Improving Biocatalytic Activity of Enzyme-Loaded Nanofibers by Dispersing Entangled Nanofiber Structure

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A simple and efficient way of dispersing hydrophobic nanofibers in aqueous solution was devised, and its utility in production and application of enzyme-loaded nanofibers was demonstrated. Polystyrene-based nanofibers were produced via an electro-spinning process. A small amount of maleic anhydride group in the polystyrene fiber was used for covalent attachment of lipase onto the fiber surface. The pristine polystyrene nanofibers are hydrophobic and aggregate in water, forming a tightly collapsed clump. These nanofibers can be dispersed in a surfactant-free aqueous solution via a simple alcohol pretreatment. The tightly aggregated electro-spun polystyrene nanofibers can be dispersed into a loosely entangled structure in aqueous alcohol solution. Once treated with aqueous alcohol solution, the polystyrene nanofibers remain dispersed even in DI water as long as the nanofibers are not dried during the washing step. The dispersion of polystyrene nanofibers increases the enzyme loading up to ~8 times and augments the steady-state conversion of a continuous flow reactor filled with enzyme-loaded nanofibers.

I. Introduction

Immobilization of enzymes on solid supports is of great interest in biocatalytic reactions and sensors because it can make the handling and recovery of enzymes much easier.[1,2] Nanostructured supports such as mesoporous materials, nanoparticles, and nanofibers are widely studied because they provide a high surface to mass ratio;[3−5] among these, polymer nanofibers offer several advantages over other nanostructures. Long and continuous polymer nanofibers can easily be made with an electrospinning process.[6,7] This technique is applicable to a variety of polymer materials. The electrospun polymer nanofibers can be structurally tailored to non-woven mats, well-aligned arrays, or membranes.[8] The large surface-to-mass ratio of nanofibers provides an advantage of higher loading per mass, and thus higher enzymatic activity per mass.[9] These electrospun nanofibers can be easily recovered from solution and re-used.[9,10]

To maximize the enzymatic activity, it is very important to disperse the enzyme-loaded supported materials in the reaction media. Because most enzymes work in aqueous solutions, hydrophilic polymers are often chosen as a support material to attain good dispersion. The electrospun nanofibers of hydrophilic polymers must be cross-linked after or during the electrospinning process to prevent the dissolution of hydrophilic polymers in aqueous solution.[11,12] The control of the degree of cross-linking still remains a challenge. In contrast, hydrophobic polymer fibers are intrinsically stable against dissolution in aqueous solution. The hydrophobic fibers may be advantageous for immobilization of enzymes that work for hydrophobic substrates such as lipase;[13] however, the hydrophobic nanofibers are not well dispersed in aqueous solution. This can pose mass transport problems during the enzyme loading on the internal surface of the non-woven mat and the reactions of the immobilized enzymes with substrates dissolved in the aqueous solution.

In this paper, we describe a simple and efficient way of dispersing polystyrene-poly(styrene-co-maleic anhydride), PS-PSMA, nanofibers in water without using conventional surfactants. The maleic anhydride groups at the nanofiber surface provide the enzyme anchoring sites, which can form covalent bonds with free amine groups at the enzyme surface (Figure 1). The PS-PSMA nanofibers are hydrophobic and do not disperse in aqueous solution. The dispersion of these hydrophobic nanofibers can be attained by washing the nanofibers with aqueous alcohol solutions before immobilizing enzymes. The well-dispersed enzyme-loaded PS-PSMA nanofibers give a higher enzymatic activity per unit mass of nanofiber than do the poorly dispersed nanofibers. Being able to disperse PS-PSMA nanofibers in aqueous solution allows the construction of continuous-flow biocatalytic nanofiber reactors.

II. Experimental Section

II-1. Preparation of Alcohol-Dispersed PS-PSMA Nanofibers. A polymer solution was prepared at room temperature by dissolving PS (M_w = 860 000) and PSMA (M_w = 224 000, maleic anhydride content = 7 wt %) with a 2:1 weight ratio in a mixture of tetrahydrofuran and acetone (7:3 ratio by volume). The total polymer concentration in the solution was 15%. The polymer solution was loaded into a 3 mL plastic syringe equipped with a 30-gauge stainless steel needle. A bias of +7 kV was applied to the needle using a high voltage supply. The solution was fed at a rate of 0.15 mL/h using a syringe pump. The electrospun fibers were collected on a clean electrically grounded aluminum foil. The distance between the needle and collecting foil was 10 cm. The nanofibers were manually peeled off, weighed, and washed with distilled water.

