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Methyl Cellulose Nanofibrous Mat for Lipase Immobilization *via* Cross-linked Enzyme Aggregates

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Abstract: Nanofibers prepared from cellulose derivatives are a suitable support for enzyme immobilization. We selected a methyl cellulose (MC) nanofibrous mat for enzyme immobilization. First, we established the electrospinning conditions for the preparation of the MC nanofibrous mat. A mixture of ethanol and water at a volume ratio of 1:1 was selected as the solvent of MC for electrospinning. An MC concentration of 5 wt% and a flow rate of 0.5 mL/h were employed to ensure stable electrospinning without bead formation. By varying the applied voltage, the sizes of the MC nanofibers could be controlled in the range of 50 to 80 nm, and an applied voltage of 18 kV was selected for further experimentation. The MC nanofibrous mats were cross-linked with glutaraldehyde under acidic conditions for 12 h in order to increase the stability of the mats in water. Lipase was directly immobilized onto cross-linked MC nanofibrous mats without a further activation step, and 34.82 μg of lipase was immobilized per mg of support. The reusability test showed an unexpected loss of activity after second reuse due to a loss of lipase during the washing procedure promoted by the surface erosion of the MC nanofiber. This problem was solved by introducing cross-linked enzyme aggregates (CLEA) to the surfaces of MC nanofibers. CLEA formation enhanced the activity of lipase per mg of support by almost 5-fold compared with the original lipase-immobilized MC nanofibrous mat. The reusability was also enhanced: more than 90% of the initial activity was retained after seven reuses.

Keywords: methyl cellulose, electrospinning, lipase, cross-linked enzyme aggregates.

Introduction

Enzymes are biocatalysts that enhance the reaction rate of every chemical reaction in living organisms. Compared with other chemical catalysts, enzymes feature high regio- and stereoselectivity, which reduces unwanted side reactions.[1] Despite this advantage, the use of enzymes at the commercial scale has been limited due to the high cost and low stability of enzymes.[2] Enzyme immobilization in a confined or localized region of space has been proposed to overcome these disadvantages. Upon immobilization, enzymes can be reused multiple times to enhance operational stability.[3] Various immobilization techniques are currently employed for immobilization, including adsorption, cross-linking, entrapment, and covalent bonding. Among these techniques, covalent bonding yields the most robust immobilization, which is required to repeatedly reuse the enzyme.[4] In general, the immobilization of enzyme *via* covalent bonding requires a solid support, whose morphology and chemical properties influence significantly the activity of immobilized enzyme.

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An ideal support for the covalent immobilization of an enzyme should not only ensure low diffusion limitations for both the substrate and product and feature some degree of hydrophilicity but also permit high enzyme loading.[5] Diffusion limitations can be solved by increasing the porosity of a support of adequate pore size.[6,7] Hydrophilicity is required to maintain the aqueous microenvironment around the enzyme, which minimizes denaturation caused by the hydrophobicity of the support.[8,9] A high enzyme loading capacity is required to compensate for the decrease in the specific activity of the enzyme after immobilization.[7,10] To this end, the enzyme may be immobilized on a support featuring a sufficient number of functional groups for covalent bonding or the surface area of the support may be increased.[11,12] Alternatively, the formation of cross-linked enzyme aggregates (CLEA) has been proposed to increase the enzyme loading on a support. The CLEA technique was initially proposed as an enzyme immobilization technique that cross-links enzyme precipitates with a cross-linker without the use of a support.[13] The new method described herein applies the formation of CLEA to the support. The enzyme was covalently attached to the surface, and the CLEA were then formed, which significantly increased the

amount of enzyme on the support.[14-16]

If the chemical properties, including number and type of functional groups, are identical, then the morphology of the support significantly affects the overall performance of the immobilized enzyme. As mentioned, the support should feature a high surface area and low diffusional limitation.[5] Porous materials are characterized by high surface areas, but porous materials of excessively small pore sizes are subject to diffusional limitations that must be overcome. Non-porous materials avoid the diffusion problem but preclude high enzyme loading. Among the various forms of supports, electrospun nanofibers may avoid both of these problems. Electrospinning allows the formation of nanofibers in a nonwoven mat using a relatively simple apparatus.[17] Due to the small diameter of each nanofiber, the nonwoven fiber mat is characterized by a high surface area, and the diffusion problem is minimized because of the open pore structure and large lateral interspace between nanofibers.[18,19] Various types of polymers have been fabricated into nanofibers by electrospinning,[20,21] and many types of enzymes have been immobilized for various applications.[22] Among carbohydrates, cellulose and its derivatives can also be electrospun by optimizing the solution and process parameters, such as the type of solvent, molecular weight (MW) and DS of the polymer, concentration of dope solution, applied voltage, and flow rate. Recently, Rezaei *et al*. summarized studies of the electrospinning of cellulosic nanofibers.[23]

