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# Nanofibrous Membranes Containing Reactive Groups: Electrospinning from Poly(acrylonitrile-*co*-maleic acid) for Lipase Immobilization

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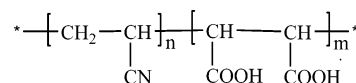
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**ABSTRACT:** Novel nanofibrous membranes containing reactive carboxyl groups were fabricated from poly(acrylonitrile-*co*-maleic acid) (PANCMA) by the electrospinning process. The morphology and fiber diameter were analyzed with field emission scanning electron microscopy. It was found that the fiber diameter could be varied from 100 to 600 nm by changing the solution concentration. Lipase from *Candida rugosa* was covalently immobilized onto the membrane surface via the activation of carboxyl groups in the presence of 1-ethyl-3-((dimethylamino)propyl)carbodiimide hydrochloride/*N*-hydroxysuccinimide. The properties of the immobilized lipases on the nanofibrous and hollow fiber PANCMA membranes were measured. It was found that, compared with the hollow fiber membrane, the enzyme loading and the activity retention of the immobilized lipase on the nanofibrous membrane increase from  $2.36 \pm 0.06$  to  $21.2 \pm 0.7$  mg/g and from 33.9 to 37.6%, respectively. The kinetic constants of the free and immobilized lipases,  $K_m$  and  $V_{max}$ , were assayed. Results indicate that the  $V_{max}$  values are similar for both immobilized enzymes, while the  $K_m$  value of the immobilized enzyme decreases from 1.36 on the hollow fiber membrane to 0.98 on the nanofibrous membrane. The studied lipase-immobilized nanofibers can be used as biocatalysts for polyester synthesis and/or in situ formation of nanofiber reinforcement composites.

## Introduction

In recent years, electrospinning has gained widespread attention since it is known to be an effective fabrication tool for preparing fibrous materials with diameters ranging from several micrometers down to tens of nanometers.<sup>1–14</sup> Among various polymers, acrylonitrile-based homopolymers and copolymers were most recently fabricated into nanofibers with reinforcing, superhydrophobic, and/or catalytic properties.<sup>6–14</sup>

As a biocatalyst, lipases have received increasing attention for biotransformation and polymer synthesis.<sup>15–30</sup> However, practical applications prefer immobilized enzymes because they offer easy catalyst recycling, feasible continuous operations, and simple product purification. Furthermore, immobilizing enzymes onto various insoluble or solid supports is a useful tool to increase their thermal and operational stabilities. In these cases, the structure of the support has a great impact on the behaviors of the immobilized enzymes. Concerning a practical application of the immobilized enzyme, large surface area-to-volume ratio from the support is always desirable, since in this way the enzyme loading per unit volume of support and the catalytic efficiency of the enzyme can be effectively improved. Recently, there is a trend to use nanostructured materials as supports for enzyme immobilization. Both nanoparticles and nanofibers were explored for this purpose.<sup>31–35</sup> In the cases of nanoparticles, nevertheless, their dispersion in reaction solution and the subsequent recovery for reuse are often difficult. On the contrary, nanofibers can be easily recovered from reaction media and be applied for continuous operations. Jia et al.<sup>33</sup> immobilized  $\alpha$ -chymotrypsin on the surface of polystyrene nanofibers (120



**Figure 1.** Schematic chemical structure of poly(acrylonitrile-*co*-maleic acid).

nm) produced by electrospinning and showed that the enzyme loading was 1.4% (w/w). Jiang et al.<sup>34</sup> studied the feasibility of lysozyme immobilization by electrospinning the enzyme-containing solution into nanofibers (200–400 nm). Biocatalytic nanofibers with high activity and stability via enzyme aggregated coating on polystyrene-based electrospun fiber ( $444 \pm 106$  nm) were describe also by Kim et al.<sup>35</sup> However, there is still great potential to enhance the enzyme loading by reducing the fiber diameter and introducing reactive groups on the nanofiber surface, which can provide more reaction sites for enzyme immobilization. On the other hand, the reaction kinetics catalyzed by the immobilized enzyme on the nanofibrous membrane need be investigated to understand the effect of support dimension on the immobilized enzyme activity more clearly.