II-2. Aqueous Alcohol Solution Treatment of PS-PSMA Nanofibers for Dispersion in Water. For dispersion of PS-PSMA nanofibers in water, the electrospun nanofiber mat was first immersed in an aqueous alcohol solution (water:alcohol = 4:1 by volume) and gently shaken for several hours. The PS-PSMA nanofibers dispersed fully in the aqueous alcohol solution. The nanofibers were then repeatedly washed without drying with a copious amount of distilled water until

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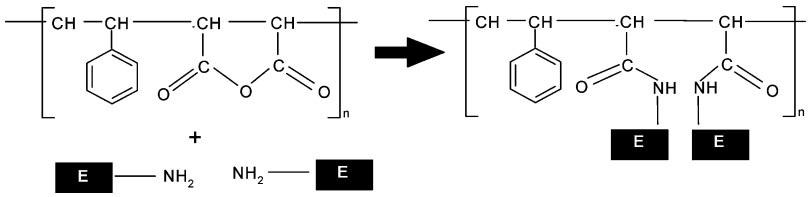

Figure 1. Schematic representation of the covalent attachment of enzymes to the maleic anhydride functional group in the PS-PSMA nanofibers. The NH_2 residues on the surface of the enzyme molecules form amide bonds with the maleic anhydride group of the PSMA (E = enzyme molecule).

alcohol was thoroughly removed from the solution phase. The dispersed PS-PSMA nanofibers were always kept in water or buffer solution until they were used for lipase immobilization.

II-3. Lipase Immobilization on PS-PSMA Nanofibers. Lipase solution used for immobilization was prepared as follows. First, 20 mg of lipase (*Mucor Javanicus*) was dissolved in 10 mL of a 20 mM sodium phosphate buffer (pH 6.5) and filtered with a series of syringe filters in the sequence of 0.45, 0.22, and 0.1 μm pore sizes. For immobilization, the PS-PSMA nanofibers were incubated in 1 mL of the filtered lipase solution at 200 rpm at room temperature for 30 min followed by overnight rocking at 4 °C. The nanofibers were then transferred to a new glass vial and washed with 20 mM phosphate buffer (pH 6.5) and 100 mM Tris-HCl buffer (pH 6.5). The Tris-HCl buffer is used to cap the unreacted maleic anhydride groups. After capping, the nanofibers were extensively washed in 20 mM phosphate buffer (pH 6.5) until no leaching of lipase was observed. The total number of washings was ~7. The amount of lipase immobilized on the surface of the PS-PSMA nanofibers was determined by measuring the initial and final concentrations of the enzyme within the incubation solution and washing solutions at 280 nm with a UV−vis spectrophotometer. Bovine serum albumin (BSA) was used as the standard to construct the calibration curve.

II-4. Activity Assay of the Free and Immobilized Lipase in a Batch Reaction Mode. The lipase activity was measured by the hydrolysis of 4-nitrophenyl butyrate (4-NB) in 20 mM sodium phosphate buffer (pH 6.5). The lipase immobilized on the PS-PSMA fibers was transferred to a new vial, and 19.6 mL of 20 mM sodium phosphate buffer (pH 6.5) containing 4-NB at concentrations varying from 0.031 to 1 mM was added to initiate the reaction. For free lipase activity measurement, 0.2 mL of filtered free lipase solution (0.26 mg/mL) was used instead of the lipase-immobilized PS-PSMA nanofibers. The vials were then shaken at 200 rpm at 35 °C, and small aliquots were removed every 5 min. The concentration of enzyme reaction product, *p*-nitrophenol, was calculated from the absorbance at 400 nm (A_{400}). The enzyme activity was calculated from the slope of the A_{400} versus time plot. One unit of the enzyme activity was defined as the amount of enzyme that catalyzed the production of 1 μmol of *p*-nitrophenol per minute. Specific activity was defined as the activity per unit mass of enzyme. The measured activity was analyzed with the Michaelis−Menten method to get the maximum activity per unit mass of nanofiber (R_{max}) and the substrate binding constant (K_m).