Methyl cellulose (MC) is a cellulose ether derivative that is highly water soluble due to the methylation of hydroxyl groups, which prevent extensive hydrogen bonding. MC remains hydrophilic unless the temperature is increased above the lower critical solution temperature (LCST) of approximately 40-70 ºC.[24] Under the LCST, even the surface of the MC film is hydrophilic because the hydroxyl groups of the polymer chain at the surface will hydrogen bond with water molecules.[25] Therefore, this material is expected to be advantageous for enzyme immobilization. One drawback of MC as a support for enzyme immobilization is the limited number of functional groups. Because some of the hydroxyl groups of cellulose are substituted to methoxy groups, the available free hydroxyl groups for enzyme immobilization are limited. The reduced number of hydroxyl groups will eventually reduce the amount of loaded enzyme. To compensate for this problem, the MC support should feature an extremely high surface area, and nanofibers can ensure such a morphology.

The objective of this study was to determine suitable electrospinning conditions of MC and to use these electrospun MC nanofibrous mats for enzyme immobilization. The electrospinning of MC has not yet been reported in a study dedicated to this subject. Frenot *et al*. described the electrospinning of MC in part of their article but reported that the fiber exhibited poor spinnability.[26] Thus, optimum parameters for MC electrospinning were not established because the electrospinning of MC was not their main objective. In this study, various solution and process parameters were investigated to determine the most suitable condition for the electrospinning of MC. Furthermore, the electrospun MC nanofibrous mat was cross-linked using glutaraldehyde (GA) under acidic conditions in order to ensure that the mat was insoluble. Lipase was selected as a model enzyme to study the applicability of MC nanofibrous mats for enzyme immobilization, and the activity and reusability of this enzyme was investigated after immobilization. The reusability of the immobilized enzyme was further enhanced by cross-linking the enzyme to the surface of the MC nanofiber.

Experimental

Materials. MC powder (DS=1.9, viscosity=190 cP) was obtained from Samsung Fine Chemicals (MECELLOSE FMC 60150, Seoul, Korea). Glutaraldehyde solution (GA; 25%), lipase (*Candida rugosa*), 4-nitrophenyl decanoate (pNPD), *N*-hydroxysulfosuccinimide (NHS), bovine serum albumin (BSA), 2-[morpholino]ethanesulfonic acid (MES) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were purchased from Sigma-Aldrich (Yongin, Korea). Hydrochloric acid (HCl; 37%) was purchased from Junsei (Japan). Amine-functionalized fluorescein isothiocyanate (Amine-FITC, FCG-456 Amine) was purchased from Bioacts (Seoul, Korea). All chemicals were used without further purification.

Preparation of MC Nanofibrous Mat. The MC nanofibrous mats were fabricated *via* electrospinning. To prepare the dope solution, a predetermined amount of MC was well dispersed in ethanol first. Then, we added the same amount of water to prepare the ethanol/water volume ratio of 1:1. The mixture was stirred at 200 rpm, and the resulting mixture was continuously mixed at 25 ºC for about 15 h. The doping solution was loaded into a 3-mL syringe and placed on a syringe pump (KD Scientific, USA) fitted with a 22-gauge needle having an inner diameter of 413 μm. The syringe needle was connected to a power supply (Chungpa EMT high voltage supply, Korea). To determine the optimal conditions of electrospinning, we varied the following parameters: the concentration of the doping solution (3, 4, and 5 wt%), the applied voltage (8, 10, 12, 14, 16, and 18 kV), and the flow rate (0.3, 0.5, and 0.7 mL/h). The tip-to-collector distance from the end of the needle to the surface of the aluminum foil connected to the ground was fixed to 13 cm. Finally, optimized processing conditions were adjusted to the concentration of doping solution of 5%, the applied voltage of 18 kV, the flow rate of 0.5 mL/h and the tip-to-collector distance of 13 cm. All of electrospinning procedures were carried out at room temperature and relative humidity of 45%. The surface morphology of the MC nanofibrous mats was evaluated using a field emission scanning electron microscope (FE-SEM, SUPRA 55VP, Carl Zeiss, Germany).

Crosslinking of MC Nanofibrous Mat. To improve the stability of the mats in water, 50 mg of the MC nanofibrous mat was immersed in 100 mL of acetone containing 2% (v/v) GA

and 0.1% (v/v) of HCl. The crosslinking reaction was performed at room temperature for various times (1, 2, 3, 6, and 12 h). After the reaction, the crosslinked MC nanofibrous mats were washed three times with acetone and three times with 0.1 M sodium phosphate buffer (pH 7.4) to remove unreacted GA. To confirm the stability of the crosslinked MC nanofibrous mat in water, the crosslinked MC nanofibrous mats were immersed in water for 1 h. The surface morphology of the crosslinked MC nanofibrous mats was evaluated using FE-SEM. Attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR, Thermo Scientific, USA) was employed to verify the success of crosslinking. The samples were examined within a wavenumber range of 700-4000 cm^{-1}, and 32 scans with 8 cm^{-1} resolution were used to obtain the spectra.

