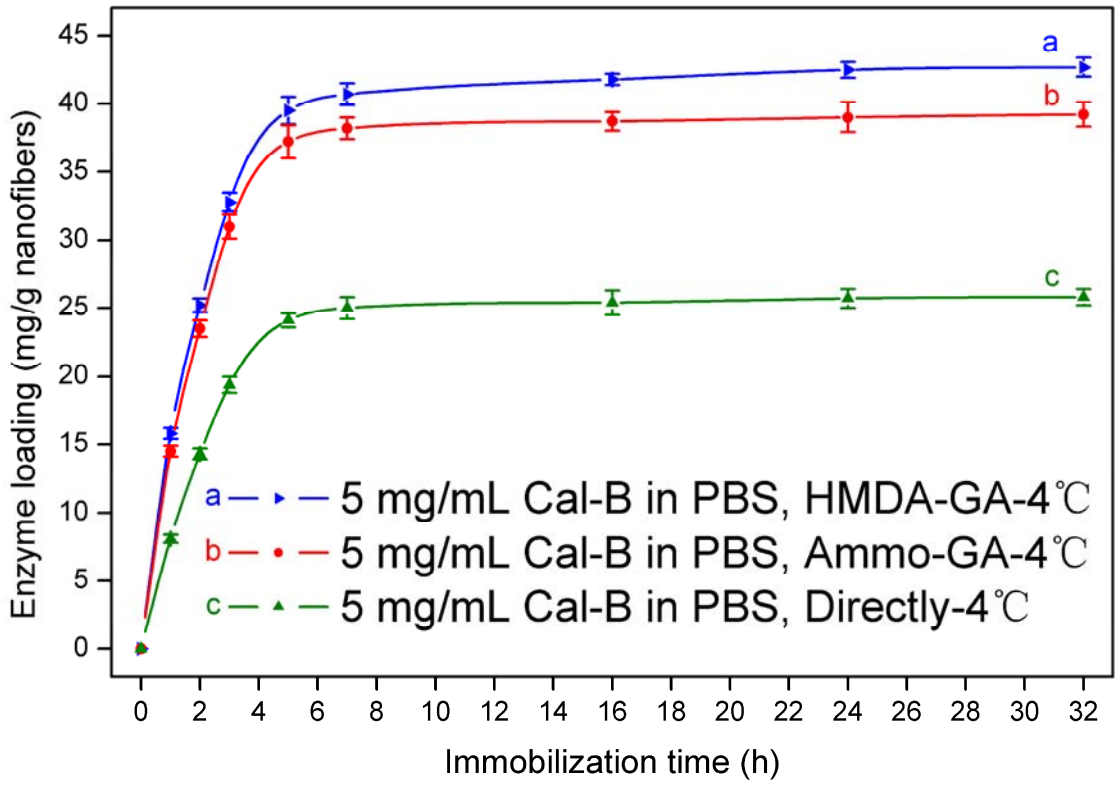
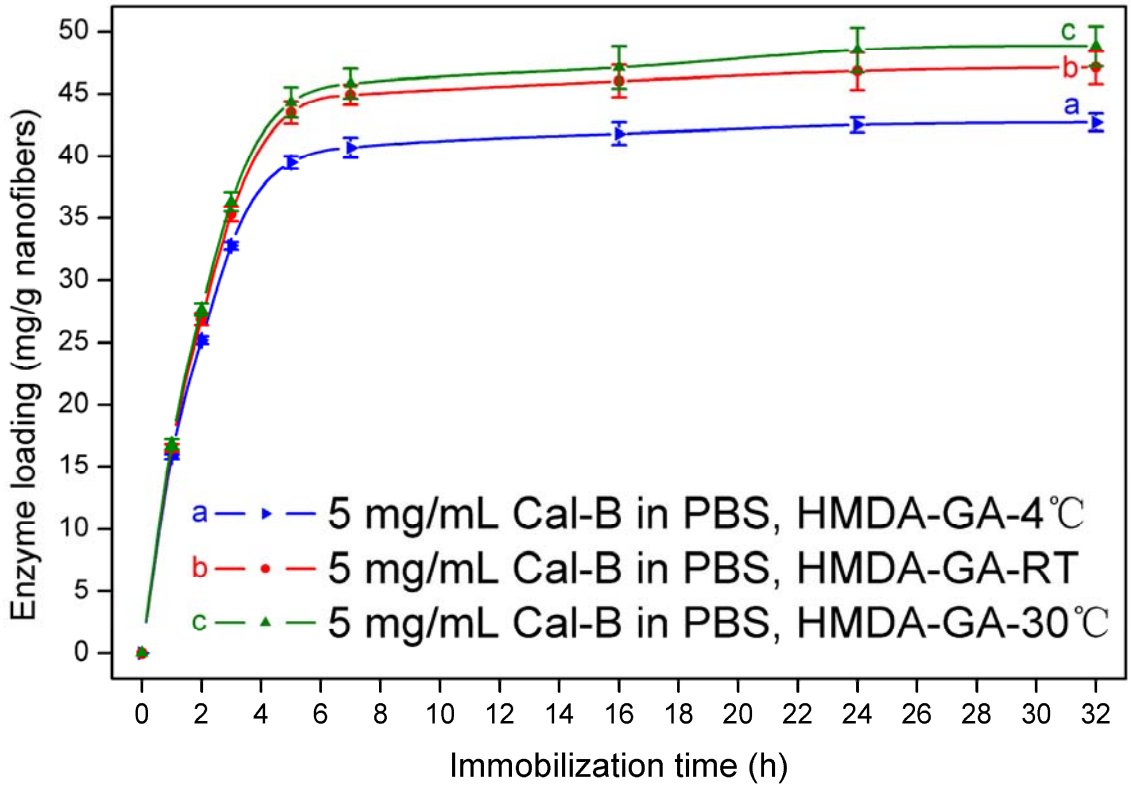


