Electrospun polylactic acid nanofiber membranes as substrates for biosensor assemblies

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Received 23 August 2005; received in revised form 12 December 2005; accepted 13 December 2005
Available online 15 February 2006

Abstract
Biotin has been successfully incorporated into polylactic acid (PLA) nanofibers through electrospinning to prepare membrane substrates for biosensors based on biotin–streptavidin specific binding. Biotin incorporated PLA nanofiber membranes were characterized with scanning electron microscopy (SEM), electron probe microanalysis (EPMA), and confocal microscopy. Under optimized conditions, small fiber size and uniform morphology were achieved for PLA nanofibers with and without biotin incorporation. Sulfur mapping indicated a non-uniform distribution of biotin on the membranes, presumably due to aggregation of biotin during the electrospinning process. Pre-blocking the membranes effectively eliminated non-specific binding between streptavidin and PLA. Preliminary biosensor assays confirmed that streptavidin immobilized on the membrane surface could capture a biotinylated DNA probe.

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Keywords: Electrospinning; Nanofiber; Biotin; Streptavidin; Polylactic acid; Biosensor

1. Introduction
A wide array of biosensors have been developed utilizing the rapid, specific, and strong binding between biotin and streptavidin [1]. In most cases, streptavidin is applied to a substrate material surface and subsequently coated with a biotinylated bio-recognition agent used to capture specific target analytes. The number of sites available for detection of the target analytes is directly related to the surface area of the sensor substrate. A variety of materials including gold surfaces of surface plasmon resonance (SPR) sensors [2–4], plastic films [5], and microfluidic devices [6] have been used as substrates for sensors based on biotin–streptavidin immobilization.

Researchers have recognized the advantage of increasing the surface area of the detector substrate to increase the number of sensing sites available without increasing the amount of overall sample required [7,8]. Polymeric membranes with high surface area can be prepared by electrospinning. Electrospinning is a fiber formation process which relies on electrical rather than mechanical forces to form fibers with sub micron diameters. These fibers (nanofibers) have exceptional properties due to their minute diameter and large surface to mass ratios [9]. Non-woven mats, collected via electrospinning have small pore size, high porosity, and large surface area. As a result, a small volume electrospun mat can provide a very large surface for sensing and easy access for contaminants to the sensing sites. Although the potential application of combining electrospun nanofiber membranes and biosensing has been recognized, limited studies have been done in this area [10,11].

Polylactic acid (PLA) has been successfully electrospun from a variety of solvents [12,13]. Additionally, a wide variety of materials have been incorporated in electrospun PLA fibers to tailor the fibers for particular end uses. Nanoscale clay particles have been incorporated in electrospun PLA fibers to control modulus and biodegradation rate for potential biodegradable packaging applications [14]. Carbon nanotubes have been incorporated in electrospun PLA fibers for potential use as bone graft materials [9]. Pharmaceutical chemicals have been included in electrospun mats for controlled release delivery. Kenawy et al. [15] incorporated tetracycline hydrochloride in electrospun non-woven fabrics of poly(ethylene-co-vinylacetate) and PLA. Tetracycline hydrochloride incorporated
in the fibers was able to diffuse to the fiber surface over time.

This paper details our efforts to incorporate biotin into electrospun PLA membranes by dispersing biotin in a PLA/chloroform/acetone solution prior to electrospinning. Electron probe microanalysis (EPMA) confirms the presence of biotin in the electrospun fibers and that the final biotin levels are proportional to the amount in the initial dispersions. Further experimentation confirms that the biotin is fixed on the PLA fibers and cannot be washed off. Preliminary biosensor assays following the method described by Baeumner et al. [16] are used to confirm that the nanofiber membrane can successfully immobilize streptavidin which in turn is used for the immobilization of biotinylated nucleic acid probes for the detection of a synthetic E. coli DNA.

2. Experimental

2.1. Materials

Polyactic acid (Mw = 186,000, Mw/Mn = 1.76) was supplied by Cargill Dow LLC (Minnetonka, MN). Chloroform and acetone were purchased from VWR Scientific (West Chester, PA). Both biotin and Streptavidin-Alexa 488 conjugate (495 nm:519 nm excitation:emission) were purchased from Pierce Biotechnology Inc. (Rockford, IL). Phosphate buffered saline (PBS) and Tween 20 were purchased from Aldrich Chemical Co. (Milwaukee, WI).

