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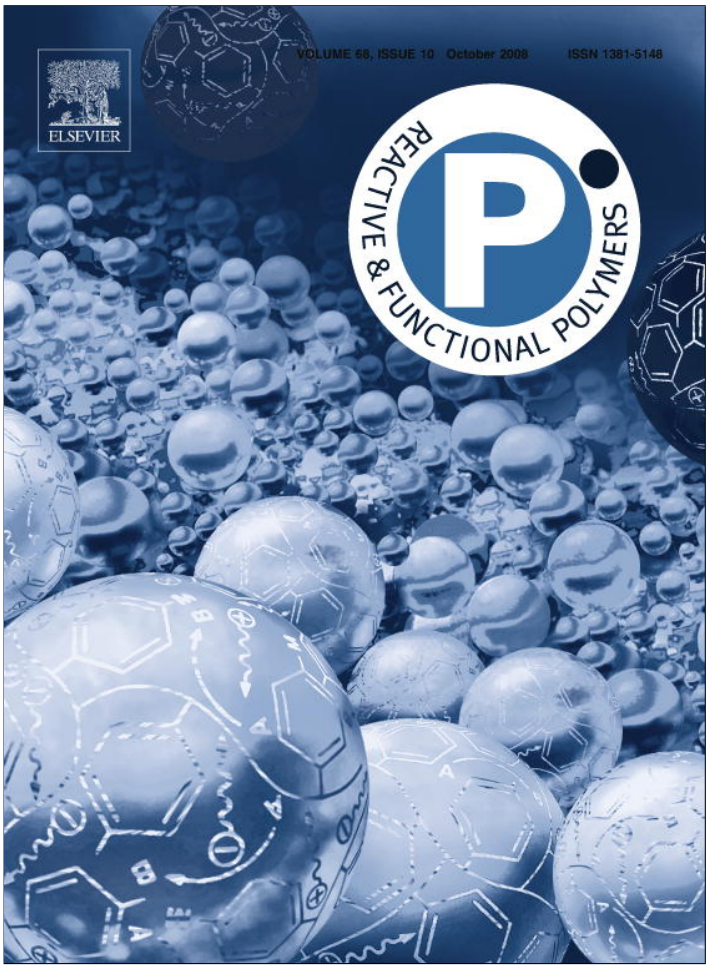

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Development of functional nanofibrous membrane assemblies towards biological sensing

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ABSTRACT

A novel research approach was investigated that has the potential to improve sample preparation for complex matrices. Generation of high surface area nanofibrous membranes with covalently attached molecular recognition elements for selective capture of target biological agents were developed using the electrospinning fabrication technique. Two types of electrospun capture membranes were fabricated containing either carboxyl (COOH) or amine (NH_2) functional groups for covalent attachment of antibodies. The carboxyl functional membrane was produced by electrospinning polyvinyl chloride (PVC) formulated to be 1.8% carboxylated. The amine functional membrane was made by co-electrospinning two polymers, water-soluble polyamine and water insoluble polyurethane. Linking of molecular recognition groups, antibodies, to the carboxylated PVC was performed using established crosslinking chemistries. Antigen/antibody experiments were tested on the electrospun membranes. Results showed that electrospun membranes, treated with a secondary antibody used as the analyte, reacted only with its complement as indicated with a chemiluminescent signal. Toxin studies with *Staphylococcal* enterotoxin B (SEB) were conducted using avidin/biotin chemistries on the electrospun membranes. Experiments were performed using a modified ELISA sandwich assay on the fibrous membranes in the following configuration: avidin–biotinylated SEB antibody–SEB toxin–SEB antibody–HRP. Results have shown that 1–100 ng/ml concentrations of toxin were detected using a chemiluminescent signal detection scheme.

