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Surface functionalization of electrospun nanofibers for detecting *E. coli* O157:H7 and BVDV cells in a direct-charge transfer biosensor

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ABSTRACT

Electrospinning is a versatile and cost effective method to fabricate biocompatible nanofibrous materials. The novel nanostructure significantly increases the surface area and mass transfer rate, which improves the biochemical binding effect and sensor signal to noise ratio. This paper presents the electrospinning method of nitrocellulose nanofibrous membrane and its antibody functionalization for application of bacterial and viral pathogen detection. The capillary action of the nanofibrous membrane is further enhanced using oxygen plasma treatment. An electrospun biosensor is designed based on capillary separation and conductometric immunoassay. The silver electrode is fabricated using spray deposition method which is non-invasive for the electrospun nanofibers. The surface functionalization and sensor assembly process retain the unique fiber morphology. The antibody attachment and pathogen binding effect is verified using the confocal laser scanning microscope (CLSM) and scanning electronic microscope (SEM). The electrospun biosensor exhibits linear response to both microbial samples, *Escherichia coli* O157:H7 and bovine viral diarrhea virus (BVDV) sample. The detection time of the biosensor is 8 min, and the detection limit is 61 CFU/mL and 10^3 CCID/mL for bacterial and viral samples, respectively. With the advantage of efficient antibody functionalization, excellent capillary capability, and relatively low cost, the electrospinning process and surface functionalization method can be implemented to produce nanofibrous capture membrane for different immuno-detection applications.

1. Introduction

Nano-structured materials, such as nanowires and nanofibers, which have unique biochemical properties, are promising candidates to fabricate biosensors with high sensitivity, rapid response and low cost. Biocompatible materials, such as nitrocellulose, polyvinylidene fluoride, and polyethersulfone, are known for their excellent binding ability to separate and capture biomolecules and pathogenic cells from biological samples. Based on these unique biochemical properties, low cost and high sensitivity biosensors have been developed based on immunochromatography and immunoassay for detecting infectious disease organisms (Lin et al., 2008), analyzing drugs of abuse (Zhang et al., 2006), veterinary testing (Beck et al., 2000), agricultural applications, environmental testing, and product quality evaluation (Wong and Tse, 2008). In recent years, many technological advances have been directed towards the development of nano-structured biosensors to obtain enhanced surface-area-to-volume ratios and reduced cross-sections, offering more effective bio-target immobilization capabilities (Basu et al., 2004; Mehdinia et al., 2009). Most development efforts are focused on conventional planar structured mesoporous layers.

New type of sensor material with one-dimensional (1D) structure, such as carbon nanotubes and metal oxide nanowires, has been introduced which provide unparalleled fast mass transfer capability of analyte molecules (Claussen et al., 2009). The 1D nanostructure has exceptional advantages in surface to volume ratio which determines the binding effect and sensor response. However, sensors based on single nanowire suffer from inherent statistical variation which induces sensing property deviation and excessive noise levels. Instead of random approach, sensor structure with multiple nanowire networks has the advantages of high reproducibility, low noise, and high reliability.

Electrospinning is a versatile and cost effective method to produce nanofibrous membranes with uniform fiber diameters in nanoscale (Wang et al., 2002). The process is based on the principle of electrostatic to draw ultrafine solid threads from material solutions, which does not require coagulation chemistry or high

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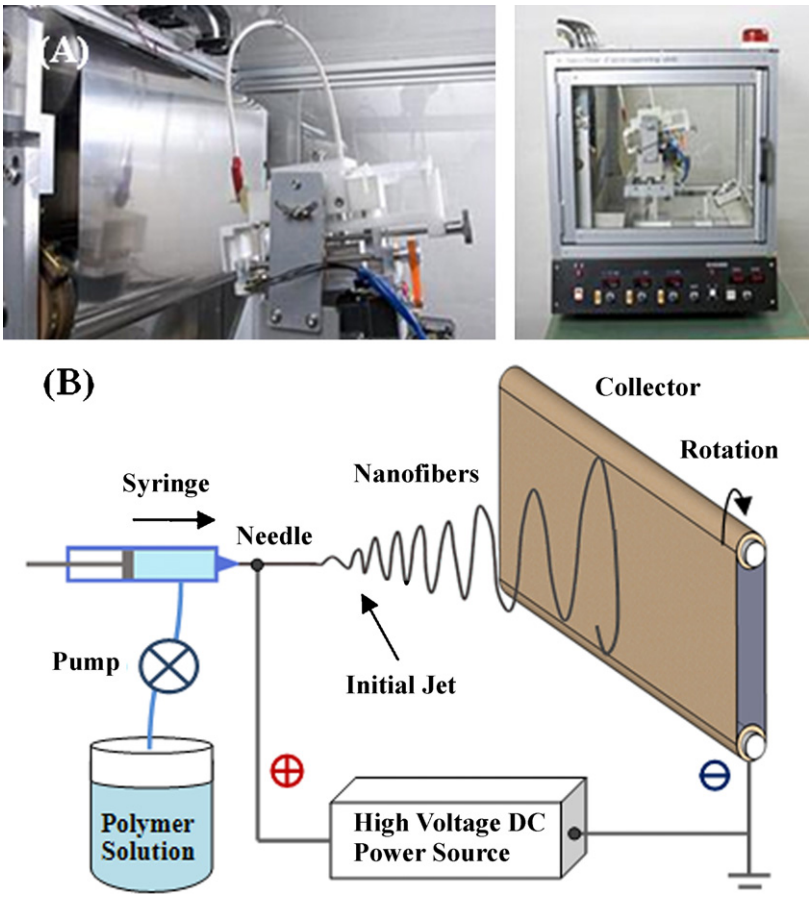