II-5. Continuous Flow Reactor Test. Two continuous flow reactor systems were constructed with the lipase-loaded PS-PSMA nanofibers (dry fiber weight = 7 mg) filled in polyethylene tubes (0.8 cm diameter, 1.2 cm long; total volume = 0.6 mL). One system was filled with the pristine nanofibers and the other with the dispersed nanofibers. The 0.5 mM 4-NB substrate solution was continuously flowed through the tube reactors using a peristaltic pump. Three different flow rates were tested: 0.7, 1.8, and 2.8 mL/min. The *p*-nitrophenol concentration of the eluted solution was obtained by measuring the absorbance at 400 nm.

II-6. Characterization. Specimens of the electrospun PS-PSMA nanofibers (with and without alcohol treatment) were analyzed by scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). For SEM, a thin layer of gold (~10 nm) was coated to prevent charging. For FTIR, the electrospun fibers were peeled off as a moderately transparent, free-standing thin film. FTIR spectrum was collected with a 4 cm^{-1} resolution.

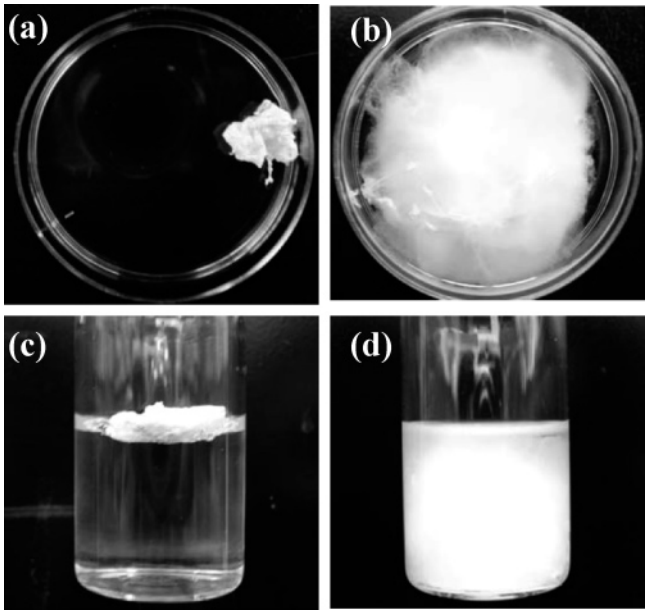

Figure 2. Visual comparison of as-spun (left column) and alcohol-pretreated (right column) PS-PSMA nanofibers immersed in water. Parts (a) and (b) are top-view images of nanofibers in a Petri dish. Parts (c) and (d) are side-view images of nanofibers in a 20 mL glass vial.

III. Results and Discussion

After the treatment with aqueous alcohol solution, the PS-PSMA nanofibers are dispersed in water. Figure 2 compares the as-spun and alcohol-treated PS-PSMA nanofibers immersed in water. The hydrophobic nature of the as-spun PS-PSMA nanofibers makes the non-woven mat form a tightly aggregated clump in aqueous solution. Even though the density of polystyrene (1.05 g/cm^3) is higher than that of water, the nanofiber aggregates float on the water. This might indicate that the nanofiber aggregate contains tiny air bubbles. In contrast, the PS-PSMA nanofiber mat pretreated with the alcohol solution is fully dispersed in water. Moreover, the nanofibers sink in water, as expected from the density of the polymer. Even after repeated rinsing with water for several days, the alcohol-pretreated PS-PSMA nanofibers remain dispersed in water. The expanded nanofiber mat does not break into pieces even after vigorous shaking because the nanofibers are physically entangled. The same dispersion effect is observed for the nanofiber mats made of PS only. So, the small amount of the maleic anhydride group as an enzyme immobilization site is not responsible for the alcohol-induced dispersion effect. The apparent volume of the entangled fiber structure was about 10 times or larger after dispersion. Further quantification of the three-dimensional change was difficult because both nondis-