All general chemicals and buffer reagents (reagent grade or above) for biosensor assay were purchased from Sigma Company (St. Louis, MO). Organic solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Predator membranes were obtained from Pall/Gelman Company (Port Washington, NY). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Sulfoethanolamine B (SRB) and streptavidin were acquired from Molecular Probes Company (Eugene, OR). Nucleic acid probes and synthetic targets, with their respective modifications, were purchased from Qiagen (Valencia, CA).

2.2. Preparation of electrospinning solutions/dispersions

PLA and in chloroform/acetone solvent (3:1 volume ratio) were mixed over night on an InnovaTM 2300 platform shaker (New Brunswick Scientific Co., NJ). For samples containing biotin, biotin was dispersed in chloroform/acetone solvent using a Branson 2510 ultrasonic cleaner (Branson Ultrasoundics Corp., CT) prior to adding PLA. Samples containing biotin were sonicated for another 60 min immediately before electrospinning to insure good dispersion of biotin. Detailed information on the solutions and dispersions that were used in this study is shown in Table 1.

2.3. Electrospinning

The electrospinning apparatus consisted of a programmable syringes pump (Harvard Apparatus, MA) and a high-voltage supply (Gamma High Voltage Research Inc., FL). Variations of the electrospinning conditions are also summarized in Table 1. Aluminum foil was used as the collector in all cases except for the electrospinning of the membranes for biosensor assay.

2.4. Instrumental analyses

Morphology and fiber size of electrospun PLA nanofibers were examined with a Leica 440 scanning electron microscope (SEM) after being coated with Au–Pd or with a LEO 1550 field emission scanning electron microscope (FESEM).

Biotin distribution on nanofiber membranes was characterized with a JEOL 8900 electron probe microanalyzer (EPMA). Both energy dispersive X-ray spectroscopy (EDS) and wavelength dispersive X-ray spectroscopy (WDS) were used to collect characteristic Kα X-ray emission of sulfur atoms in the biotin molecules. As a semi-quantitative means, Ratemeter X-ray counting was used to correlate the amount of biotin in the initial dispersion with the amount that has been incorporated into the membranes.

A Leica TCS SP2 laser confocal scanning microscope was used to track the biotin–streptavidin specific binding by imaging the fluorescence of streptavidin-Alexa 488 conjugate treated nanofiber membranes. Scanning was performed starting from the top layer of each membrane towards the deeper layers and stopped when no fluorescence could be detected. To prepare the samples for confocal analysis, 50 μL of diluted streptavidin-Alexa 488 conjugate solution was applied to a ∼4 mm × 4 mm nanofiber membrane that was pre-wet out with PBS/Tween 20 buffer. Treated membranes were subjected to repeated wash with PBS/Tween 20 buffer to remove any extra streptavidin-Alexa 488 conjugate before being placed onto a glass slide with a cover glass for confocal analysis.

Table 1

<table>
<thead>
<tr>
<th>Run</th>
<th>PLA (wt%)</th>
<th>Biotin (wt%, relative to PLA)</th>
<th>Voltage (kV)</th>
<th>Feed rate (μL/min)</th>
<th>Needle size (mm)</th>
<th>Ground distance (cm)</th>
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<tr>
<td>6-0-1</td>
<td>6</td>
<td>0</td>
<td>15</td>
<td>10</td>
<td>0.26</td>
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<tr>
<td>6-0-2</td>
<td>6</td>
<td>0</td>
<td>15</td>
<td>5</td>
<td>0.26</td>
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<td>0.41</td>
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<td>8-0-1</td>
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<td>8-0-2</td>
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SEM image analysis was conducted using Scion Image Beta 4.02 (Scion Corporation, Frederick, MD, www.scioncorp.com) to obtain the size range of nanofibers for each membrane.

2.5. Biosensor assay

For biosensor assay, PLA membranes were electrospun onto a copper-backed laminate from a solution of 8 wt% PLA in the solvent chloroform/acetone (3:1 volume ratio) and an applied voltage of 15 kV. The PLA membranes were cut into 3 cm × 0.4 cm strips and pre-wet with PBS/Tween 20 buffer. Twenty picomoles streptavidin dissolved in Na2CO3/NaHCO3 buffer, pH 9.0, containing 5% methanol (20 pmol/μL) was deposited on PLA strips to form a capture zone. The membranes were put into the vacuum oven at 103.421355 °C for 20 min. The exact composition of washing buffer (40% formamide, 8× SSC, 0.2% Ficoll, 0.2 M sucrose) was added to the test tubes to provide enough liquid to wet out the entire length of the strip. The color of the strip was measured with a BR-10 reflectometer (ESECO Company, Cushing, OK) to detect the presence of SRB in the capture and background zones.