Fig. 1. (A) The Nanofiber Electrospinning Unit (NEU, Kato Tech Co., Japan) uses a rotating drum as collector for high yield fiber production, (B) schematic diagram of electrospinning system for nanofibrous membrane fabrication.

temperatures. This non-invasive method is particularly suited to fabricate nanofibers using biological materials. The surface area is significantly increased by the novel nanostructure compared to conventional planar materials, which enhance binding effect and reaction rate (Huang et al., 2003). Moreover, the electrospinning process provides many attractive advantages to develop high performance nano-structured materials for biomedical and biosensor applications, including inherent stability, compatibility with micro-fabrication process, high production yield and relatively low cost (Kim et al., 2006). The electrospun nanofiber with biomimetic structures has been developed in different morphologies for tissue engineering (Li et al., 2006), drug delivery (Zeng et al., 2003), and artificial organ implant applications (Venugopal et al., 2008). The excellent binding capability improved the performance in molecular absorption (Nama et al., 2009) and cell adhesion (Hou et al., 2009). In recent years, more research efforts have been directed towards biosensor applications, such as biomolecular detection and enzyme functionalization (Wang et al., 2009; Ren et al., 2006; Sawicka et al., 2005). However, its application in whole cell detection of microbial or viral pathogens has not been reported in the literature.

This paper presents electrospinning method to produce nitrocellulose nanofibrous membrane, and its optimization and functionalization with biological treatment for biosensor applications. A biosensor based on the electrospun capture membrane is designed by integrating magnetic separation, capillary immunoassay, and direct-charge electrical measurement for rapid and quantitative detection of bacterial and viral pathogens. In this experimental study, the process condition and parameters of electrospinning and biological functionalization were optimized to improve capillary action and biomolecular capture capability to achieve enhanced sensor response and sensitivity. The electrospun nanofibrous membrane was synthesized from nitrocellulose polymer solution with ultrafine fiber diameter around 150 nm. And surface functionalization method was developed and optimized according to the specific pathogen target. The high surface area of the material allowed more biological events to occur and facilitated assay kinetics. The electrospun biosensor was tested using both bacterial and viral samples to verify the binding and separation performance of the bio-modified nanofibrous membrane.

2. Materials and methods

2.1. Electrospun material synthesis

The electrospun nanofibrous membrane was fabricated using the Nanofiber Electrospinning Unit (NEU, Kato Tech Co., Japan), which is shown in Fig. 1A. The electrospinning process uses the principles of electrostatic generated by high voltage power source between the needle spinneret and collector to produce its fine polymer jet from polymer or composite material liquid (Fig. 1B).

The electrospun material was fabricated using nitrocellulose polymer due to its excellent biocompatibility and solubility in common solvents. The electrospinning process conditions were optimized and controlled by the self-contained NEU in order to generate ultrafine nanofibers. The nitrocellulose polymer of 8 wt% was dissolved in a mixed solvent system containing tetrahydrofuran (THF) and dimethylformamide (DMF) of 60:40, which provided optimum viscosity and surface tension for electrospinning (Nartker et al., 2005). A 20-mL syringe was used to extrude the polymer