Immobilization of Lipase. The crosslinked MC nanofibrous mats (45 mg) were immersed in 8 mL of lipase solution (2 mg/mL) at 4 °C with gentle shaking (100 rpm). After an overnight incubation (15 h), the mats were washed three times with cold 0.1 M sodium phosphate buffer (pH 7.4) containing 0.5 M sodium chloride followed by three washes with 0.1 M sodium phosphate buffer (pH 7.4) without sodium chloride. Finally, the mats were washed three times with distilled water to remove unbound enzymes. The lipase immobilization capacity was defined as the amount of protein per milligram of MC nanofibrous mat. The amount of immobilized protein was calculated using Eq. (1):

$$\text{Bound protein } (\mu g/mg) = (C_0\text{-}C) \times V/W \quad (1)$$

where C_0 and C are the initial and final concentration of the protein (μg/mL), respectively. V is the volume of the solution (mL), and W is the weight of the MC nanofibrous mat (mg). The concentration of lipase was determined by measuring the absorbance at 280 nm with a UV spectrophotometer (Optizen POP, Mecasys, Korea). Molar extinction coefficient was adopted as 6,350 M^{-1} cm^{-1} for p-nitrophenol (p-NP), which was determined, prior to measurements, from the absorbance of standard solutions of p-NP in the reaction medium.

Preparation of CLEA on MC Mat. The crosslinked MC nanofibrous mats were functionalized with CLEA by adding 320 μL of GA to 8 mL of 2 mg/mL lipase solution, which contained the lipase-immobilized crosslinked MC nanofibrous mat (45 mg). The mixture was allowed to react under gentle stirring for 15 h at 4 °C. The mat was then washed three times each with cold 0.1 M sodium phosphate buffer (pH 7.4) followed by distilled water. The effectiveness of CLEA formation was confirmed by confocal microscopy (SP8 X STED, Leica, Germany). Here, amine-FITC-labeled BSA (FITC-BSA) was synthesized using an EDC/NHS coupling reaction and used as a model protein instead of lipase. Specifically, 8 mg of BSA was dissolved in 4 mL of a solution that contained 0.1 M MES and 0.5 M NaCl. Subsequently, 1.6 mg of EDC and 2.4 mg of NHS were directly added to the solution. This activation reaction of BSA was carried out for 15 min at room temperature. After the reaction, the pH of solution was adjusted to 7.2 using sodium bicarbonate buffer. The amine-FITC dissolved in distilled water (1 mg/mL) was added to the solution containing activated BSA and reacted for 2 h at room temperature. The reaction mixture was dialyzed for 3 days in a dark container and used directly in subsequent steps. The immobilization and CLEA formation of FITC-BSA was carried out onto crosslinked MC nanofibrous mats using the same procedure described previously for lipase.

Determination of Enzyme Activity. The activity of lipase was measured based on the rate of 4-nitrophenyl decanoate (pNPD) hydrolysis. For the lipase-immobilized MC nanofibrous mat, 4 mg of the mat was added to 4 mL of substrate solution. The substrate solution contained 1 mM pNPD, 0.32% Triton-X and 4% acetone in 50 mM sodium phosphate buffer (pH 7.0). The reaction was carried out for 10 min at 37 °C, and the absorbance of the supernatant at 348 nm was then measured using UV-Vis spectrometer. The reusability of the lipase-immobilized MC nanofibrous mat was evaluated by successively measuring the activity of immobilized lipase eight times within one day. After each test, the lipase-immobilized MC nanofibrous mat was washed three times with 0.1 M sodium phosphate buffer (pH 7.4) to eliminate any residual substrate.

Results and Discussion

Electrospinning of MC. Electrospinning relies on the competition between an external force to stretch the polymer and the counteracting force of the polymer to resist this stretching once the polymer has been ejected from the meniscus of the polymer solution at the tip of the syringe. When the external force, *i.e.*, applied voltage, exceeds Rayleigh's limit, the polymer solution will be ejected from the meniscus of polymer solution to form a polymer jet. The polymer jet will be whipped due to instability as it travels in the air and become thinner as the solvent evaporates. Simultaneously, the electrical repulsion will increase, and the jet will ultimately split into thinner jets. Finally, the solvent will completely evaporate during the flight, leaving micro- and nanofibers behind on the collector. During the entire process, the viscosity of the polymer will counteract the external force. The viscosity of the polymer is a result of polymer entanglement and determined primarily by the concentration and MW of the polymer if the temperature is fixed. If the viscosity of the polymer is sufficiently high, the polymer will withstand the external force, and the fiber will be formed. At low viscosities, the polymer jet will disintegrate into droplets, resulting in bead formation, whereas beaded fibers will form at moderate viscosities.