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1. Introduction

Outbreaks in food-borne illness both on the international and local scale bring rapid sensing diagnostics of biological pathogens to the forefront as a necessity to keep the food supply safe [1]. Detecting pathogens in foods and environmental samples is a difficult task. In these complex sample matrices, pathogens can be present in small numbers along with large numbers of background microflora in a complex sample matrix. Additionally, the type of food being analyzed, e.g., fruits vs. vegetables or smooth surface vs. an irregular surface, and pH of food matrix in the extraction solution can affect the attachment, release and recovery of pathogenic organisms [2]. Sample complexity can also interfere with the chemistries and signal transduction associated with emerging rapid technologies slowing down the detection process. To compensate for these negative effects, food and environmental samples are diluted to minimize interfering substances that are inherent to many products. Therefore, appropriate sampling procedures for concentrating and separating bacterial pathogen from the matrix must be developed and integrated with novel biosensor technologies in order to provide rapid and accurate pathogen detection tests [3].

Electrospinning is a process by which a polymer in solution/melt becomes charged by the addition of high static voltage resulting in the production of an interconnected

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membrane-like web of small fibers with diameters ranging from 20 to 1000 nm (Fig. 1). These nanofibrous membranes can have surface areas approximately one to two orders of magnitude higher than those found in continuous films. This technique has been studied since the 1930s [4] and has been used to make nanofibers of polymers such as polyethylene oxide, polyacrylonitrile, collagen and even calf thymus DNA [5–7]. Recently, electrospinning has been looked at as a versatile process tapping into properties inherent in nanofibers such as increase in surface area to volume ratio and decrease in pore size. Electrospun nanofiber use ranges from tissue scaffolds for wound dressing applications, nanofiber meshes impregnated with molecules for drug delivery and nanofiber clothing to filters that deal with chemical and biological threats [8–9].

The association of molecular recognition elements (MREs e.g., antibodies, aptamers, peptides, PNAs (peptide nucleic acids), liposaccharides, etc.) with high surface area electrospun membranes presents the opportunity for developing both novel sampling devices that can also be tailored to fit biosensor detection platforms [10]. Because the capture and detection platform are the same, these electrospun membranes will not require flow devices or pumps allowing for the development of smaller biosensor foot prints. It is expected that the available surface area demonstrated by this technique may provide a one to two log increase in sensitivity and capture efficiency, and a less than 2 h response time needed in sensing applications. In recent literature [11–14] scientists looked at surface functionalized nanofibers containing primary amine groups for covalent immobilization of biological molecules (chymotrypsin and lysozyme). They found that the nanofibrous mesh could immobilize a greater amount of active molecule compared to that of a cast film when looking at lysozyme activity. In this study, nanofibrous membranes containing amine and carboxyl functional groups were prepared. Antibodies were covalently attached to the functional groups on the membranes and an assay was developed for capturing and detecting *Staphylococcus* enterotoxin B (SEB). The development of electrospun platforms can be used in diagnostics to both capture and detect biomolecules and have the potential to improve sampling and identification of biological pathogens from complex matrices.

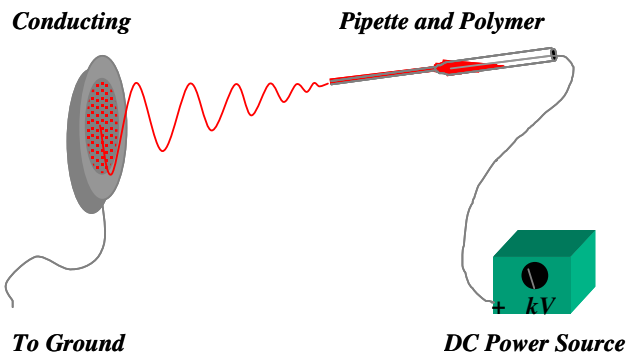

Fig. 1. Diagram showing electrospinning.

2. Experimental

2.1. Electrospinning device

The electrospinning apparatus used consisted of a DC power source (Gamma High Voltage Research, Inc. Model HV ES 30P/100) where the charged electrode wire was immersed in a polymer solution (Fig. 1). The polymer solution was drawn into a disposable glass pipette positioned a few degrees down from horizontal. A second ground wire from the power source was attached to a conducting target where the fibers were collected. The electrospinning target used in making the sensing platform was a stainless steel screen in which an electrospun fibrous coating was applied (Fig. 2).

