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Fabrication of cellulase protein fibers through concentric electrospinning

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ABSTRACT

Cellulase catalyzes the hydrolysis of cellulose to glucose and is of great interest for biofuel production. Cellulase fibers with diameters ranging from 100 to 500 nm were fabricated for the first time by core–shell electrospinning. The enzymes in the core were cross-linked using glutaraldehyde and the PEO outer shell washed away using water and methanol to produce a free standing nonwoven enzyme fiber mat. The activity of the cross-linked protein fibers was determined to be 24% that of the free enzyme for hydrolysis of an insoluble paper substrate. Also, the cellulase was complexed with Ca^{2+} and electrospun for EDX analysis. Elemental mapping of Ca^{2+} showed fibers that cross-linked cellulase fibers maintained the fibrous morphology after washing away the PEO shell.

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1. Introduction

Interest in alternative fuels has recently experienced a surge globally due to the reduction in fossil fuel reserves and increased environmental concerns. Cellulose is a promising source for renewable energy due to its high abundance. The conversion of biomass to biofuels involves a series of complex catalytic processes facilitated by the enzyme cellulase [1]. Cellulases are a series of enzymes that collectively facilitate the hydrolysis of β (1–4) linkages of cellulose to glucose. The three activities that constitute a complete cellulase are from endoglucanase, exoglucanase, and β-glucosidase. The widespread use of enzymes such as cellulase is possible by innovations in enzyme immobilization that stabilizes and improves enzyme performance [2]. Enzyme immobilization provides many advantages which include thermal and environmental stability while allowing for reuse and easy processing [3,4]. We have previously shown that mesoporous metal oxides and more recently hybrid frameworks are effective support for immobilizing enzymes [5–11]. Such systems require the substrate to diffuse into the pores to access the enzyme. Therefore, these supports would not be appropriate for cellulase because of the limited diffusion of the larger cellulose fiber substrate into these mesoporous supports. With these considerations, the present study focuses on cross-linked cellulase enzyme fibers which permit improved contact with substrate.

Electrostatic spinning, or electrospinning, is a process in which a polymer solution is converted to a solid fiber mat. Interest in electrospinning was revived in the early 1990s when it was shown that nanofibers could be fabricated from a broad range of organic polymers [12]. In addition to the simplicity and versatility of electrospinning, the electrospun fibers can be collected after accumulation as a free standing paper, which allows for easy handling. Electrospinning has also gained attention as a technology for immobilizing and controlled release of enzymes [13–24]. It has been reported that a protein encapsulated in polymer electrospun fibers can exhibit up to six times the activity as that of a cast thin film of the same solution [19]. Through this technique enzyme fibers have been produced, not directly, but through the use of core–shell fibers for the first time. Formation of core–shell fibers is possible through a modified approach to the conventional method of electrospinning. Core–shell, or concentric, electrospinning is utilized with polymer solutions that phase separate as the solvent is evaporated. In this process, two coaxially aligned capillaries are employed for the distribution of the polymer solutions [25]. This method has opened up the possibility of fabricating composite materials that may exhibit novel properties and functionalities for nanoscale devices. Furthermore, by dissolving the inner core material hollow nanofibers can be produced. Also, a material that is not typically fiber forming can be fed into the core and the outer shell removed, leaving the inner core in a fibrous morphology [26]. By electrospinning a polymer that readily forms fibers in the outer core we are able to electrospin many different types of materials in the inner core. The outer sheath material serves as a barrier to keep the morphology of the inner core material, which can be further processed. It has been reported that cross-linked cellulase exhibits better heat and pH stability as well as longer sustained activity compared to free enzyme [4]. Additionally, electrospun cellulase poly (vinyl alcohol) composites has been shown to have high reusability rates with up to 36% retained activity after six cycles of reuse [27].

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A membrane composed of nanofibrous enzyme is of particular interest as they provide easy handling and separation of biocatalyst and substrate as well as the high surface area. The present study involves a novel method to fabricate cross-linked cellulase fibers through the method of concentric electrospinning. The cellulase used in this study, *Trichoderma reesi*, is able to hydrolyze crystalline, amorphous, and chemically derived celluloses to glucose quantitatively. Thus such a membrane could be incorporated in a process to generate glucose for biofuel production.