The choice of solvent is essential for the success of electrospinning. MC is a water-soluble polymer, but water is unfortunately not a good solvent for electrospinning because of its low volatility at room temperature. To increase the volatility of the solvent, we selected ethanol as a co-solvent. Specifically, the boiling point of a 1:1 (v/v) mixture of water and

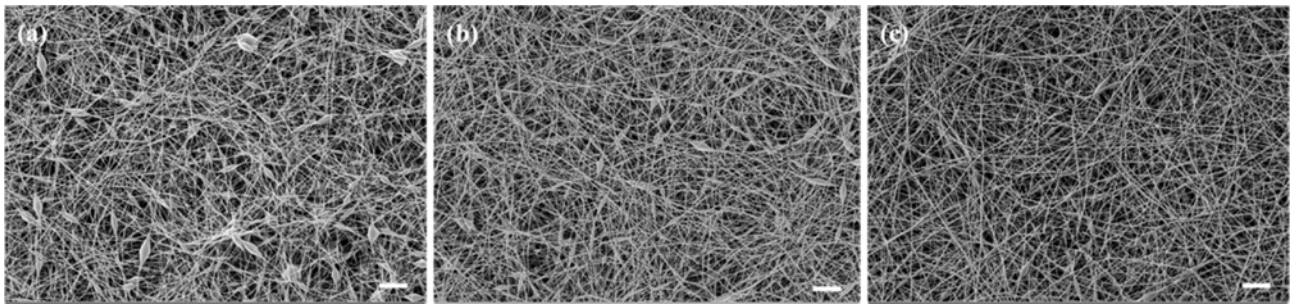

Figure 1. FE-SEM images of MC nanofibrous mat prepared using various concentrations of MC: (a) 3%, (b) 4%, and (c) 5% (magnification: 100,000×, scale bar: 2 μm).

ethanol is 81.2 ºC, which is close to the boiling point of pure ethanol.[27] This solvent combination has previously been used to electrospin MC but failed to yield solid and separated nanofibers, likely due to the low concentration of MC (2.86 wt%).[26] Moreover, the solubility of MC in the ethanol/water solvent was not affected at concentrations below 60% (v/v). Therefore, we used this 1:1 mixture of ethanol and water as a solvent. To determine the optimum electrospinning conditions using this solvent, we manipulated three experimental parameters: the concentration of MC, flow rate and applied voltage. Based on previous findings, the effect of the MC concentration was investigated starting at 3% MC.[26] As shown in Figure 1, bead formation negatively correlated with the MC concentration, and beads were absent at 5 wt% MC. Specifically, increasing the concentration of MC will increase the viscosity of MC, which will provide a cohesive force during polymer jet stretching and reduce bead formation. MC solution concentrations higher than 6% were difficult to prepare or could not be electrospun due to the high viscosity of the MC solution. The flow rate was fixed at 0.5 mL/h because the ejection of polymer solution below this level was intermittent, whereas higher rates caused solvent ejection. Only the applied voltage significantly affected the size of the electrospun MC nanofibers. Figure 2 shows the FE-SEM images of electrospun MC nanofibers at various applied voltages. The ejection of the polymer jet can be observed starting at 8 kV, but bead formation was observed at this applied voltage, which indicates that the applied voltage was insufficient to stretch the polymer jet. Furthermore, increasing the voltage significantly reduced bead formation. The size of MC nanofibers is presented in Figure 3. From applied voltages of 8 to 12 kV, the size of MC nanofibers decreased from 80 nm to 50 nm, whereas it increased when the applied voltage was further increased beyond 12 kV. Thus, increasing the applied voltage can either decrease or increase the size of the nanofibers. The decrease in the size of nanofibers can be attributed to the increase in