2.2. Polymer solutions for electrospinning

Membranes containing either amine (NH_2) or carboxyl (COOH) functional groups were fabricated. The amine functionalized electrospun membranes were made by the co-electrospinning of two polymers, polyamine and polyurethane. The PAA-H-10C (poly (2-propen-1-amine), Nitto Boseki, LTD) 10% solution in water was used as the amine fraction of the co-polymeric membrane. Thirty to 150 mg of the PAA-H-10C was dried down in a vacuum to a concentrated solution for inclusion in the spin dope prior to electrospinning. The polyurethane used was Pellethane 80AE (Dow Chemical, Midland, MI) was solubilized 10% by weight in *N*,*N*-dimethyl formamide (DMF) and the fraction of the PAA-H-10C was added with vigorous stirring keeping both polymers in solution. The membranes were electrospun quickly to avoid precipitation by either the polyamine or polyurethane. Electrospinning was conducted for approximately 10 min on the target screen. Amine functional groups were determined after electrospinning, by cutting out uniform sizes (0.5 cm diameter) on the stainless steel screen with a dye punch to minimize size and weight variances. The polyurethane/polyamine membranes combining both a water-soluble and water insoluble polymer were tested for amine activity. The cut out fibrous membranes were then dialyzed in water for

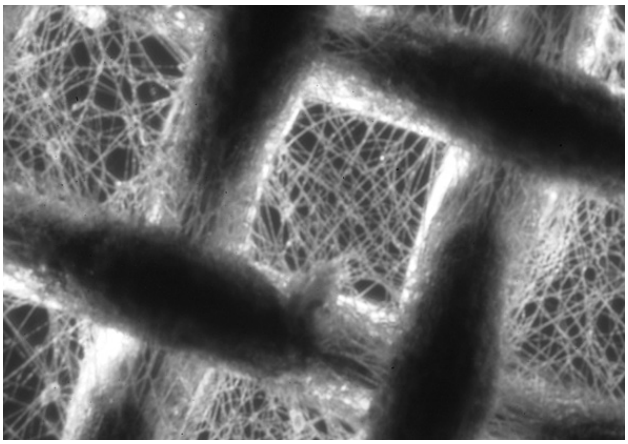

Fig. 2. Electrospun membrane shown on a stainless steel screen as seen with a microscope under polarizing light.

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24 h to remove non-associated water-soluble amine functional polymer. The amines associated in the fibrous membranes were labeled using Fluorescein-EX kit protein labeling kit (Molecular Probes, Eugene, OR). The fluorescent signal given off by fluorescein attachment to the amines was measured using the blue laser detection mode (wavelength excitation 450 nm, emission 520 nm) on the STORM 860 (Molecular Dynamics, Sunnyvale, CA).

The carboxylated polymer used to fabricate electrospun membranes was polyvinyl chloride 1.8% carboxylated (PVC–COOH) (Aldrich Chemical, St. Louis, MO). This polymer was solubilized at 10% by weight in 80% DMF and 10% tetrahydrofuran (THF) w/w.

2.3. Antibody attachment to electrospun membranes

Two crosslinking chemistries were studied for antibody attachment to the electrospun membrane. One process covalently linked the antibodies to the carboxylated PVC using *N*-hydroxysulfo-succinimide (sulfo–NHS) and the dehydrating agent 1-ethyl-3-(3-Dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Pierce Chemical Company, Rockford, IL). In this procedure the membrane was treated as a carboxylated protein to be linked with an antibody. The second crosslinking chemistry studied used avidin and fluorescein labeled biotin. The avidin was attached on the PVC–COOH membranes using EDC carbodiimide (Pierce Biotechnology, Rockford, IL) covalent chemistry. Again, the membrane was treated as the initial carboxylated protein in 1 mL of reaction buffer pH 5.0, 0.1 M 2-[*N*-morpholino] ethane sulfonic acid (MES)/0.1% tween 20. The second protein, avidin, was added at a concentration of 0.5 mg/mL to the reaction buffer to which 1 mg of EDC was added to initiate the conjugation reaction to the membrane. The carboxylated membrane and avidin reacted 2 h at room temperature. After the conjugation reaction the membranes were washed 3× with pH 7.2 phosphate buffered saline/0.1% tween 20 (PBST) to allow buffer equilibration. Biotin–fluorescein conjugate was added to the membranes 0.5 mg/mL in the same buffer and allowed to react for 1 h. The membranes were then washed again 3× with the PBS buffer and the fluorescent signal was detected by using blue laser detection mode on the STORM 860.