2. Experimental

2.1. Materials

All chemicals were purchased from Sigma–Aldrich and used as received. Cellulase (from *Trichoderma reesi*) was prepared by dissolving in phosphate buffer (0.1 M, pH 6.1) and used as inner core solution. For calcium complexing, calcium chloride was added to cellulase solution at a 4:1 molar ratio, mixed overnight at room temperature, and then filtered. Polyethylene oxide (PEO 400,000 mw) in methanol (5%, w/v) was used as outer shell material. The spinneret for inner core and outer shells were flat tipped B-D 22 gauge and 16 gauge needles respectively. Glutaraldehyde used to cross-link was 50% (v/v) in water. Glass filter paper, Whatman glass microfiber filter 934-AH^{TM}, was affixed to a rotating foil drum with a paper clip.

2.2. Experimental

For electrospinning, two independently operated mechanical syringe pumps controlled both inner core and outer shell solutions in the ranges of 0.1–0.3 ml/h and 0.3–1.0 ml/h respectively. During electrospinning, humidity was controlled in the electrospinning apparatus by purging with nitrogen gas and kept constant at 31% relative humidity. A voltage of 15 kV was applied to the spinneret from a variable high power voltage supply (ES50P-5W, Gamma High Voltage Research). Fibers were collected with a distance of 13 cm measured from the spinneret to the grounded rotating foil collector. Samples collected on a rotating foil drum and glass filter paper were subsequently cross-linked with glutaraldehyde vapor by suspending the fibers above glutaraldehyde heated to 100 °C for 10 min. The resulting cross-linked fibers were then washed with a water and methanol solution in a soxhlet extractor.

2.3. Characterization

The fiber morphology was examined by SEM (Leo 1530 VP field emission scanning electron microscope) on Au/Pd coated samples. Energy dispersive X-ray (EDX) analysis (Zeiss SUPRA 40 field emission scanning electron microscope) was used in elemental mapping of carbon coated fibers. Enzyme fiber activity was determined using a dinitrosalicylic acid reducing sugar assay [28,29].

2.4. Assay

The enzymatic activity of concentrically formed and cross-linked cellulase fibers was assayed using a standard filter paper assay for saccharifying cellulases as described by Ghose [28]. Sections of 1 × 1 cm fiber samples were fastened in close contact with the Whatman no. 1 filter paper within a nylon envelope and then incubated for two hours at 60 °C. Cellulase activity was terminated in a boiling water bath for 15 min. Termination steps were also done for standards and a free cellulase blank. The amount of glucose produced was measured at 540 nm by UV–vis spectroscopy.

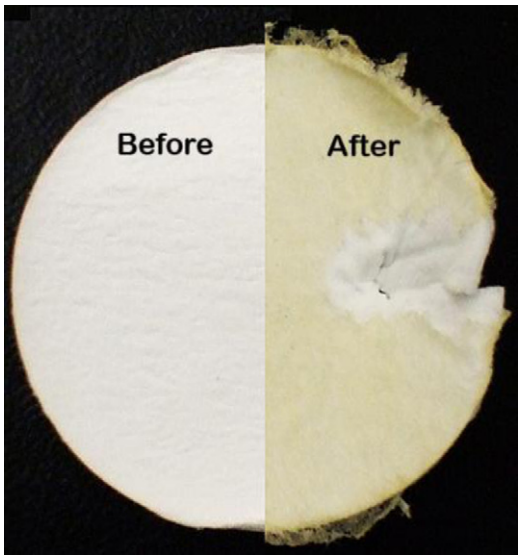

Fig. 1. Digital image of glass filter paper before and after coated with concentrically electrospun fibers.

3. Results and discussion

3.1. Electrospun fibers

Concentrically electrospun PEO–cellulase fibers were fabricated with diameters ranging from 100 nm to 500 nm as shown as a digital image in Fig. 1 and in SEM Fig. 2a–c. Fiber diameter was controlled by adjusting the flow rates of the individual inner core and outer shell materials. When the flow rate was 0.1 ml/h and 0.3 ml/h inner core and outer shell respectively, fibers generated were 100 nm in diameter while increasing the flow rates to 0.3 ml/h and 1.0 ml/h fibers generated fibers closer to 500 nm in diameter. The fibers generated are uniform in diameter with little beading throughout electrospun samples. The fibers composed of a PEO shell are not highly stable under the electron beam at high magnification SEM and thus cracking can occur as seen in the SEM image in Fig. 2c. PEO was chosen as the outer sheath material because it easily forms fibers when electrospun and can be dissolved with water during the washing process. Fibers that exhibit non-uniform diameters and beading adversely affects the specific activity of the protein fiber by further increasing blocked or restricted active sites by aggregation. Therefore, fibers with uniform diameters are necessary to ensure homogeneous activity by reducing excessive active site restriction of enzymes confined to the inner portion of the fiber. Beading, in concentrically electrospun fibers, signifies a local aggregation of inner core materials and is undesirable as they increase the amount of enzymes constrained within the inner portion of the aggregate. These morphological features negatively influence the efficiency of electrospun protein fibers and can be reasonably controlled through the electrospinning process. Thus the target fibers would exhibit uniform morphology and smaller fiber diameter. This combination should be ideal for maximizing enzymatic activity because of the high specific surface area for enzyme activity and allow for better substrate diffusion.