Figure 2. FTIR spectrum of neat (curve (a)) and Cal-B immobilized (curve (b)) PANGMA nanofibers, immobilized nanofibers prepared by HMDA and GA activation and immobilization in 5mg/mL Cal-B/PBS solution at 4 ℃



(a)



(b)

Figure 3. Comparison of enzyme loading of Cal-B immobilized PANGMA nanomats: (a) via different immobilization routes; (b) at different immobilization temperature

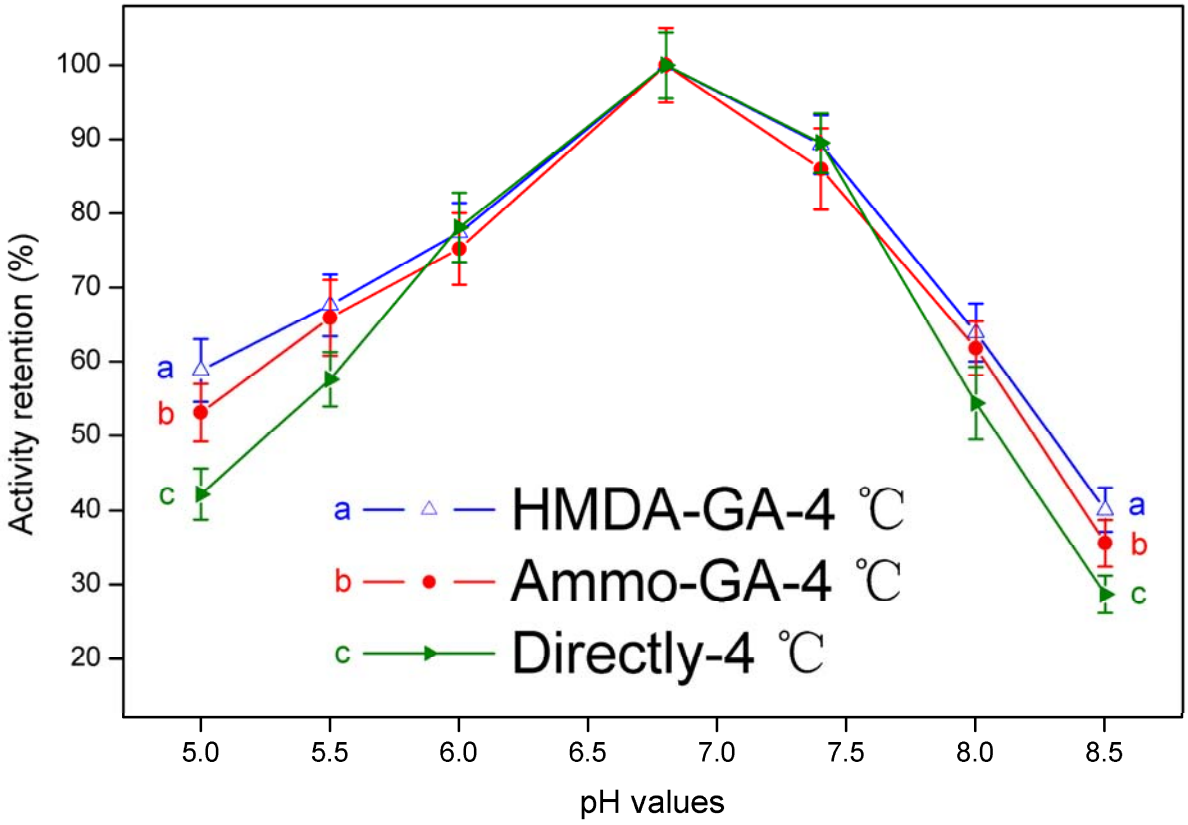


Figure 4. Hydrolytic activity of Cal-B immobilized PANGMA nanomats versus pH value of the buffer solution used during immobilization

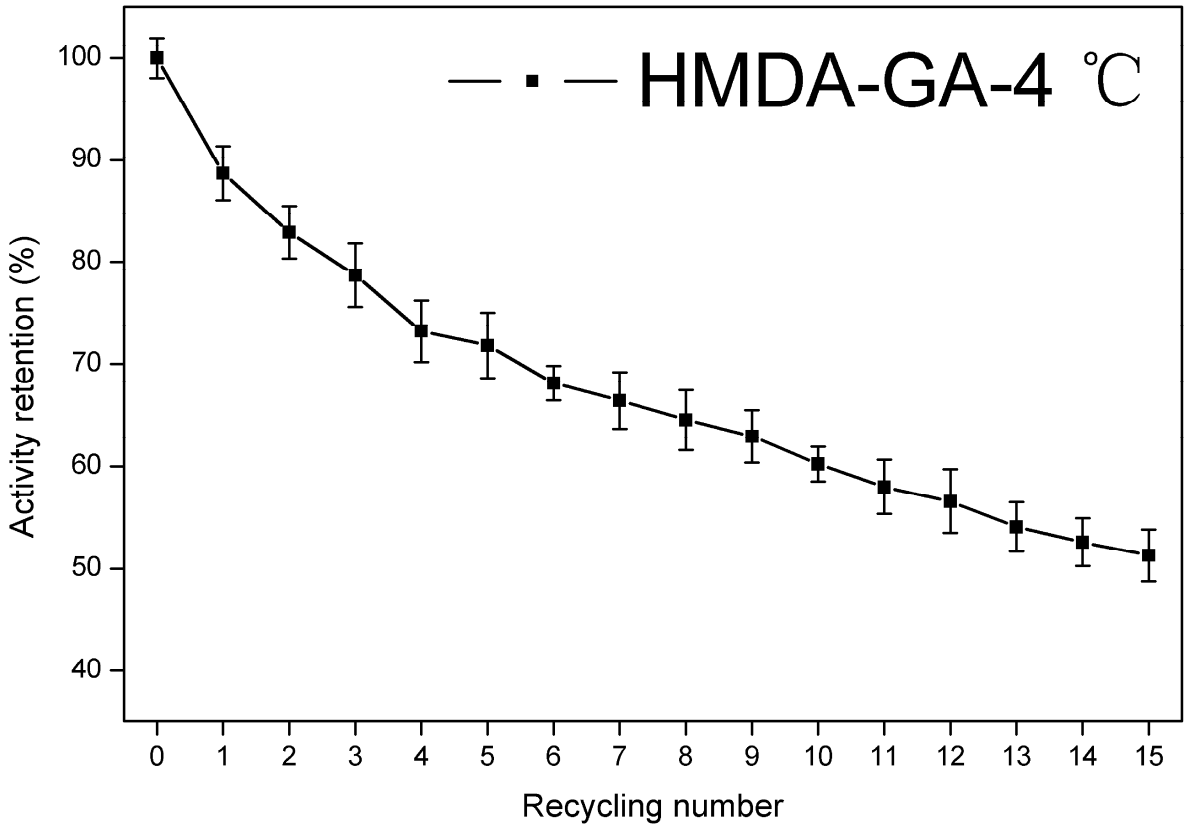


Figure 5. Reusability of the Cal-B immobilized PANGMA nanomats (activated with HMDA and GA, immobilized in PBS buffer at 4 ℃)