In our previous work,<sup>36,37</sup> poly(acrylonitrile-*co*-maleic acid) (PANCMA) (its chemical structure is shown in Figure 1) was synthesized and used to fabricate ultrafiltration hollow fiber membrane. Then the hollow fiber membrane containing reactive carboxyl groups was used as support for enzyme immobilization.<sup>38,39</sup> In this work, PANCMA nanofibrous membranes were prepared with electrospinning and were used as supports for enzyme immobilization. Lipase from *Candida rugosa* was immobilized on these nanofibrous membranes by activating the carboxyl groups on the membrane surface with 1-ethyl-3-((dimethylamino)propyl)carbodiimide hydrochloride/*N*-hydroxysuccinimide (EDC/NHS). Effects of the fiber diameter on the enzyme loading, the bioactivity, and the kinetic parameters of the enzyme were studied. Results were compared with those of

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lipase immobilized on the corresponding hollow fiber membrane.

## Experimental Section

**Materials.** Poly(acrylonitrile-*co*-maleic acid), designated as PANCMA in the following text, was synthesized in our laboratory with a water-phase precipitation copolymerization process.<sup>36</sup> The molar fraction of maleic acid in the copolymer is 7.5%. Lipase (from *Candida rugosa*, protein concentration is 7 wt %), Bradford reagent, bovine serum albumin (BSA, molecular mass: 67 000 Da), 1-ethyl-3-((dimethylamino)propyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), *p*-nitrophenyl palmitate (*p*-NPP), and 2-morpholinoethanesulfonic acid (MES) were purchased from Sigma and used as received. All other chemicals are of analytical grade and used without further purification.

PANCMA hollow fiber ultrafiltration membrane was fabricated in our lab according to the reported process.<sup>36,37</sup> The outer and the inner diameters of the hollow fiber ultrafiltration membrane are 850 and 545  $\mu\text{m}$ , respectively, with water flux of 146 L/(m<sup>2</sup> h atm), BSA rejection of 96%, and breaking strength of 135 N/cm<sup>2</sup>.

**Preparation of the PANCMA Nanofibrous Membrane via Electrospinning.** PANCMA was dissolved in dimethylformamide (DMF) at room temperature with gentle stirring for 12 h to form homogeneous solution. After air bubbles were removed completely, the solution was placed in a plastic syringe (50 mL) bearing an 1 mm inner diameter metal needle which was connected with a high voltage power supply (DW-P303-1AC, Tianjin Dongwen Co., China). A grounded counter electrode was connected to the tinfoil collector. Typically, electrospinning was performed at 8.5 kV voltage, 180 mm distance between the needle tip and the collector. The flow rate of the solution was controlled by a syringe pump (WZ-50C2, Zhejiang University, China) to maintain at 0.3 mL/h from the needle outlet. It usually took 5 h to obtain sufficiently thick membrane that can be detached from the tinfoil collector. The membrane on tinfoil was dried under vacuum at 60 °C before it was detached. The morphology of the electrospun nanofibrous membrane was examined under a field emission scanning electron microscope (FESEM, FEI, SIRION-100). The viscosity of the PANCMA solution was measured with rotational viscometer (NDJ-79, Electrical Machinery Co., Tongji University, China).