3.2. Cross-linking of fibers

After the core shell PEO/cellulase fibers were collected, either on glass filter paper or foil, they are cross-linked with glutaraldehyde vapor. Glutaraldehyde is a common cross-linking agent that cross-links enzyme through external lysine residues [30,31]. The heated glutaraldehyde vapor is able to permeate the outer PEO sheath and

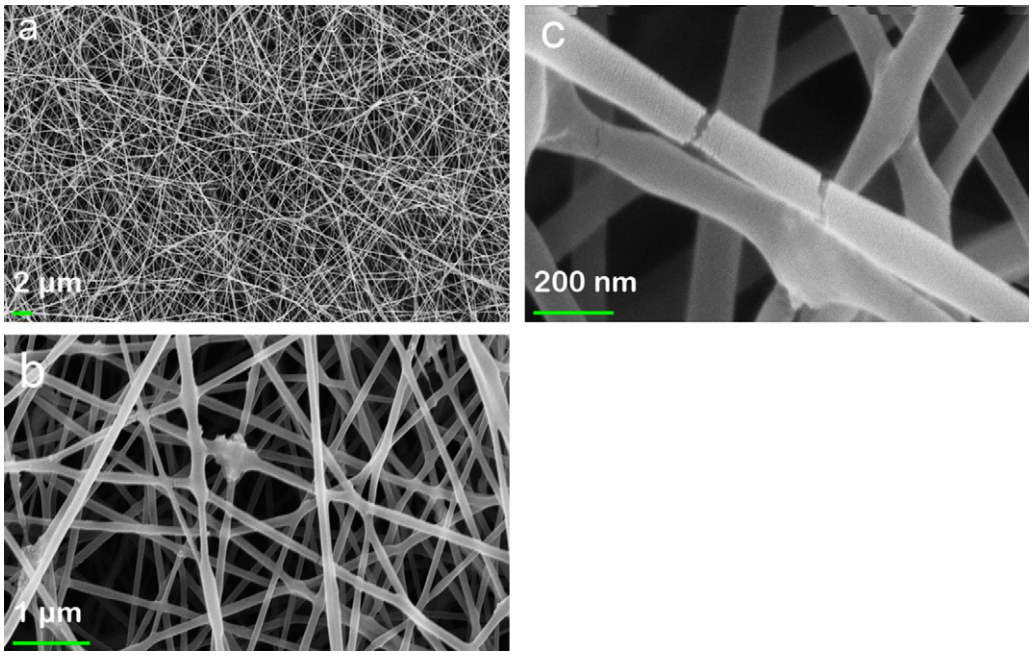

Fig. 2. (a–c) SEM images at 5k, 25k and 60k magnification respectively of concentrically electrospun PEO-cellulase fibers collected on a rotating foil drum before washing. Fibers exhibit uniform diameters of 100–500 nm.

crosslink the inner core cellulase. The cross-linked fibers were then washed in a soxhlet extractor with water and methanol to remove the outer shell PEO and excess glutaraldehyde. ATR-FTIR spectroscopy was used to track the removal of PEO from the cellulase fibers. Fig. 3A shows the spectra of PEO and cellulase fibers, which largely resemble the outer shell PEO material as expected. Fig. 3B shows the spectrum of the fibers after partial washing. The broad 3400 cm^{-1} peak is assigned to the vibrational stretching mode of the –OH groups. The weak bands near 1600 cm^{-1} are consistent with the C–N and C=O stretching modes found in peptide bonds. These peaks are more apparent in the fully washed fibers (Fig. 3C). These cellulase fibers were then assayed and imaged.