2.4. Capture studies

Antigen/antibody experiments were performed to determine the potential for producing electrospun membranes with capture specificity. Two different primary antibodies were used for the experiment. Rabbit anti-Staph and Goat anti-Rat, were each covalently attached to two separate electrospun membranes using the EDC and sulfo–NHS crosslinking chemistries above. The same secondary antibody, Rabbit anti-Goat–horse radish peroxidase (HRP) conjugate, complement to the Goat anti-Rat primary antibody only, was reacted with each of the membranes. The chemiluminescent signal produced using Super Signal (Pierce Chemical Company) was detected on the CHEMIGENIUS 2 Bio-imaging system (Syngene, Frederick, MD).

Experiments were conducted to detect staph entero toxin B (SEB) using electrospun PVC–COOH membranes with attached anti-SEB antibodies to capture and concentrate the toxin. Highly purified SEB, Rabbit anti-SEB biotinylated and HRP antibodies (Toxin Technology, Sarasota, FL) were used for this study. Biotinylated Rabbit anti-SEB was attached to the avidin crosslinked membrane in a 1/2000 dilution for 1 h at room temperature using a rocking platform (Labnet Shaker, Woodbridge, NJ) setting dial at 4. The membrane was then blocked with 0.2% non-fat dry milk (NFDM) in PBST. NFDM blocked electrospun PVC–COOH membranes without antibodies were used as controls to determine extent of non-specific binding in the experiment. Membranes with and without Biotinylated SEB antibodies were reacted with SEB concentrations in PBST at 0, 1, 10, and 100 ng of SEB toxin per mL with shaking at 360 rpm on a LabLine Microtiter plate shaker Model 4265 for 1 h in 24 well polystyrene titer plates (Dow Corning, Midland MI). All experimental and control samples were done in triplicate. The membranes were washed 5× with 1 mL PBST with shaking at 470 rpm for 5 min. The membranes were then reacted with 1 mL of a 1/10,000 dilution of SEB–HRP conjugate antibody in PBST for 1 h with shaking at 360 rpm. After binding the membranes were then washed 5× with 1 mL PBST with shaking at 470 rpm to remove excess labeled antibody. The membranes were removed from the 24 well titer plates and placed into individual wells of a 96 well microtiter black plate to which 100 uL of super signal reaction mixture was added for detection of chemiluminescent signal. The membranes that bound SEB were determined using a membrane biosensor fitted with modifications [14]. Light generated from the peroxidase-chemiluminescent reaction was measured by positioning a fiber optic probe above the membrane surface in the absence of light. The probe was connected to a A5-2021A Lumi-Tec luminometer (St. Johns Associates, Beltsville, MD) for measurement of chemiluminescence signal in millivolts.

Additional membranes were tested to determine an initial level of sensitivity for the detection of SEB in PBST. Following the same capture and detection procedure above, membranes were tested at the 0 and 1 ng/mL level of SEB concentration in PBST.

2.5. Statistical analysis

Statistical analysis was performed on the capture studies using the mixed model analysis of variance procedure of Statistical Analysis Systems software (SAS Institute, Inc., Cary, N.C.). The fixed effects in the model statement included the treatment (with and without avidin) and concentration of SEB in ng/mL ($n = 3$ per each concentration of SEB tested). Significant differences ($P < 0.05$) in least square means of signal emitted from the chemiluminescence reaction were determined by the least significant difference test. Sensitivity testing data was analyzed to determine significant differences between the treatments ($n = 9$ per each concentration of SEB tested) 0 and 1 ng/mL of SEB using the mixed model analysis of variance procedure of SAS and differences were determined by least significant difference test. The fixed

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effects in the model statement included repetition and concentration of SEB in ng/mL. Confidence levels for limits of detection were set on a signal to noise ratio of 2 standard deviations higher than the average of the control signal.