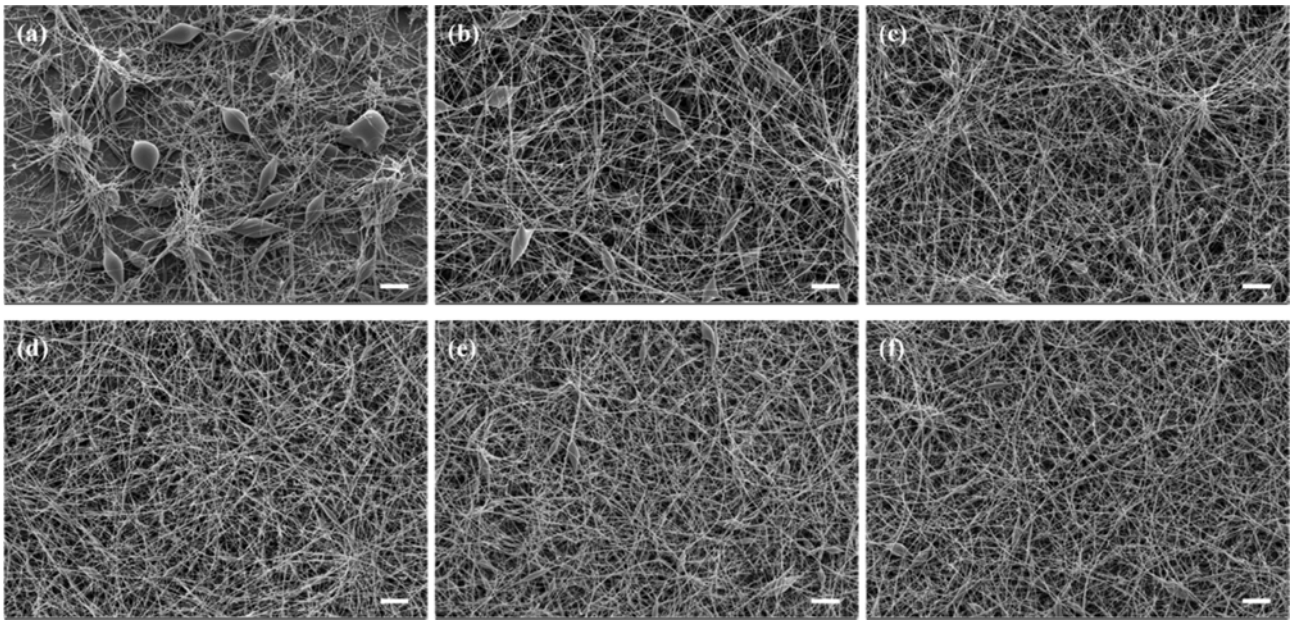

Figure 2. FE-SEM images of MC nanofibrous mat prepared using various applied voltages: (a) 8 kV, (b) 10 kV, (c) 12 kV, (d) 14 kV, (e) 16 kV, and (f) 18 kV (magnification: 100,000×, scale bar: 2 μm).

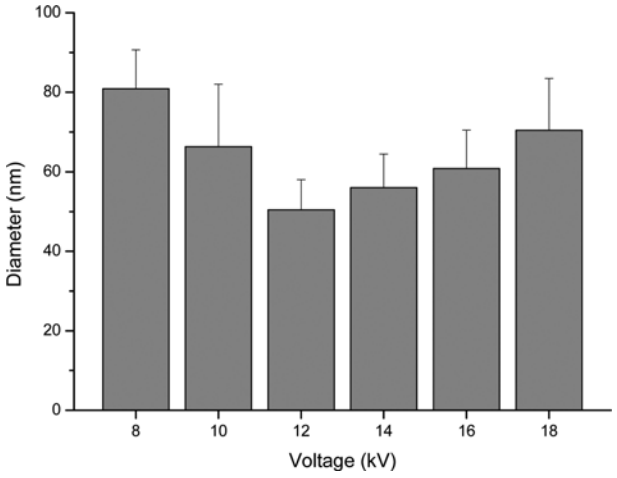

Figure 3. Effect of applied voltages on fiber diameter size distributions.

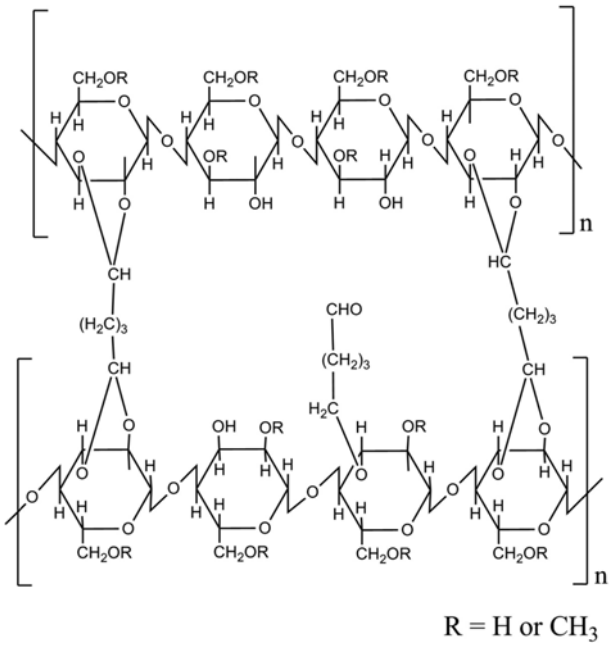

Figure 4. Crosslinking reaction of MC with GA.

electrostatic repulsion of the polymer jet in response to increasing voltages.[28] This situation favors the disintegration of the polymer jet, resulting in fibers of smaller diameter. Increasing the applied voltage may also increase the size of nanofibers due to spontaneous increases in the ejection power.[29] In other words, more polymer can be ejected for the meniscus of the polymer solution at the tip of the syringe. Therefore, the initial size reduction of MC nanofibers below 12 kV is due to the increased electrostatic repulsion of the polymer jet. When this electrostatic repulsion is saturated, further increases in the applied voltage will increase the ejection power of the MC, resulting in thicker MC nanofibers. Therefore, the following electrospinning conditions were employed for further experiments: applied voltage of 18 kV, MC concentration of 5 wt% and flow rate of 0.5 mL/h.