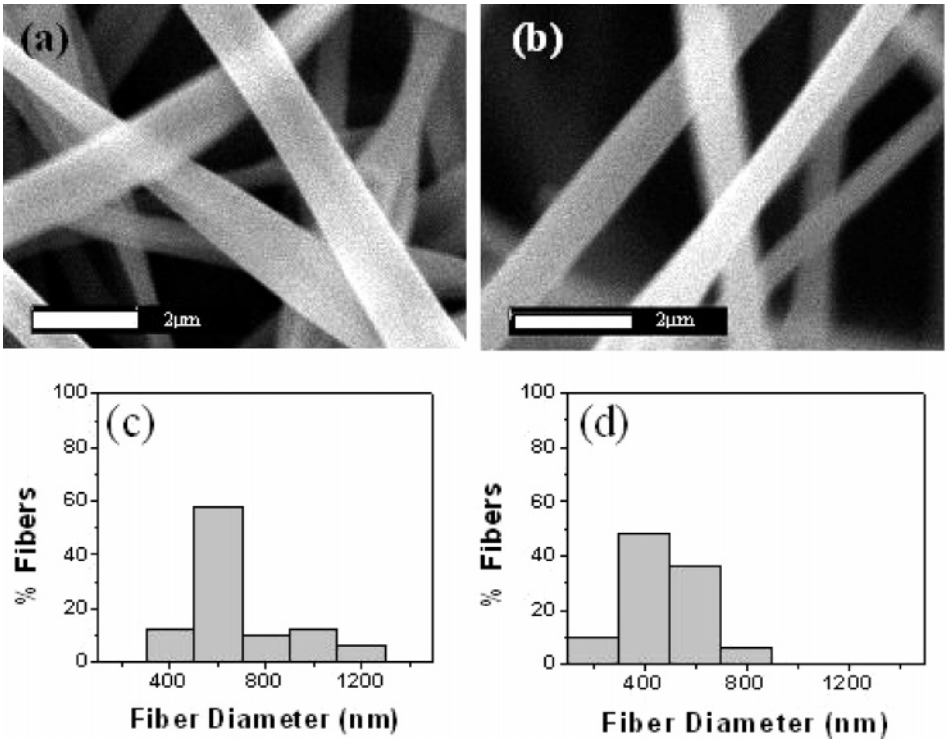

Figure 3. SEM images of (a) as-spun PS-PSMA nanofibers and (b) alcohol-pretreated PS-PSMA nanofibers. SEM images were taken after washing nanofiber mats with water and drying in air. Size distribution of (c) as-spun and (d) alcohol-pretreated PS-PSMA nanofibers. The distributions are obtained from diameter measurements for 50 nanofibers.

persed and dispersed nanofibers had irregular shape and were floating in the solution.

The alcohol-induced dispersion does not depend strongly on the nature of alcohol. We have tested the dispersion effect for methanol, ethanol, 1-propanol, 1-butanol, and 1-pentanol. As the alkyl chain length gets longer, the initial dispersion of the PS-PSMA mat in alcohol solution becomes easier, occurring even at gentle agitation. Once the PS-PSMA nanofiber mats are fully dispersed in the alcohol pretreatment solution, they remain fully dispersed in pure water regardless of the alkyl chain length of the alcohol used in the pretreatment process. In the rinsing step, the only precaution is that the PS-PSMA nanofibers should be kept in wet conditions at all times. Once the dispersed PS-PSMA nanofibers are dried in air, they do not redisperse upon immersion into water. The dried nanofibers should be treated again with alcohol solution. It seems that a critical alcohol concentration is required to disperse the PS-PSMA mat structure into the loosely entangled structure. When the alcohol concentration is lower than ~20 vol %, the PS-PSMA nanofiber mat does not get fully dispersed or expanded even with vigorous shaking.

The alcohol solution pretreatment does not alter the physical and chemical feature of the PS-PSMA nanofibers. Figure 3 compares the SEM images of the as-spun PS-PSMA nanofibers and the alcohol-pretreated PS-PSMA nanofibers. The SEM images were taken after the wet nanofibers were completely dried. The data show that the alcohol treatment does not induce any morphological changes of the PS-PSMA nanofiber surface. The average diameters of the as-spun nanofibers and alcohol-pretreated PS-PSMA nanofibers were calculated to be 619 ± 175 and 575 ± 202 nm, respectively. The diameter of the nanofibers is the same within one standard deviation range.