3. Results and discussion

3.1. Aminated electrospun membranes

Results from aminated pellethane electrospun membranes are shown in Fig. 3. The amines associated with the membranes were labeled with fluorescein isothiocyanate and monitored over time for relative amounts of amines present. The graph shows fluorescein counts corresponding to overall concentrations of polyamine co-electrospun with the pellethane polymer. Due to water solubility of the polyamine polymer, there was concern that the amines would not be part of the membrane under aqueous conditions needed for covalent molecular recognition attachment. The membranes were washed with water over time and the primary labeled amines associated with the nanofibrous membrane were monitored. Results show that fluorescein counts differed significantly with the amounts of polyamine added to the initial spin dope solution prior to electrospinning. Washing over time did seem to have a small effect for removal of fluorescein but the majority of initial amine concentration was still present. These results show ability to control the amount of functional groups within the electrospun fibrous membrane by varying the initial concentration of the amine polymer added. This could allow one to "tailor" the process of membrane fabrication according to what is needed for biomolecule attachment.

3.2. Carboxylated electrospun membranes

The PVC–COOH polymer was studied due to the known presence of carboxyl groups available for attachment of molecular recognition chemistries. These membranes with specific antibodies attached could then be used to demonstrate the capture and concentration concept in the electrospun membrane configuration described in this paper. Fig. 4 illustrates the antigen/antibody experiment that was performed on the electrospun membranes to demonstrate the capture specificity potential of the membrane. Different primary antibodies were attached by EDC and sulfo–NHS crosslinkers to separate membranes. The carboxylated membranes possessing the different primary antibodies were then both reacted in exactly the same manner to the secondary HRP labeled antibody. The two membranes on the left side (Fig. 4) are the results of the electrospun membranes to which Rabbit anti-Staph antibody was attached. This antibody was used as the control since it is not the complement to the secondary HRP labeled antibody. As would be expected, there was only a slight chemiluminescent signal produced which may have been due to non-specific binding of the antibody to the membrane. In contrast to the control, the results of the membrane to which Goat anti-Rat were attached had a strong chemiluminescent signal produced, demonstrated by the two membranes on the right side (Fig. 4) marked as experimental. These membranes contained the complement antibody to the secondary HRP labeled Rabbit anti-Goat antibody. The results from this experiment demonstrated the capability of utilizing the electrospun membrane architecture for specifically capturing antigens from solution.

Shown in Fig. 5 are the results of the avidin/biotin binding studies conducted on PVC–COOH electrospun membranes. The results indicate that the biotin–fluorescein conjugate does not strongly associate when avidin is not chemically crosslinked to the electrospun membranes. In addition, the biotin–fluorescein molecule does not adsorb or "stick" by itself to the membrane as well. This indicates that little or no nonspecific binding occurred with the avidin/biotin coupling strategy. With this result in mind we adopted the biotin/avidin scenario for the SEB toxin

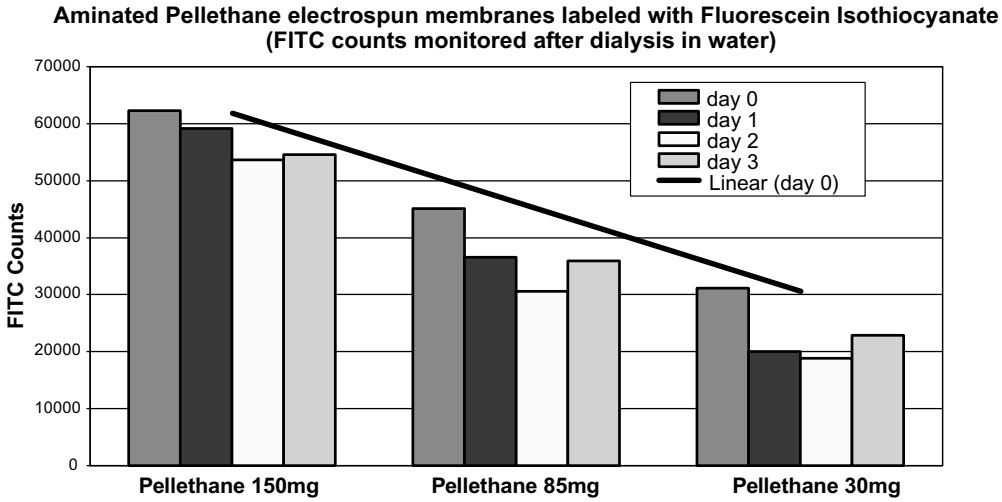

Fig. 3. Shows graph of fluorescein labeled free amines associated with the electrospun membrane. Membranes were washed with milli-Q water and measured on the STORM 860 for fluorescence. Milligram amounts correspond to the dry weight of the polyamine polymer added to a 10% Pellethane spin dope prior to electrospinning.