Cross-Linking of the MC Nanofiber. To immobilize enzymes onto the MC nanofibrous mats, the mats should be insoluble. Among the various available cross-linking agents, we selected GA as a cross-linking agent. GA exhibits pH dependent reactivity with hydroxyl and amine groups. At acidic conditions, it reacts with hydroxyl groups, whereas it reacts with amine groups at basic conditions. The reaction between GA and MC is illustrated in Figure 4. Because the DS of MC is 1.9, at least one hydroxyl group of each AGU unit will remain intact on average. GA will react with these hydroxyl groups to cross-link the MC molecules. Figure 5 shows the FE-SEM images of MC nanofibrous mats cross-linked with GA for various times. The stability of the MC nanofiber in water positively correlated with the cross-linking time. However, the swelling of the MC nanofiber was unavoidable and increased the MC nanofiber diameter after immersion in water. The cross-linking of MC was verified by the FTIR spectra (Figure 6). After cross-linking with GA, the peak of MC at 3400 cm^{-1} broadened due to the stretching vibration of the methylene groups of GA. In addition, C=O stretching vibrations of GA were also evident at 1640 cm^{-1}. The intensity of this peak positively correlated with the cross-linking time, which indicates that one of the aldehyde groups of GA remained unreacted after cross-linking (see Figure 4). This finding prompted us to directly immobilize the enzyme to these free aldehyde groups without a further activation step.

MC Nanofibrous Mat as an Enzyme Immobilization Support. After crosslinking the MC nanofibrous mat, it was directly used to immobilize the enzyme *via* an aldehyde-amine coupling reaction. Table I shows the amount and activity of the bound lipase. The amount of the bound lipase was 34.82 μg per mg of MC nanofiber. Because we initially loaded 355.5 μg of lipase per mg of MC nanofibrous mat, the enzyme-loading efficiency should be approximately 9.8%. The apparent

Table I. Activity of Lipase Immobilized onto MC Nanofibrous Mat

Sample	Bound Protein (μg/mg)	Apparent Activity (μmol/min)	Specific Activity (μmol/min/mg)	
			Per mg Enzyme	Per mg Support
Free Lipase	0.1 mg[a]	0.0540 ± 0.0048	0.539 ± 0.047	
Lipase-MC	34.82 ± 1.02	0.0695 ± 0.0043	0.051 ± 0.006	0.00147 ± 0.00015
CLEA-MC		0.0285 ± 0.0027		0.00713 ± 0.00066

[a]Amount of free enzyme.