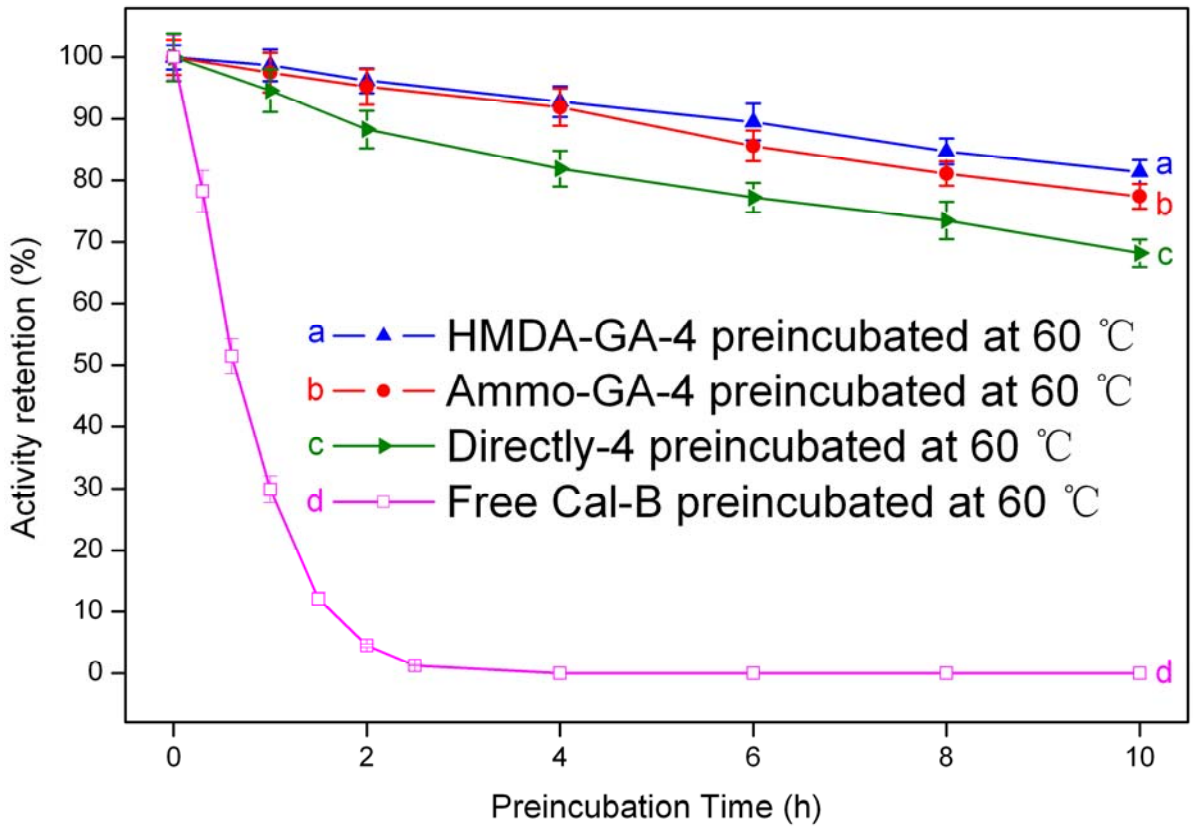


Figure 6. Thermal stabilities of Cal-B immobilized nanomats (curve a,b,c) and free Cal-B (curve d), preincubated in PBS buffer at 60 ℃