3.3. Processing of fibers to remove the shell

In order to expose the cellulase at the core of the fibers, the PEO shell must be dissolved. The PEO outer shell material was present in a much higher amount that the enzyme, such that the fiber left after washing are significantly smaller. Since the processing and recovery of the cellulase fibers from the free standing core shell proved difficult, all subsequent fibers were electrospun on foil or glass filter paper as a support. Fig. 1 shows a glass filter paper before and after electrospun fibers were collected. The noticeable brown color is from cellulase encapsulated in the inner core of the fibers.

The electrospun core–shell fiber diameters (100–200 nm) are smaller than the typical cross-linked enzyme aggregates (CLEAs). CLEAs are immobilized enzymes generated by the cross-linking of enzyme aggregate precipitates and can comprise of different enzymes that are each available for catalysis. These enzyme aggregates can range from 1 to 100 μm. Cellulase based CLEAs have been reported to be on average 15 μm in size [32,33]. CLEAs have the advantage of high activity, sometimes slightly higher than the free enzymes, and can be precipitated and centrifuged for recovery. Without a supporting matrix, CLEA recovery can be difficult to integrate in certain systems where products, un-reacted substrate, and CLEA separation is not easy. Electrospun enzyme fibers could prove to be advantageous for these situations.

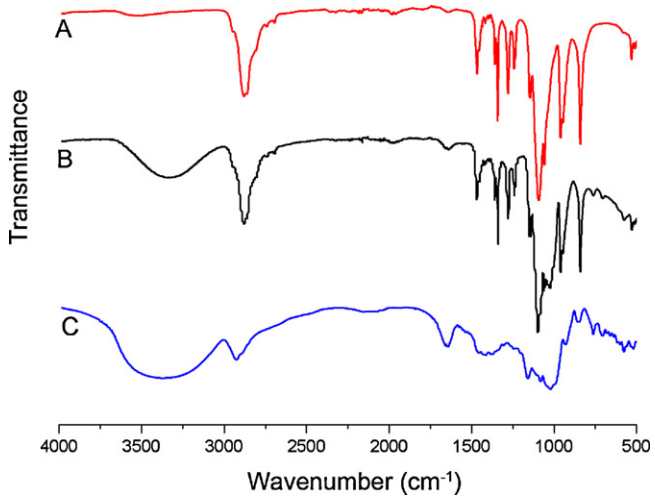

Fig. 3. (A–C) ATR-FTIR of PEO and cellulase composite fibers. (A) Fibers as spun, (B) fibers after partial washing, and (C) fibers after removal of PEO and excess glutaraldehyde.

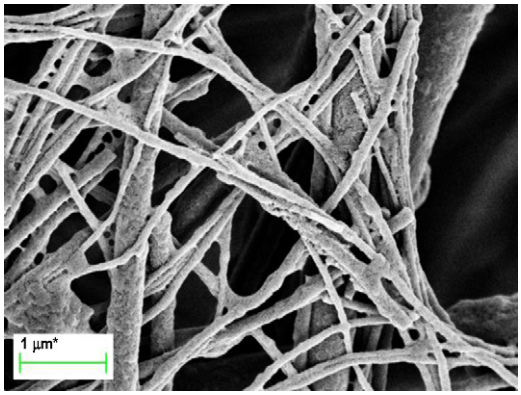

Fig. 4. SEM image of electrospun PEO/cellulase fibers on glass filter paper.