3. Results and discussion

3.1. Electrospun PLA nanofibers

SEM images of PLA nanofibers electrospun under different conditions are shown in Fig. 1. Sample 6-0-1 (Fig. 1a) shows a typical beaded-fiber morphology, which is mostly attributed to the relatively low concentration of PLA in the electrospinning solution. The beaded-fiber morphology is not affected by feed rate even when the feed rate is decreased by a factor of two, as seen from the image of sample 6-0-2 (Fig. 1b). The fiber uniformity improves slightly when the nozzle size is increased with other conditions held the same. Nanofibers electrospun with a needle of 0.41 mm in diameter are shown in Fig. 1c. Under these conditions, the bead-fiber morphology has been replaced by spindle-fiber morphology.

A noticeable change in uniformity occurs when the concentration of PLA is increased from 6% to 8% and the ground distance is increased by 2 cm, as seen in Fig. 1d, without significantly increasing fiber size. Fiber diameters range from 200 nm to 5 μm. The fiber size can be made slightly smaller when the feed rate is decreased from 10 μL/min at 8% concentration (compare Fig. 1d with Fig. 1e), but decreasing feed rate doubles the time for electrospinning the same amount of nanofibers. The change in fiber size as a function of PLA concentration agrees with previous studies [17]. Fibers reported here were spun from the same solvent system with the same polymer concentration but using a larger molecular weight PLA (Mn = 186,000 versus Mn = 48,000). Increased molecular weight is associated with increased viscosity of the spinning solution, which in turn is associated with increased electrospinning fiber diameter [18].

Fig. 2 shows the SEM images of PLA nanofibers spun from solutions with different amounts of biotin dispersed in the spinning solution. Fiber diameters ranged from 150 nm to 5 μm as measured by image analysis and were independent of biotin concentration. The addition of biotin in the amount of up to 3% relative to PLA weight in the electrospinning dope does not make a significant difference in fiber size and uniformity.

3.2. EPMA analysis

EDS spectra of PLA nanofiber membranes with and without biotin are shown in Fig. 3 before and after being subjected to PBS buffer wash. Enrichment of sulfur is observed in PLA-biotin nanofibers. Because sulfur atoms are solely attributed to biotin molecules in this system (see Scheme 2 for the structure of biotin molecules), the presence of the sulfur peak in EDS spectra indicates the successful incorporation of biotin molecules in
nanofibers through electrospinning. Secondly, the sulfur peak shown in Fig. 3d indicates that biotin is not removed when the membrane is subjected to PBS buffer wash. Also seen from Fig. 3 is that buffer wash causes two major changes in the spectra: the increased background and the appearance of some new peaks such as Na, Cl, K, and P. These peaks are introduced by PBS buffer wash and do not overlap with the sulfur peak located at 2.3 keV.

Further characterization using X-ray counting of PLA nanofiber membranes with and without biotin is shown in Fig. 4. The X-ray counts of the sample without biotin are the background signal level [19]. Subtracting the background, the X-ray
counts of the biotin incorporated PLA samples are proportional to the amount of biotin dispersed in the electrospinning dopes, which provides us a semi-quantitative estimate of the level of biotin incorporated into the membranes. Comparing the X-ray count of the membrane before PBS buffer wash and that after, we can see a much higher background generated in the membrane after PBS wash (260 versus 667). Subtracting the background from the control that is subjected to PBS wash, the PLA membrane electrospun from the dispersion with 3% biotin shows that about 2/3 of the amount of biotin initially dispersed in the spinning dope remains on the membrane. The reason for examining the biotin retention on the membrane after PBS wash is to confirm that biotin will not be removed by PBS buffer wash during biosensor assays.