**Immobilizing Lipase on the Nanofibrous PANCMA Membranes.** Lipase was immobilized onto the nanofibrous PANCMA membranes by an EDC/NHS activation procedure. An appropriate amount of the membrane was thoroughly washed with deionized water and then rinsed with MES buffer (50 mM, pH 6.0). After this, the pretreated membranes was submerged into the EDC/NHS solution (20 mg/mL in MES buffer, 50 mM, pH 6.0, the molar ratio of EDC to NHS = 1:1) and shaken gently for 6 h at room temperature. The activated membranes were taken out, washed several times with PBS (50 mM, pH 5.5), and submerged into the enzyme solution (2 mg/mL in PBS, pH 5.5). The immobilization process was carried out at 4 °C in a shaking water bath for 1 h. Finally, the membranes were taken out, thoroughly rinsed with PBS (50 mM, pH 5.5), and then rinsed with deionized water. The amount of immobilized enzyme on the membrane was determined by measuring the initial and final concentrations of protein within the enzyme solutions and washings using Coomassie Brilliant Blue reagent following Bradford's method,<sup>40</sup> and at least three experimental results were averaged to get a reliable date. BSA was used as a standard to construct the calibration curve. The immobilization capacity of the enzyme on the membrane was defined as the amount of protein (milligrams) per gram of the nanofibrous membrane.

**Activity Assay of the Free and Immobilized Lipases.** The reaction rate of the free and immobilized lipase preparations was determined according to the method reported by Chiou et al. with only minor modification.<sup>41</sup> In the standard conditions, the reaction mixture was composed of 1.0 mL of ethanol containing 14.4 mM *p*-NPP and 1.0 mL of PBS (50 mM, pH 7.5) in an Erlenmeyer flask. The reaction was started by addition of 0.10 mL of free lipase preparation (or 2 mg of immobilized lipase preparation). The

mixture was incubated at 37 °C under reciprocal agitation at 120 strokes/min. After 5 min of reaction, agitation was stopped, and then the reaction was terminated by adding 2.0 mL of 0.5 N Na<sub>2</sub>CO<sub>3</sub> followed by centrifuging for 10 min (10 000 rpm). The supernatant of 0.50 mL was diluted 10-fold with deionized water and measured at 410 nm in an UV-vis spectrophotometer (UV-1601, Shimadzu, Japan) against a blank without enzyme and treated in parallel. The reaction rate was calculated from the slope of the absorbance vs time curve. A molar extinction coefficient of  $14.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  for *p*-nitrophenol (*p*-NP), which was determined from the absorbance of standard solutions of *p*-NP in the reaction medium, was used.