Figure 4 displays the transmission infrared spectra of PS-PSMA nanofibers after different treatments. The vibration peaks characteristic of polystyrene are the C−H deformation of the polymer backbone at 1450 cm^{-1}, the deformation of the aromatic ring at 1490 and 1590 cm^{-1}, and the C═C stretch at 1605 cm^{-1}. The characteristic bands of the maleic anhydride group are the symmetric and antisymmetric C═O stretch at 1780 and 1860 cm^{-1}, respectively. The alcohol treatment or rinsing with the buffer solution do not change the C═O stretching peak, indicating that the maleic anhydride functional group is intact (not hydrolyzed) and still available for enzyme immobilization. The reaction of the maleic anhydride group of the PS-PSMA nanofiber with free amine is checked by exposing the nanofibers to the buffer solution containing *n*-propyl amine. The infrared spectrum shown in Figure 4d shows the decrease of the maleic anhydride peak at 1780 and 1860 cm^{-1} and the growth of the amide bond peak at 1557 and 1642 cm^{-1}. This proves the feasibility of the covalent bonding of enzymes via the reaction between the surface amine group of the enzyme and the maleic

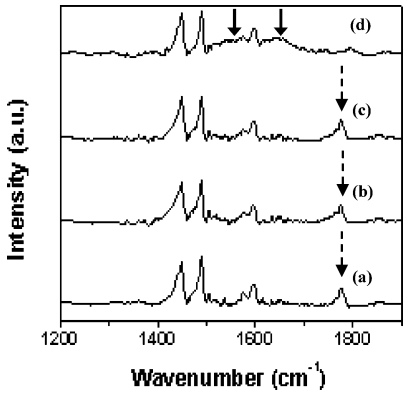

Figure 4. FTIR transmission spectra of (a) as-spun PS-PSMA nanofibers, (b) alcohol-pretreated PS-PSMA nanofibers, (c) as-spun PS-PSMA nanofibers washed with distilled water, and (d) PS-PSMA nanofibers reacted with *n*-propyl amine. The vibration peaks corresponding to the maleic anhydride group are marked with dashed lines. The C═O stretching (1642 cm^{-1}) and the in-plane N−H bending (1557 cm^{-1}) vibration peaks are marked with arrows.

Table 1. Activity and Kinetic Parameters for Free Lipase in Solution and Lipase Immobilized on As-Spun and Alcohol-Dispersed PS-PSMA Nanofibers (35 °C, pH = 6.5)

sample	immobilized protein[a] (μg/mg nanofiber)	specific activity (U/mg lipase)[b]	K_m (mM)	R_{max} (U/mg nanofiber)
free lipase in solution		187.2 ± 34.1	0.381 ± 0.106	
as-spun nanofiber with lipase	5.6 ± 2.2	31 ± 6.9	0.344 ± 0.085	0.17 ± 0.04
dispersed nanofiber with lipase	42.4 ± 18.5	31 ± 7	0.481 ± 0.116	1.30 ± 0.29

[a] The amount of lipase is measured as the BSA-equivalent mass in the 280 nm absorbance measurement. [b] U = 1 μM of substrate reacted per minute.

anhydride group of the PS-PSMA nanofiber surface (Figure 1). Therefore, enzymes can be immobilized directly to the nanofibers without any surface activation process.

The lipase immobilization to the PS-PSMA nanofibers and the reactivity of the lipase-loaded nanofibers were measured for both pristine and alcohol-dispersed nanofibers. The total loading of the lipase enzyme is significantly increased by the dispersion of the PS-PSMA nanofibers. The immobilized protein (lipase) content was determined to be 5.4 ± 2.2 μg (BSA-equivalent) per mg of nanofiber for the as-spun PS-PSMA system, while it was 42.4 ± 18.5 μg (BSA-equivalent) per mg of nanofiber for the alcohol-pretreated PS-PSMA system. The increase of a factor of ~8 in the loading is attributed to the increase of the effective surface area of nanofibers available for enzyme loading upon dispersion. When the PS-PSMA nanofibers are tightly aggregated together, only the maleic anhydride groups at the outer region of the aggregate will be available for the enzyme immobilization. Small droplets of air trapped in the hydrophobic nanofiber aggregate can also block the transport of the enzyme into the deeper region of the aggregate. Upon alcohol-induced dispersion of the PS-PSMA nanofibers, more surface area of the nanofibers is exposed to the solution and more maleic anhydride groups on the nanofiber surface will be used for enzyme immobilization. The enhanced protein loading clearly demonstrates the importance of the dispersion of PS-PSMA nanofibers via alcohol treatment.