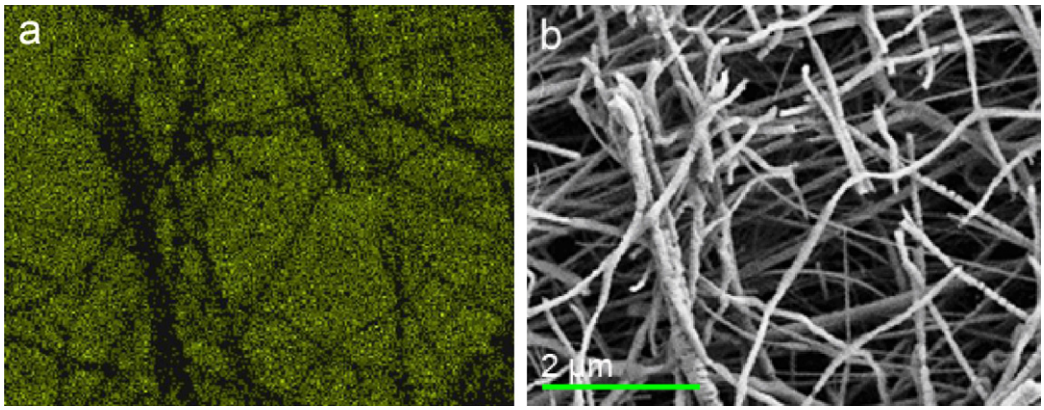

Fig. 5. (a, b) EDX mapping and corresponding SEM image of electrospun and washed fibers on glass filter paper. Silicon signal denoted in yellow (a) with corresponding SEM image (b). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.4. Fiber imaging and EDX analysis

The resulting cellulase fibers were analyzed by SEM and EDX. Fig. 4 shows the cellulase fibers deposited on glass paper. The largest fiber (>200 nm) is consistent with the glass paper, while the smaller nanofibers are likely the cellulase fibers. While there is clearly two different fibers present in the image, the size along does not confirm which one is cellulase. Therefore, to verify which fibers are silica and which ones are protein, EDX analysis and elemental mapping were performed.

The cellulose binding domain (CBD) of cellulase has been extensively studied and found to have three distinct Ca^{2+} binding domains which are essential for optimal activity as well as aids in stability of certain cellulase complexes strains [34,35]. Therefore, cellulase was complexed with calcium, electrospun, and processed under similar conditions. EDX mapping of the resulting fibers collected on glass filter paper and foil are shown in Figs. 5 and 6 respectively. The glass filter paper and cellulase fibers are difficult to distinguish in the SEM image (Fig. 5b). However, a map of silicon, shown in Fig. 5a, results yellow background arising from the glass filter paper. The dark areas where there is no silicon signal reveal the cellulase fibers that can be compared to Fig. 5b. The calcium signal was not seen in these image maps due to the overwhelming silicon signal from the glass filter paper. However, when the core shell fibers that were electrospun on aluminum foil, both calcium and chloride, from the calcium chloride used to complex cellulase, was observed before washing (not shown). Due to the lateral resolution of SEM-EDX, micron-sized fibers were prepared for