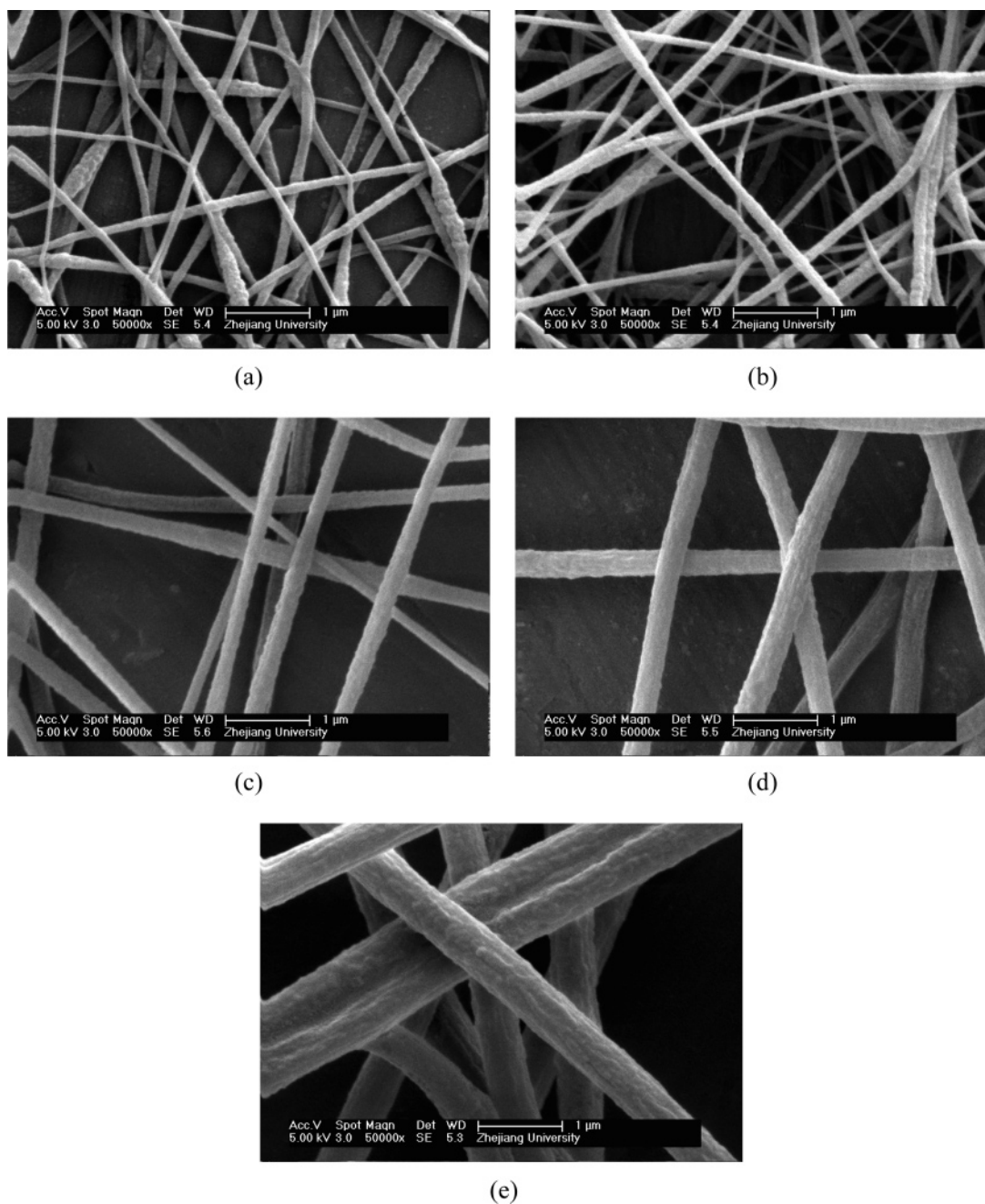
One enzyme unit was the amount of lipase liberating 1.0  $\mu\text{mol}$  of *p*-NP per minute in these conditions. Activity was defined as the enzyme units per gram of support. Specific activity was defined as the enzyme units per milligram of protein. Activity retention was defined as the ratio of the activity of the amount of the enzyme coupled on the fibrous membrane to the activity of the same amount of free enzyme.

## Results and Discussion

**Fabrication and Characterization of the Fibrous PANCMA Membranes.** Electrospinning has been proven effective in preparing polymeric nanofibers.<sup>1-14</sup> The solution properties, such as viscosity, surface tension, and net charge density, can significantly influence the size and morphology of the fibers.<sup>2,3</sup> In this study, PANCMA solutions with different concentrations were prepared and were used for electrospinning. It was found the solution viscosity increases from 6.0 to 308 mPa·s when PANCMA concentration rises from 2 to 10 wt %. From SEM analysis on the prepared fibers, it was observed that stable jets could be formed when the voltage is above 8500 V for all of the solutions. To compare the effect of PANCMA concentration on the formation of electrospun membranes, the voltage was fixed at 8.5 kV, the solution flow rate was set at 3 mL/h, and the distance between the electrode and the collecting plate was maintained 180 mm for all of the samples. Figure 2 shows the SEM photographs of the fibrous membranes prepared from different PANCMA concentrations. When a low concentration of PANCMA (2 wt %, its viscosity is 6.0 mPa·s) is used, as presented in Figure 2a, beads are deposited on the collector and thin fibers coexist among the beads. As the PANCMA concentration is increased to 4 wt % (its viscosity is 20.5 mPa·s), relatively homogeneous fibers with an average diameter of 100 nm could be spun (Figure 2b). In fact, one of the most significant parameters influencing the fiber morphology is the solution viscosity. When the solution viscosity is low, the jets of polymer solution are unstable and likely to break up and form droplets, which are the precursors of the beads observed in the electrospun fibrous membranes. For high-viscosity solutions, the jets would not break up rather than travel and split into filaments to the collecting plate and form fibers.<sup>42</sup> Furthermore, it was also observed that the fiber diameter increases from about 100 to 600 nm when the concentration of PANCMA solution changes from 4 to 10 wt %.