The maximum activity per unit mass of nanofiber (R_{max}) and substrate binding constant (K_m) are obtained from the Michaelis−Menten analysis of the kinetic data (Table 1). The R_{max} value of the lipase immobilized on the nondispersed PS-PSMA nanofibers is 0.217 ± 0.049 U per mg nanofiber, while that of the lipase immobilized on the dispersed PS-PSMA nanofibers is 1.51 ± 0.28 U per mg nanofiber. The difference in R_{max} is consistent with the total amount of the lipase loaded per unit mass of the nanofibers. So, the apparent specific activity, r_{sp} (=R_{max} divided by the BSA-equivalent mass of the loaded lipase), is almost the same, regardless of the nanofiber dispersion. The r_{sp} values of the immobilized lipase are much lower than the specific activity measured for the free lipase (Table 1). This is commonly observed for other immobilized enzyme systems.[14,15] This could be due to several reasons. One possibility is the denaturation or blocking of the active sites during the immobilization of the enzyme via the covalent attachment to the maleic anhydride group.[16]

The substrate binding constant, K_m, is slightly lower for the lipase loaded on the pristine nanofibers than for the lipase loaded on the dispersed nanofibers, although the difference is not significantly larger than the experimental error range. If this difference is real, it might be due to the preferential segregation

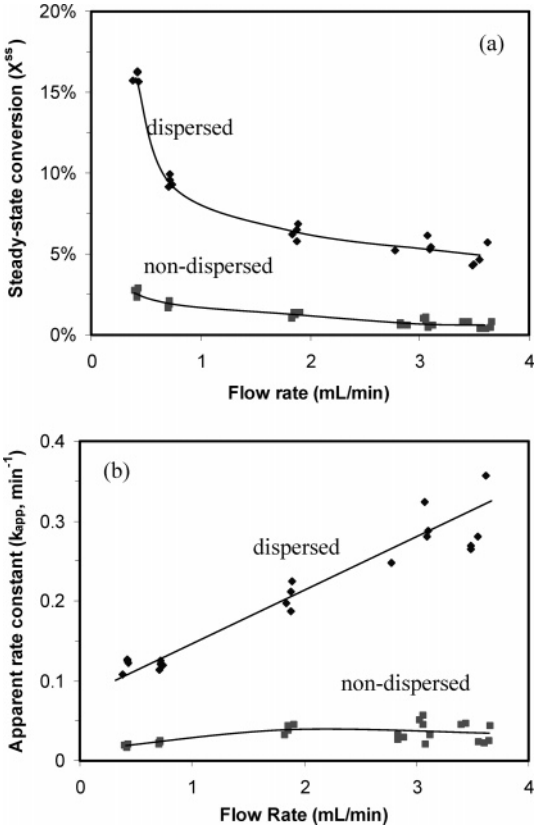

Figure 5. (a) Conversion and (b) apparent pseudo-first-order reaction rate constant of the continuous flow reactors constructed with the as-spun and dispersed PS-PSMA nanofibers loaded with lipase.

of the hydrophobic substrate (4-NB) to the hydrophobic nanofiber surface in water, which would increase the local substrate concentration near the immobilized lipase to a value higher than the bulk concentration. Another possibility could be the different agitation behavior between the tightly aggregated nanofiber clump and the dispersed but physically entangled nanofiber body under the shaking condition.

When a continuous-flow reactor is constructed using nanofibers, it is important to attain the highest dispersion of nanofibers. We constructed two continuous-flow reactors using the nondispersed and dispersed nanofibers. The 7 mg dispersed nanofibers filled the entire flow reactor (V = 0.6 mL), while the nondispersed nanofibers of the same weight partially filled the flow reactor. The flow rate dependence of the steady-state conversion (X^{ss}) of these reactors is shown in Figure 5a. The decrease of X^{ss} at the higher flow rate is mainly due to the decrease of the residence time ($\tau = V/F$) in the reactor. Regardless of the flow rate, X^{ss} is always significantly larger for the dispersed nanofibers than for the pristine nanofibers. This could be ascribed to the enzyme loading difference of these two nanofibers.