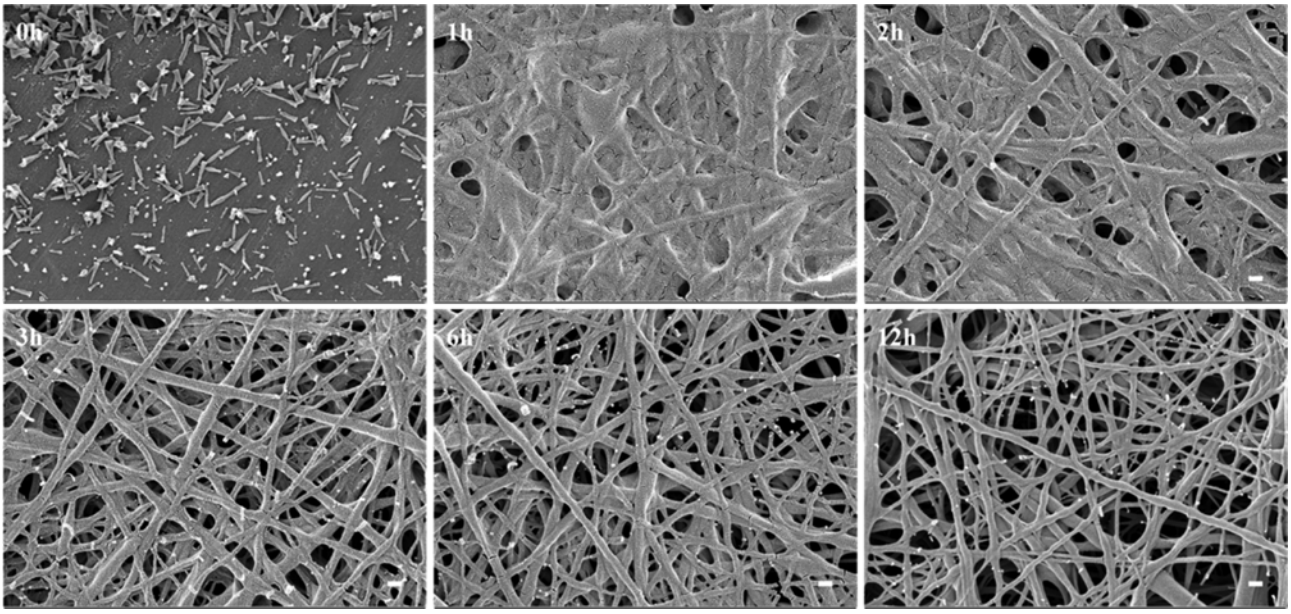

Figure 5. FE-SEM images of MC nanofibrous mat prepared using different crosslinking times after immersion in water for 1 h: crosslinking time of 0, 1, 2, 3, 6, and 12 h (magnification: 500,000×, scale bar: 200 nm).

activity of immobilized lipase was higher than that of free lipase, but its specific activity was only one tenth that of the free enzyme. These changes are typical of enzyme immobilization and due to non-site-specific covalent bonding. The specific activity of the enzyme is high if the active site of the enzyme is well exposed to the substrate. However, the active site of the enzyme can be blocked by the support unless a site-specific immobilization strategy is employed. The major advantage of enzyme immobilization is that the immobilized enzyme can be used multiple times, which compensates not only for the low specific activity of the enzyme but also for the high cost of the enzyme. Therefore, we assessed the relative activity of lipase after 7 repeated uses (Figure 7). Unfortunately, the results of this test were disappointing because the activity of the enzyme significantly decreased after the first use. Such a dramatic loss of activity is usually observed if the attached enzyme detaches from the support during repeated uses.[11] Therefore, we assessed the robustness of enzyme immobilization onto the MC nanofibrous mat. First, we treated the crosslinked MC nanofibrous mat with Tris-HCl buffer solution in order to reduce the free aldehyde groups into hydroxyl groups prior to lipase immobilization. In this case, the lipase will physically adsorb onto the MC nanofibrous mat instead of covalently bonding to the substrate. As shown in Figure 7, this configuration exhibited similar dramatic losses in activity after repeated uses due to the loss of lipase from the MC nanofi-

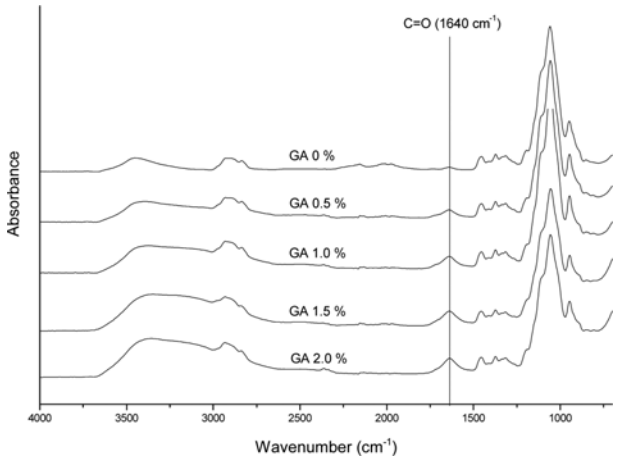

Figure 6. FTIR spectra analysis of MC nanofibrous mat after crosslinking with different concentrations of GA.

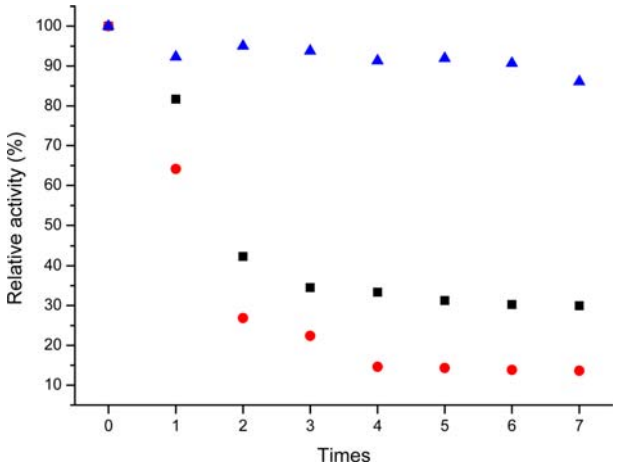

Figure 7. Stabilities of lipase immobilized on MC nanofibrous mat by covalent bonding (■), adsorption (●), and CLEA (▲).