**Lipase Immobilization.** To covalently immobilizing lipase on these membranes, a two-step process was employed. First, the carboxyl groups on the PANCMA membrane surfaces were activated with EDC/NHS. Second, the condensation reaction of the amino groups of lipase with the activated carboxyl groups was carried out. During the condensation reaction, amide bonds are formed between the amino groups of the enzyme and the carboxyl groups of the membranes.

As described above, a series of electrospun fibrous membranes with the fiber diameters ranging from 100 to 600 nm could be obtained by varying the PANCMA concentration.



**Figure 2.** SEM photographs of the electrospun fibrous PANCMA membranes. Concentration of the PANCMA solution is (a) 2, (b) 4, (c) 6, (d) 8, and (e) 10 wt %.

**Table 1. Activity and Kinetic Parameters of the Free and Immobilized Lipases under Optimum Reaction Conditions**

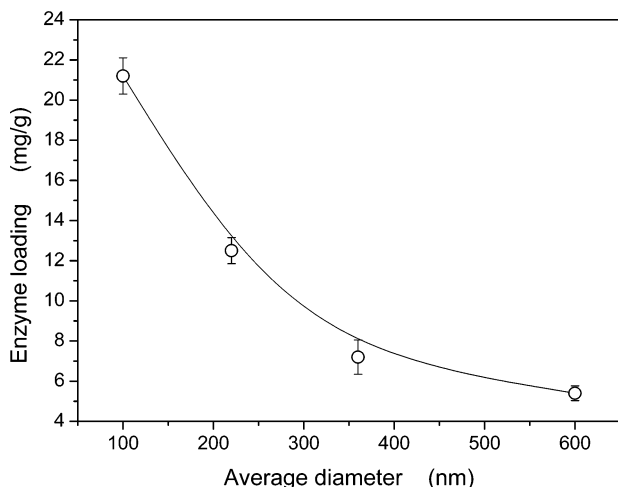
samples	temp (°C)	pH	bound protein (mg/g)	specific activity (U/mg protein)	$V_{\max}$ (U/mg)	$K_m$ (mM)	activity retention (%)
free lipase	37	7.5		42.1	46.4	0.45	100
lipase immobilized on the hollow fiber membrane	45	7.5	$2.36 \pm 0.06$	14.3	16.1	1.36	$33.9 \pm 1.6$
lipase immobilized on the nanofibrous membrane	45	7.5	$21.2 \pm 0.71$	15.8	16.5	0.98	$37.6 \pm 1.8$

These electrospun fibrous membranes were employed as supports for lipase immobilization, and the effect of the fiber diameter on the amount of immobilized protein is given in Figure 3. It was found that the amount of immobilized protein increases from  $5.46 \pm 0.12$  to  $21.2 \pm 0.7$  mg/g as the fiber diameter decreases from 600 to 100 nm. This result is largely due to the high specific surface area of the electrospun fibrous membrane and the migration/concentration of carboxyl group on the fiber surface<sup>37</sup> with the reduction of the fiber diameter,

which can provide more potential reaction sites for covalent coupling with lipase. It indicates that the reduction of the geometric size of the enzyme support can effectively improve the efficiency of enzyme loading since the specific area can be remarkably enhanced. Therefore, the nanofibrous membrane with the fiber diameter of 100 nm was chosen and applied in the next experiments.

Table 1 shows the activity and kinetic parameters of the free and immobilized lipases under the optimum reaction conditions.





**Figure 3.** Effect of the diameter of the electrospun fiber on the enzyme loading.

The amounts of bound protein are  $2.36 \pm 0.06$  mg/g on the corresponding hollow fiber membrane and  $21.2 \pm 0.7$  mg/g on the nanofibrous membrane. In comparison with the free enzyme, the immobilized lipase under its optimum reaction condition retains  $33.9 \pm 1.6\%$  of the activity on the hollow fiber membrane and  $37.6 \pm 1.8\%$  on the nanofibrous membrane.