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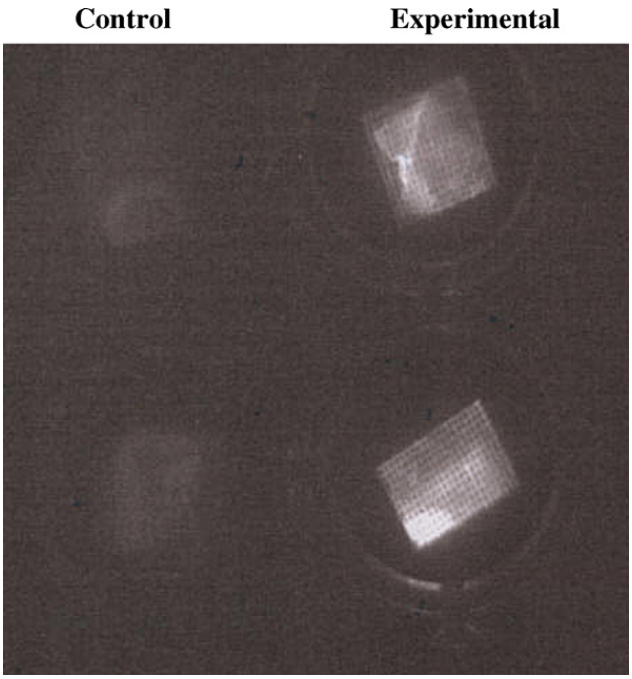

Fig. 4. Crosslinkers: EDC and sulfo–NHS; control: primary attached antibody = Rabbit anti-*staph*; experimental: primary attached antibody = Goat anti-Rat secondary label antibody = Rabbit anti-Goat HRP conjugate.

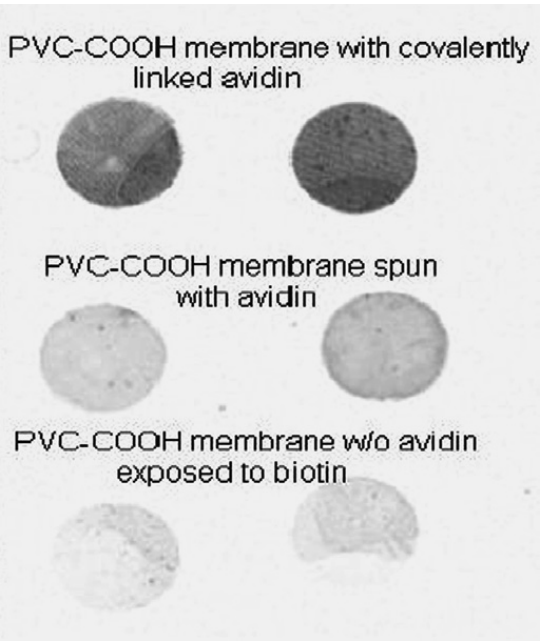

Fig. 5. Avidin/biotin studies conducted on PVC–COOH electrospun membranes.

study looking to minimize the occurrence of nonspecific binding.

The PVC–COOH membranes used in the SEB modified ELISA sandwich assay [15] study were produced at a diameter to fit the well of a standard 96 well microtiter plate (0.5 cm). One set of membranes had avidin chemically crosslinked to the membrane coupled with biotinylated anti-*staph* antibodies. The control membranes used in this study were electrospun PVC–COOH without avidin and the biotinylated antibody. Both membrane sets were reacted in similar fashion. The controls for both membrane sets were only exposed to the anti-*staph* HRP labeled antibodies. All test samples were first reacted with known concentrations of SEB and then the secondary HRP labeled antibody. The test was designed to see if the electrospun membranes with antibodies specific for SEB could bind, capture and detect the toxin. Data in Table 1 demonstrates the capability of the membrane configuration to potentially capture down to a 1 ng/mL concentration level of SEB under the current assay conditions. This was concluded based on the 1 ng/mL sample having an average of more than 2 standard deviation greater than the average of the control ($n = 3$). However, the detection of 1 ng/mL was not statistically significant ($P > 0.05$) in these series of experiments due to high within sample variability. SEB concentrations greater than 10 ng/mL, were statistically significant ($P < 0.05$) in this study for detection of the toxin.