Further kinetic analysis of the conversion data reveals another important difference between two reactors with different degrees of nanofiber dispersion (Figure 5b). The apparent pseudo-first-order reaction rate constant (k_{app}) of the lipase-loaded nanofiber reactor is calculated from the following design equation of the continuous flow reactor: $k_{app} = -\ln(1 - X^{ss})/\tau$. Note that k_{app} is the rate constant of the reactor loaded with 7 mg of nanofibers, not the rate constant of enzymes. The dispersed nanofiber reactor shows a linear increase of k_{app} from ~0.12 min^{-1} at F = ~0.4 mL/min to ~0.32 min^{-1} at F = ~2.8 mL/min. In the case of the nondispersed nanofiber reactor, the k_{app} value increases marginally from ~0.021 min^{-1} at F = ~0.4 mL/min to ~0.039

min^{-1} at $F = \sim 1.9$ mL/min and then levels off or decreases slightly with further increase of the flow rate.

The large flow rate dependence of k_{app} for the dispersed nanofiber reactor is due to a more efficient supply of the substrate into the reactor at a higher flow rate. This can be estimated using the Weisz modulus, $\Phi = k_{app} \times \tau$. By definition, this modulus shows the competition between the enzyme reaction and substrate transport rates in the reactor.[17,18] When Φ is less than ~ 0.1, the reaction kinetics is governed by the steady-state conversion because the substrate supply into the system is much faster than the enzyme reaction in the system. If Φ is larger than ~ 0.1, the substrate supply rate (flow rate) becomes comparable to the reaction rate of the reactor and the steady-state conversion becomes a function of the flow rate. The Φ modulus of the dispersed nanofiber reactor varies from 0.05 at $F = 3.5$ mL/min to 0.18 at $F = 0.4$ mL/min. In other words, the enzymatic reaction in the reactor is fast enough to reach the flow-rate-dependent regime.

In contrast, the Φ modulus of the nondispersed nanofiber reactor is only 0.01−0.03. This explains the weak dependence of k_{app} for the nondispersed nanofiber reactor. It should be noted that the k_{app} value of the nondispersed nanofiber reactor levels off or slightly decreases at a flow rate higher than ~ 1.9 mL/min. This decrease must be related to the change in the fluid flow pattern inside the reactor. Because the nondispersed nanofibers fill the reactor only partially, some fraction of the substrate solution can pass through the flow reactor without encountering the enzyme-loaded nanofibers. This fraction will increase as the flow rate increases, lowering the apparent rate constant.

The experimental data presented in this paper accentuate the importance of the dispersion of enzyme-loaded nanofibers for biocatalytic applications. The pretreatment of hydrophobic PS-based nanofibers with aqueous alcohol solutions can make the nanofibers dispersed in water, enhancing the efficiency of enzyme loading and the enzymatic activity per unit mass of nanofibers. The origin of alcohol-induced dispersion is not fully elucidated yet. However, it is understood that the dispersion is not due to the presence of the maleic anhydride group because the pure PS nanofibers show the same alcohol-induced dispersion behavior. Also, the bulk PS and PSMA materials do not dissolve or swell in the pure alcohols tested in this study. The Hansen solubility parameter for ethanol is ~ 19, which is much larger than the interaction radius of PS (12.7). This indicates that ethanol cannot swell or dissolve polystyrene. To dissolve PS, the solvent Hansen parameter must be smaller than 12.7 (like THF = 9.7).

Ruling out these effects, one can speculate that alcohol molecules may behave like a surfactant at the interface between the hydrophobic polystyrene and water. Because the alcohol molecule is composed of a hydrophobic alkyl group and a hydrophilic hydroxyl group, it can be preferentially adsorbed at the polystyrene/water interface with the alkyl group facing the polystyrene surface and the hydroxyl group facing the water side.[19] This interfacial ordering of alcohol molecules can make the hydrophobic nanofibers disperse in water. The alcohol molecules at the polystyrene/water interface do not seem to be removed by simple rinsing because the alcohol-pretreated nanofibers remain dispersed in water after the alcohol is removed in the bulk liquid phase.

IV. Conclusion

This paper demonstrates the dispersion of hydrophobic polymer nanofibers by simple pretreatments with aqueous alcohol solutions, which increases the enzyme loading and thus the enzymatic activity per unit mass of the nanofiber support. The improved enzyme loading and activity will help to develop efficient biocatalytic systems for bioconversion, bioremediation, and biosensing. In addition, the easy control of nanofiber structure and surface chemistry can open up the potential for more versatile and efficient use of hydrophobic polymer nanofibers in bio-applications including drug delivery and tissue engineering.

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