To examine the distribution of biotin on nanofiber membranes, sulfur mapping was performed using EPMA. In Fig. 5,
the increased number of blue spots in the PLA-biotin nanofiber membrane over the PLA only membrane confirms dispersion of biotin throughout the PLA-biotin nanofiber membrane. On the other hand, several large bright spots on the EPMA map of the PLA-biotin nanofiber membrane indicate that biotin is heterogeneously distributed on the mapping area. EMPA mapping of sulfur content in one of the bright spots is shown in Fig. 6 at a higher magnification. Under this magnification it is evident that the distribution of sulfur signals matches the fibers alignment. This pattern match further confirms the incorporation of biotin into PLA nanofibers.

3.3. Confocal microscopy analysis

To confirm that biotin incorporated in PLA nanofiber membranes is available to bind with streptavidin, specific binding
between the incorporated biotin and a fluorescent dye-Alexa 488-labeled streptavidin conjugate was studied via confocal microscopy. Fig. 7 shows the top view of the overlapped fluorescence images of both a PLA nanofiber membrane and the PLA-biotin nanofiber membrane. Also shown in Fig. 7 is the first frame of the optical images of both membranes. The color bar beside each image indicates the location of fluorescence signals on the scanned layers. The purple and blue nanofibers are located on the layers closest to the surface of the membrane, while the red and pink nanofibers are located on the layers farthest away from the surface. The PLA-biotin membrane exhibits greater fluorescence at all depths than the PLA only membrane. More importantly, however, fluorescence on the PLA-biotin membranes follows the pattern of the electrospun fibers. Biotin incorporated in the fibers appears to be successfully bound to fluorescence labeled streptavidin along the length of the fibers.

The difference between PLA membrane and PLA-biotin membrane is also seen in the profile images shown in Fig. 8b and c. The travel distance of fluorescence is about twice as long in the biotin incorporated PLA membrane than in the pure PLA membrane. On the other hand, the control membrane treated only with PBS buffer (Fig. 8a) shows no fluorescence at all. Such difference is certainly due to the incorporation of biotin.

First of all, the fluorescence resulted from the pure PLA membrane is simply caused by the non-specific binding between streptavidin and the substrate, or the physical absorption of the protein onto the membrane (Fig. 8b). The incorporation of biotin into PLA membrane on the one hand introduces the active sites for specific binding with streptavidin while on the other hand decreases the hydrophobicity of the PLA membrane (see the contact angle measurements in Fig. 9). Both the introduction of active binding sites and the increased hydrophilicity of the membrane contribute to the increase in streptavidin penetration.

Also shown in Fig. 8 is the difference in fluorescence between blocked and non-blocked PLA membranes. Comparison of Fig. 8b and d confirms that pre-blocking have effectively suppressed the non-specific binding of streptavidin-Alexa 488 conjugate. As seen from Fig. 8d, blocked PLA membrane has little or no fluorescence, the same appearance as the control (Fig. 8a) sample. Blocked PLA-biotin membrane (Fig. 8e), on the other hand, has fluorescence, indicating that biotin is accessible to bind with streptavidin after the membrane is blocked. Blocked PLA-biotin membrane shows approximately the same penetration depth along the profile as does the sample without pre-blocking.
Fig. 6. High magnification sulfur mapping of biotin incorporated PLA nanofiber membrane.

(Fig. 8c) Fig. 8d and e clearly shows the difference between PLA and PLA-biotin membranes when non-specific binding is effectively suppressed by pre-blocking.

The confocal results indicate two important properties of the PLA-biotin nanofiber membranes. First, fluorescence signal appears along the length of individual fibers, indicating that biotin incorporated into individual fibers is accessible for streptavidin binding. The heterogeneous distribution of biotin evident from EPMA mapping (Fig. 5) does limit the availability of sites for biotin–streptavidin binding. Second, although the biotin incorporated into PLA membrane is accessible to streptavidin to bind with, only the biotin located approximately 10 μm depth of the entire thickness of the membrane has specifically bound with streptavidin in confocal experiments. The hydrophobicity of PLA nanofibers may limit the wicking of streptavidin through the membrane under aqueous buffer environment.