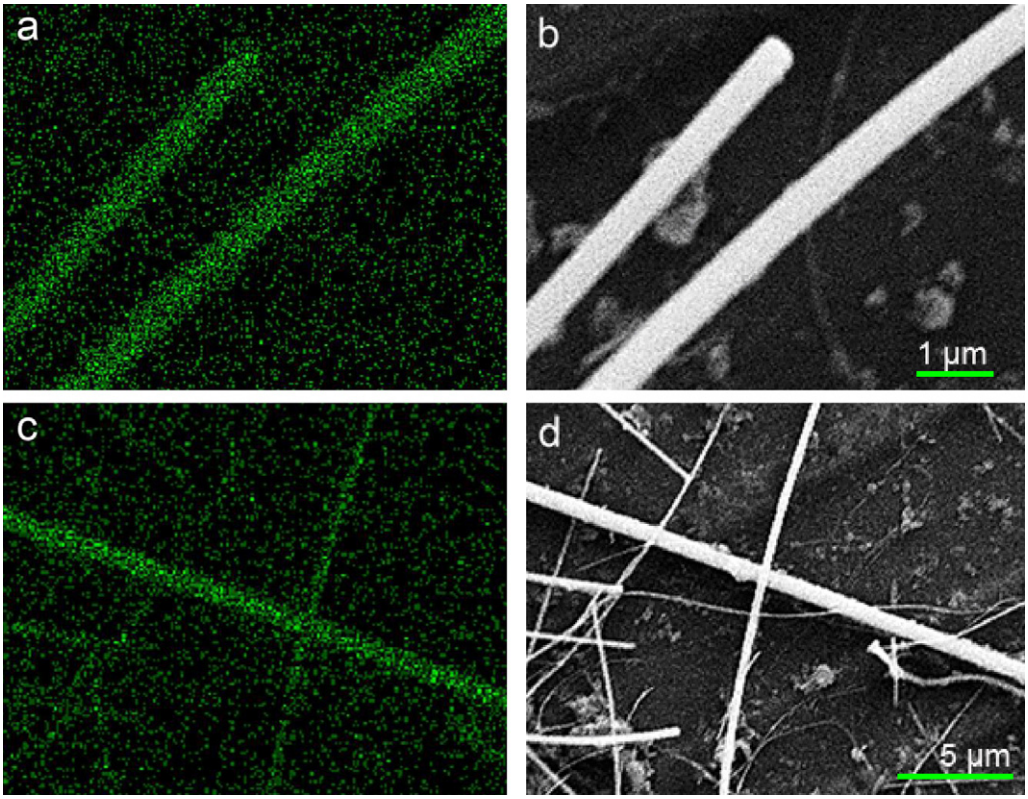

Fig. 6. (a–d) EDX mapping and corresponding SEM image of cross-linked cellulose fibers after washing. Calcium signal is clearly seen in green (a and c) in fibrous morphology of corresponding SEM image (b and d) whereas chloride signal is dispersed or absent. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

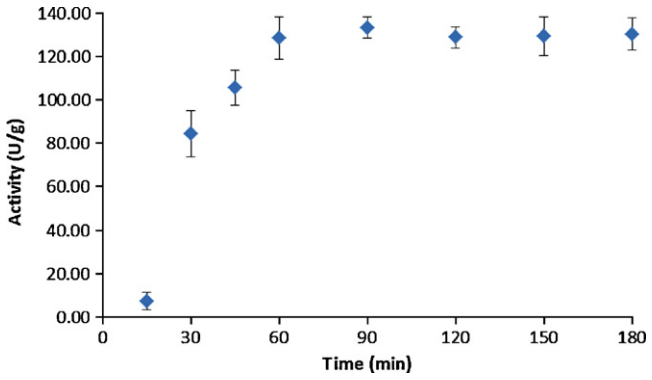

Fig. 7. Activity assay of cellulase fibers after washing.

EDX mapping. These fibers were generated by adjusting the syringe pump feed rates. After washing away the PEO shell, the calcium signal (Fig. 5a and c) shown in green remained intact in the cellulase fibers (SEM images from Fig. 5b and d) while chloride completely gone (not shown).

3.5. Activity assay

A variety of cellulose feedstocks could be employed for biofuel production. This could range from soluble polysaccharide to insoluble paper or particles. The activity on immobilized cellulase is often tested with soluble substrates. However, in the present study, an insoluble paper substrate was selected to better challenge the system. A limitation of this approach is the insoluble substrate must be in contact with the cross-linked cellulase fibers for activity. Thus the resulting activity only reflects the activity of these cellulase fibers in direct contact with the cellulose substrate. For a 1×1 cm sample it was determined that fibers constituted 1.8 mg of cellulase fibers calculated from amount of cellulase used and total area of sample collected. Fibers used in the assay were 100 nm in diameter. When assayed, the cellulase fibers activity reached 130($\pm$7) U/g, there a unit (U) is defined as μmol/min of glucose liberated from cellulose. When compared to the activity of the free enzyme (522 U/g), this represents 24% of the activity of equivalent free enzyme. This value is comparable to other glutaraldehyde cross-linked cellulases [36]. The k_m and V_{max} values were calculated to be 9.88×10^{-3} mol/min mg and 1.81×10^{-4} M respectively. Although the lower activity may be a result of cross-linking it is more likely that not all the cellulase is in contact with the substrate. Enzymes fiber surface and top of the nonwoven mat have a greater access to the substrate and higher specific activity, while enzymes with unfavorable active site orientations require substrate diffusion. The lag time observed in the plot of Fig. 7 can be attributed to the mass flow limitation of the solid cellulose substrate in the static configuration for this assay. The supported cellulase nanofiber mats might be employed as screens or filters in batch or flow reactors for biofuel production. However, practice applications may require some pretreatment of the cellulose to facilitate substrate contact and diffusion to the immobilized enzyme.

4. Conclusions

In this study cellulase fibers have been concentrically electrospun as free standing papers for the first time. The crosslinked cellulose nonwoven mat was challenged with hydrolysis of an insoluble paper substrate. While the contact between enzyme and substrate limited activity, the full potential of this novel system might be realized with a soluble substrate. The application of the espun enzyme mats as screen s or filters in either flow or batch type reactors for biofuel production would allow recovered and separation of the expensive biocatalysts. We are currently examining core–shell cellulase fibers where the shell is composed of mesoporous acidic polymers to further assist with endoglucanase and exoglucanase activity. The concentric electrospinning technique described for cellulose should be applicable to many different enzymes for biocatalysis, sensors and energy production.

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