Obviously, compared to that on the PANCMA hollow fiber membrane, there is a sharp increase of the amount of bound protein on the nanofibrous membrane. This phenomenon is largely attributed to the presence of the remarkable high specific area of the nanofibrous membrane with the reduction of the fiber diameter, which can provide more potential reaction sites for covalent coupling with protein. Functionalized polystyrene nanofiber produced by electrospinning was used by Jia et al.<sup>33</sup> to immobilize  $\alpha$ -chymotrypsin, and they found that the enzyme loading was about 1.4 wt %. In our case, the enzyme loading on the nanofibrous PANCMA membrane is up to 2.12 wt %. There are two reasons to explain this result. On one hand, our PANCMA nanofiber can provide more potential reactive sites for enzyme immobilization than the functionalized polystyrene nanofiber. On the other hand, the diameter of the PANCMA nanofiber used in this work is 100 nm, while that of the functionalized polystyrene nanofiber is 120 nm. Therefore, the PANCMA nanofiber shows higher specific surface area, which results in higher enzyme loading.

After enzyme immobilization, a decrease of enzyme activity has been commonly observed. This is due to the minor modification in the enzyme three-dimensional structure that may lead to the distortion of amino acid residues involved in catalysis, the presence of random immobilization which causes the analytic approach to the active site of the enzyme hindered, and the limitations imposed by slow mass transfer of substrate or product to or from the active site of the enzyme. In this work, it is very interesting to find that the activity retention of the immobilized enzyme on the nanofibrous PANCMA membrane ( $37.6 \pm 1.8\%$ ) is higher than that on the hollow fiber PANCMA membrane ( $33.9 \pm 1.6\%$ ). The main reason for the increase of the activity retention could be the remarkable reduction of the diffusion resistance for the immobilized lipase on the nanofibrous PANCMA membrane.

The kinetic parameters  $V_{\max}$  and  $K_m$  are listed in Table 1 from the double-reciprocal plot.  $V_{\max}$  defines the highest possibility velocity when all the enzymes are saturated with substrate. Therefore, this parameter reflects the intrinsic characteristics of the immobilized enzyme but may be affected by diffusional

constraints. As shown in Table 1, the  $V_{\max}$  values of lipases demonstrate a decrease upon immobilization from 46.4 U/mg for the free lipase to 16.1 and 16.5 U/mg for the immobilized lipases on the hollow fiber membrane and on the nanofibrous membrane, respectively. In general, the  $V_{\max}$  values of enzymes show a decrease upon immobilization. It was found that the  $V_{\max}$  values of both the immobilized lipases are similar, while it is interesting to see that the  $K_m$  value of the immobilized lipase on the nanofibrous membrane (0.98 mM) is lower than that on the hollow fiber membrane (1.36 mM). These results support the explanation discussed above; i.e., the remarkable reduction of the diffusion resistance for the immobilized lipase on the nanofibrous PANCMA membrane is the main reason for the increase of the activity retention. Furthermore, there are two factors to explain this reduction of the diffusion resistance. First, compared to the hollow fiber membrane, the nanofibrous membrane with the large specific surface area can create a more favor interface for the mass transfer of substrate or product to or from the active site of the enzyme. Second, the hollow fiber membrane is a porous material, which usually results in a much greater diffusional limitation.

## Conclusion

Nanofibrous PANCMA membranes were prepared by electrospinning and used for lipase immobilization. The fiber diameter can be modulated from 100 to 600 nm by changing the solution concentration. The enzyme loading and the activity retention of the immobilized lipase on the nanofibrous membrane are much higher than those on the hollow fiber PANCMA membrane. The main reason for the activity increase is due to the decrease of  $K_m$  value of the immobilized enzyme. Thus, the process investigated in this work presents a convenient approach to fabricate nanofibrous membranes containing reactive groups for enzyme immobilization such as lipase. These lipase-immobilized nanofibers with high enzyme loading and bioactivity may have great potentials as biocatalysts for polymer synthesis and/or in situ formation of nanofiber reinforcement composites.

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