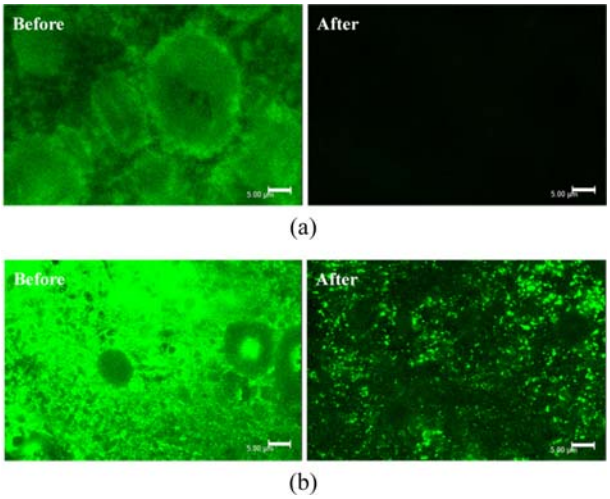

Figure 8. Confocal microscopy images of methylcellulose nanofibrous mat containing immobilized FITC-labeled protein before and after washing; (a) simple immobilization and (b) cross-linked enzyme aggregates (magnification: 200×, scale bar: 5 μm).

brous mat. However, this loss in activity was more severe than that of the covalently bonded lipase because the hydrophobic interaction between lipase and MC is low. The loss of enzyme after repeated uses could also be microscopically observed using FITC labeling. We used FITC-BSA instead of lipase because BSA is a more versatile protein than lipase. Figure 8(a) shows the confocal microscopy images of the FITC-BSA-immobilized MC nanofibrous mat before and after washing with buffer solution. Whereas the mats initially showed strong green fluorescence, fluorescence was absent after washing, indicating a loss of protein during the washing procedure. Because GA-mediated covalent bonding is the most common means of immobilizing an enzyme to a support,[30] the cleavage of GA-mediated covalent bonding was unexpected. In general, the reaction between an aldehyde and amine group forms an unstable Schiff base, which requires further reduction into a stable form. However, in the case of GA, such a reduction step is not mandatory because GA has been proposed to react with amines *via* a Michael addition due to the presence of complex GA derived oligomers.[30,31] Therefore, the loss of protein from the MC nanofibrous mat could be due to the dissolution of MC at the surface. We have shown that crosslinking greatly enhanced the water stability of MC nanofibrous mats (Figure 5). However, it seems that the bulk erosion of the MC nanofibrous mat was inhibited, but the surface erosion of the MC nanofibrous mat could not be prevented. Therefore, because the lipase was immobilized to the surface of the MC nanofibers, it may have also been washed out during the multiple washing steps, resulting in a loss of activity after repeated uses.

Lipase Immobilization *via* CLEA Formation. To overcome this problem, we used a CLEA strategy, which relies on cross-linking the lipase on the surface of the MC nanofiber (Figure 9). Here, lipase was immobilized onto the MC nanofibers followed by further crosslinking between lipases *via* adding additional lipase and GA. In Table I, we present the activity of the CLEA on the MC nanofibrous mat (CLEA-MC). In the case of CLEA, the exact amount of bound lipase was not easily measured due to self-crosslinking of the enzyme which increases the turbidity of solution.[32] Therefore, we expressed the specific activity of CLEA-MC as a hydrolysis rate of substrate per weight of MC nanofibrous mat. The apparent activity was low compared with the activity of the original lipase-immobilized MC nanofibrous mat. However, the specific activity of CLEA-MC was increased by almost 5-fold compared with the activity of the original lipase-immobilized MC nanofibrous mat. The increase in the specific activity of CLEA-MC may be due to the increase in bound lipase per support area. The reusability of CLEA-MC was also significantly improved; more than 90% of the initial activity was retained after 7 reuses (Figure 7), indicating the stable immobilization of lipase. The robustness of protein immobilization *via* CLEA formation is also evident in Figure 8(b). Specifically, the green fluorescence of FITC-BSA persisted after the washing step. The formation of CLEA on the surface of MC nanofibers might yield coating the surface of MC nanofibers result in prevention of the surface erosion of MC nanofibers.[15,16]

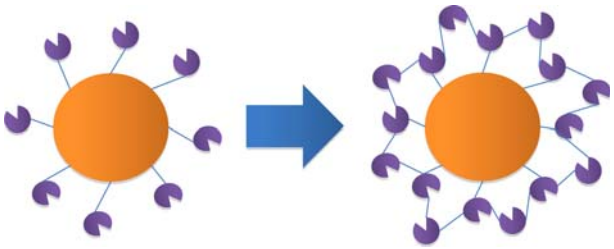

Figure 9. Image of a lipase cross-linked enzyme aggregates formation on lipase immobilized methyl cellulose nanofiber.

Conclusions

The MC nanofibrous mat was successfully prepared by selecting proper electrospinning conditions. Crosslinking with GA improved the water stability of the MC nanofibrous mat, but surface erosion was unavoidable. This erosion led to the loss of lipase after immobilization, which resulted in the poor reusability of the immobilized lipase. To overcome this problem, we adopted a CLEA strategy, which consisted of coating the MC nanofiber with lipase. This strategy significantly increased the reusability and specific activity of the lipase per mg of support. We propose the application of CLEA-MCs in biodiesel production, which utilizes lipase for the transesterification of vegetable oils into biodiesel.

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