3.4. Biosensor assay

To demonstrate that the analyte solution can travel through nanofiber membranes and that the target E. coli DNA can be captured by streptavidin on the nanofiber surface a biosensor assay was performed. An electrospun PLA nanofiber membrane was used as the substrate as shown in Scheme 1. Filter paper is attached to one end of the strip to collect excess liposomes that are not specifically bound with the deposited streptavidin during the wicking process. For comparison, a parallel biosensor assay using regular PES membrane as the substrate was also performed. Shown in Fig. 10 are the photographs of typical biosensor assay results from both membrane substrates. The test solution wicked successfully through the PLA nanofiber membrane, capture probes were successfully trapped where streptavidin had been deposited and excess solution wicked past the capture zone to the filter paper. Specific signals for the clpB synthetic target sequence in the capture zones were 57 for the PES sensor and 69 for the PLA sensor. Signals of the corresponding background zones were 26 and 59. This indicates that streptavidin was successfully immobilized on the PLA membrane through deposition and that it was capable of capturing biotinylated DNA capture probes hybridized to the target sequence-liposome complex, thus proving the validity of our concept in nanofiber membrane-based biosensors.
Fig. 7. Pattern match between the distribution of fluorescence and that of PLA nanofiber membranes with (bottom, 8-3) and without (top, 8-0-1) biotin. Refer to Table 1 for sample information.

(a) Non-blocked PLA membrane applied only with PBS buffer (Control)
(b) Non-blocked PLA membrane treated with streptavidin Alexa 488 conjugate
(c) Non-blocked PLA-biotin (8%-3%) membrane treated with streptavidin Alexa 488 conjugate
(d) Blocked PLA membrane treated with streptavidin Alexa 488 conjugate
(e) Blocked PLA-biotin (8%-3%) membranes treated with streptavidin Alexa 488 conjugate.

Fig. 8. Fluorescence image profiling of streptavidin-Alexa 488 conjugate treated PLA and PLA-biotin nanofiber membranes. (a) Non-blocked PLA membrane applied only with PBS buffer (control). (b) Non-blocked PLA membrane treated with streptavidin-Alexa 488 conjugate. (c) Non-blocked PLA-biotin (8%-3%) membrane treated with streptavidin-Alexa 488 conjugate. (d) Blocked PLA membrane treated with streptavidin-Alexa 488 conjugate. (e) Blocked PLA-biotin (8%-3%) membranes treated with streptavidin-Alexa 488 conjugate.
Biotin has been successfully incorporated into PLA nanofibers through electrospinning without significantly changing the morphology and size of the resulting nanofibers. Nanofiber membranes were characterized with EPMA to confirm biotin incorporation in the fibers. Although biotin is dispersed throughout the nanofiber membranes the distribution is heterogeneous and regions of locally high biotin concentration are evident. Confocal microscopy measurement indicates that both specific and non-specific binding occurs between streptavidin and the biotin incorporated PLA nanofiber membranes. With blocking reagent, non-specific binding can be suppressed effectively. Fluorescence labeled streptavidin binds along the surface of PLA-biotin fibers providing evidence that biotin incorporated via electrospinning is available at the fiber surface. Biosensor assay experiments confirm that the PLA nanofiber membranes can successfully transport analyte solutions via wicking. A biotinylated DNA probe was successfully captured by immobilized streptavidin in a preliminary biosensor assay using the PLA nanofiber membranes. With and without biotin.

4. Conclusions

Biotin has been successfully incorporated into PLA nanofibers through electrospinning without significantly changing the morphology and size of the resulting nanofibers. Nanofiber membranes were characterized with EPMA to confirm biotin incorporation in the fibers. Although biotin is dispersed throughout the nanofiber membranes the distribution is heterogeneous and regions of locally high biotin concentration are evident. Confocal microscopy measurement indicates that both specific and non-specific binding occur between streptavidin and the biotin incorporated PLA nanofiber membranes. With blocking reagent, non-specific binding can be suppressed effectively. Fluorescence labeled streptavidin binds along the surface of PLA-biotin fibers providing evidence that biotin incorporated via electrospinning is available at the fiber surface. Biosensor assay experiments confirm that the PLA nanofiber membranes can successfully transport analyte solutions via wicking. A biotinylated DNA probe was successfully captured by immobilized streptavidin in a preliminary biosensor assay using the PLA nanofiber membrane as substrate. Further efforts are being made in (1) optimizing the nanofiber substrate by decreasing the fiber diameter and increasing biotin loading and (2) functionalizing the electrospin nanofibers for specific capture and detection of environmental pathogens or other contaminants.

Acknowledgements

The project was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service (grant number 2005-35603-15298). The authors acknowledge the contribution made by Ms. Barbara Leonard and Ms. Jamie Mullally.

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