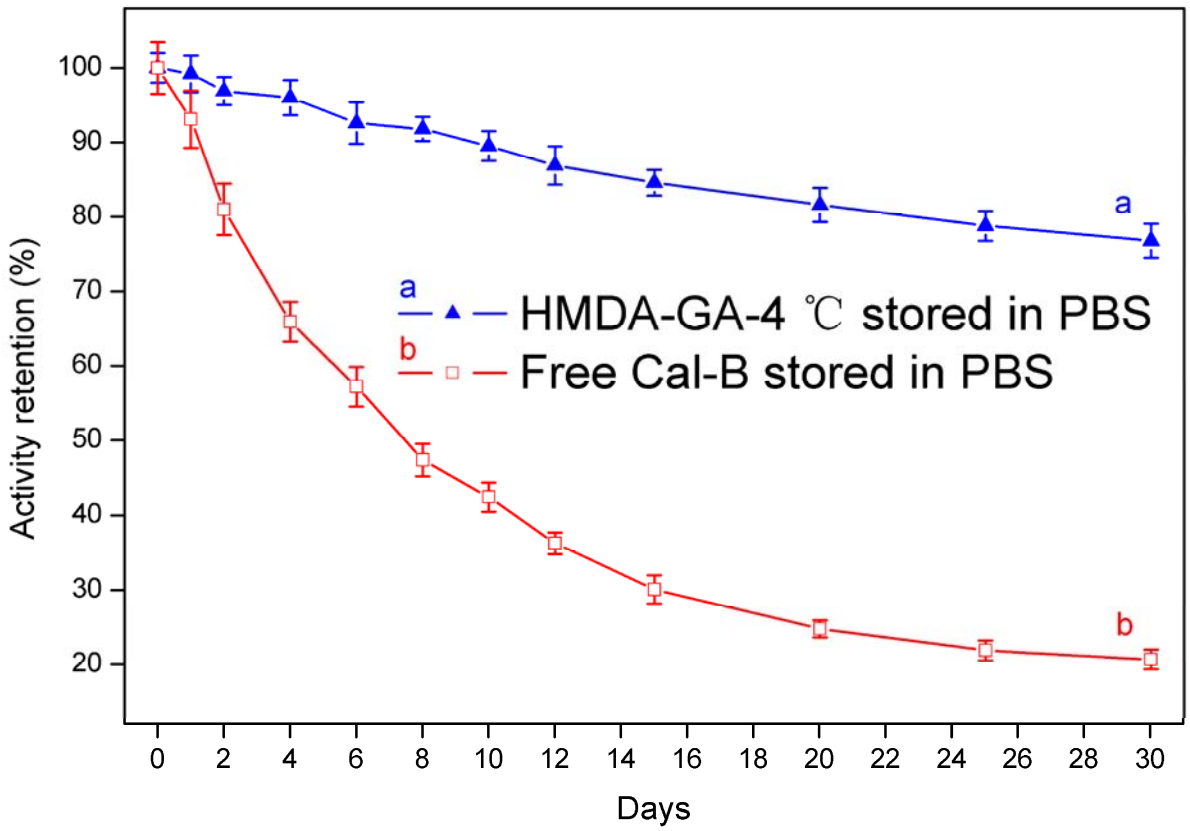


Figure 7. Storage stability of Cal-B immobilized nanomat (curve a) and free Cal-B (curve b) in PBS buffer at 4 ℃ for 30 days

Table 1. Cal-B immobilization on PANGMA nanomats obtained by different immobilization routes and at different immobilization temperatures with immobilization for 24 h: enzyme loading and hydrolytic activity

Catalyst	**Modification**	**Activation**	**Immo. temp. (℃)**	**Enzyme loading (mg/g fiber)**	**Hydrolytic activity (nmol *p*NP/min mg Cal-B)**
H-G-4	Hexamethylenediamine	Glutaraldehyde	4	42.1 ± 0.7	2285.6 ± 45.1
H-G-RT	Hexamethylenediamine	Glutaraldehyde	20	46.0 ± 1.3	2403.3 ± 72.5
H-G-30	Hexamethylenediamine	Glutaraldehyde	30	47.3 ± 1.6	2555.2 ± 60.6
A-G-4	Ammonia	Glutaraldehyde	4	38.6 ± 0.9	1995.3 ± 57.4
A-G-RT	Ammonia	Glutaraldehyde	20	42.5 ± 1.4	2037.4 ± 48.9
A-G-30	Ammonia	Glutaraldehyde	30	43.7 ± 1.1	2144.5 ± 69.3
D-4	/	/	4	25.7 ± 0.6	1691.9 ± 65.2
D-RT	/	/	20	28.8 ± 0.8	1775.2 ± 55.0
D-30	/	/	30	29.5 ± 0.8	1836.1 ± 81.6
Cal-B powder	/	/	/	/	1551.8 ± 32.9
Novozyme 435	/	/	/	200[50]	2457.9 ± 29.9

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It is very clear from the graph that after 30 days storage in the PBS buffer, the immobilized enzyme (curve a) still maintains about 80% of its original catalytic activity, while the free enzyme (curve b) has almost lost all of its catalytic activity. The outstanding improvement of the storage ability of enzyme proves that the immobilization of enzyme on electrospun nanofibrous mat is a good and effective method to extend the useful life of enzyme.