Although the detection of 1 ng/mL was not statistically significant in the first series of experiments (Table 1), it was later determined in follow on studies by increasing the number of membranes replicates at the 1 ng/mL toxin level in PBST, statistical significance could be achieved using this detection scenario. Further studies (Table 2) were conducted to determine the potential for detecting SEB at concentrations as low as 1 ng/mL in PBST. These studies, conducted on three separate days, confirmed that 1 ng/mL concentrations were detected and significantly different ($P < 0.05$) than 0 ng/mL back ground signal. However, using the nanofibrous membrane configuration significant variability existed between experimental days due to membrane differences and dark noise differences of the A5-2021A Lumi-Tec luminometer. This generated higher than expected experimental error when averaged across the 3 experimental days ($n = 9$). Within each day, the experimental error was low across the replicates, indicating membrane difference per batch. Issues like these will be addressed during future optimization studies involving the nanofiber membrane biosensors.

Table 1
Membranes with avidin linker and biotinylated antibody

SEB (ng/ml)	Millivolts Avg.	Standard error of the mean	LOD (mV)	Detection of SEB
0	0.67	0.15	1.17	
1	2.47	0.57		Yes
10	10.63	2.00		Yes
100	96.23	8.55		Yes

Table 2
Multiple day studies of membranes sensitivity at 1 ng/ml SEB concentration in PBST

SEB (ng/ml)	Millivolts Avg.	Standard error of the mean	LOD (mV)	Detection of SEB
0	15.01	5.00	57.00	
1	60.10	28.33		Yes

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Table 3
Membranes without avidin linker and biotinylated antibody

SEB (ng/ml)	Millivolts Avg.	Standard error of the mean	LOD (mV)	Detection of SEB
0	0.73	0.33	1.36	
1	0.86	0.03		No
10	0.30	0.15		No
100	0.13	0.06		No

Another important detail identified in the study was that both the toxin and the secondary antibody did not bind non-specifically to the nanofibrous surface of the electrospun membrane, Table 3. Even at the highest concentration of SEB toxin, 100 ng/mL, there was no significant difference in chemiluminescent signal over background produced by the membranes without the biotinylated antibody specific for SEB toxin. This investigation showed primary biotinylated antibody was necessary in order to bind the SEB toxin on the electrospun membrane, and the signal to be generated upon the binding event of the HRP labeled antibody to the toxin.

4. Conclusions

A nanofibrous membrane concept was developed to specifically concentrate and detect the analyte of interest from extracted and dilute samples. Generating high surface area membranes through the use of electrospinning, unique polymeric membranes were formulated with defined functionalities that allow covalent attachment of molecular recognition elements for the selective capture of target analytes. The high surface area membranes could improve the sensitivity needed for sensor development increasing the probability of finding and detecting analytes present at low concentrations.

Studies shown in this paper indicate polyurethane membranes can be tailored to include water-soluble polyamine, providing the necessary functional primary NH_2 for attachment of biomolecules. Fluorescence measurements show primary amines can be added in a concentration dependent manner to the spin dope prior to membrane formation. Electrospun PVC–COOH membranes used for the biomolecule attachment studies, through covalent chemistries to include antibodies and the avidin/biotin coupling strategy, have shown conclusively that sensor elements can be linked to the membrane and that one can design the membranes according to detection needs. Immunoassays using antibodies demonstrated potential for antigen specificity. The modified ELISA sandwich assay used in the SEB toxin studies demonstrated the capture of toxin down to the 1 ng level.

Functionalizing the surface of electrospun fibers provides extensive possibilities in designing the high surface membranes with a variety biological capture and detection possibilities to include multiplexing capabilities by arranging multiple molecular recognition elements on a single electrospun membrane. Exposure of electrospun capture membranes to larger sample volumes provides potential for increasing the sensitivity for detection of biological agents when present in very low or dilute concentrations as might be found in environmental and/or food detection scenarios. The electrospinning method provides a process for developing both capture and detection methods within the same platform.

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