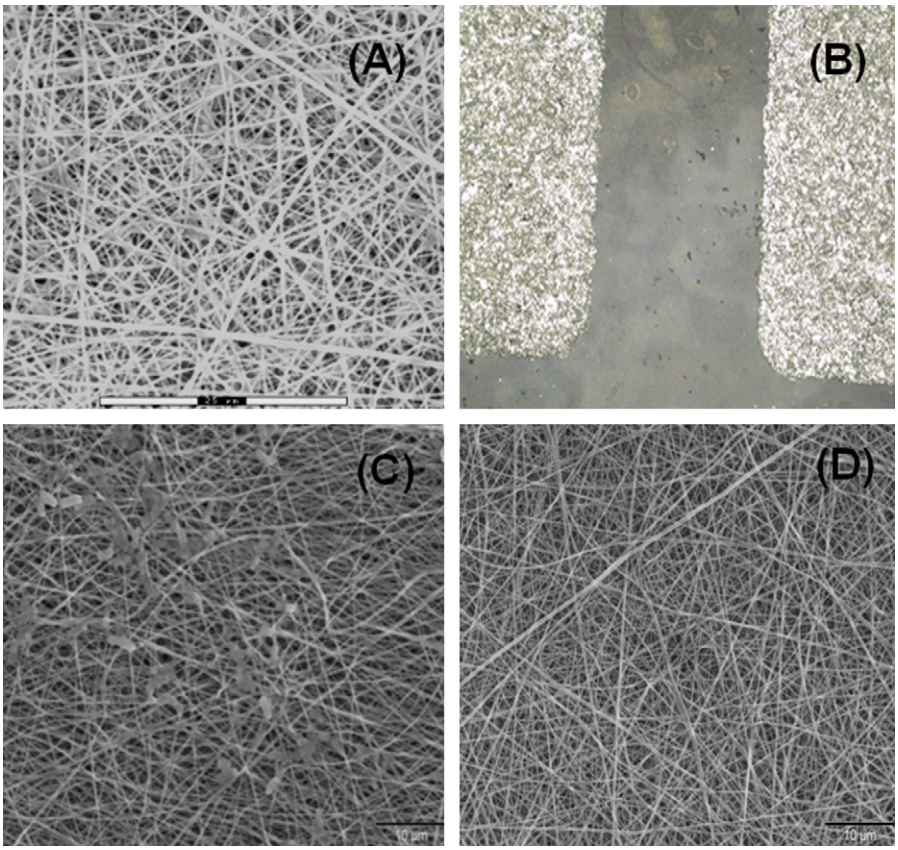

Fig. 2. (A) Scanning electron microscopy image of electrospun nitrocellulose nanofibers and (B) silver electrodes fabricated on the electrospun membrane using spray deposition method. SEM image of electrospun samples after bacteria test (C) *E. coli* O157:H7 effectively captured on functionalized fiber mat, (D) no bacteria are observed in the nanofiber mat without treatment.

solution at 0.2 mL/h via an 18-gauge needle. The applied voltage between the needle and collector was tuned to be 10 kV at a distance of 6 cm. The polymer solution was electrospun on polyvinylidene chloride (PVDC) substrate into nanofibrous unwoven membrane. The quality of fiber sample was observed using a scanning electron microscope (SEM) as shown in Fig. 2A. This electrospinning fabrication system yielded reproducible nanofibrous membrane which consisted of defect-free smooth fiber with uniform diameter of approximately 150 nm.

In order to enhance capillary performance, the electrospun nanofiber mat was plasma treated using 120 W RF generated O_2 plasma at 13.6 MHz with O_2 at 250 mTorr to remove the surface nitrate groups, which was verified using XPS spectrum analysis. After plasma enhancement, the material becomes more hydrophilic which was verified by capillary contact angle test. The contact angle between water droplet and nanofiber mat was improved from 135° to 56° after the plasma treatment.

2.2. Sensor architecture and detection principle

A conductometric lateral flow biosensor based on the electrospun material was designed to consist of three porous membranes: application pad, capture pad, and absorption pad (Pal et al., 2007). The electrospun nitrocellulose mat was implemented as the capture pad due to its capillary properties. The fiber surface was bio-modified for antibody attachment using glutaraldehyde as cross-linker. A pair of electrodes was fabricated on the capture membrane 0.5 mm apart using a spray deposition of silver paint as shown in Fig. 2B. The chemical composition was carefully chosen to prevent the erosion of nanofibers. The application pad was used to control the flow of the sample onto the capture pad. The excess solution was absorbed in the absorption pad which modulated the capillary action. The cellulose membranes with flow rate of 180 mL/min (Millipore, MA, USA) were used for the application and absorption pads due to their excellent filtration and sopping properties (Quadrat et al., 1998). After cleaning with sterilized and deionized (DI) water, the application and absorption pads were cut into 7 mm × 5 mm and 10 mm × 5 mm, respectively. The capture membrane had a dimension of 23 mm × 5 mm which was optimized according to sample volume and flow rate. The overall dimension was 40 mm × 5 mm. Finally, the biosensor was assembled by attaching the three membrane pads onto the PVDC substrate via polystyrene adhesive backing as shown in Fig. 3A.

The pathogen detection principle of the biosensor is demonstrated in Fig. 3B. The antibody modified conductive magnetic nanoparticles are mixed and incubated with the test sample for target conjugation. After magnetic separation (Pal and Alocilja, 2009), the purified sample with the conductive label is dispensed on the application pad. The porous structure modulates the flow and filters large particle size impurities. By capillary action, the electrospun capture pad tethers the target pathogen onto the immobilized secondary antibody and forms a sandwich complex, which accumulates to produce an electron transport path across the silver electrodes. Unbound materials are subsequently separated into the adsorption pad by capillary flow. After capillary flow equilibrium, the direct-charge transfer between the electrodes is proportional to captured sandwich complex which can be used to determine the pathogen concentration.

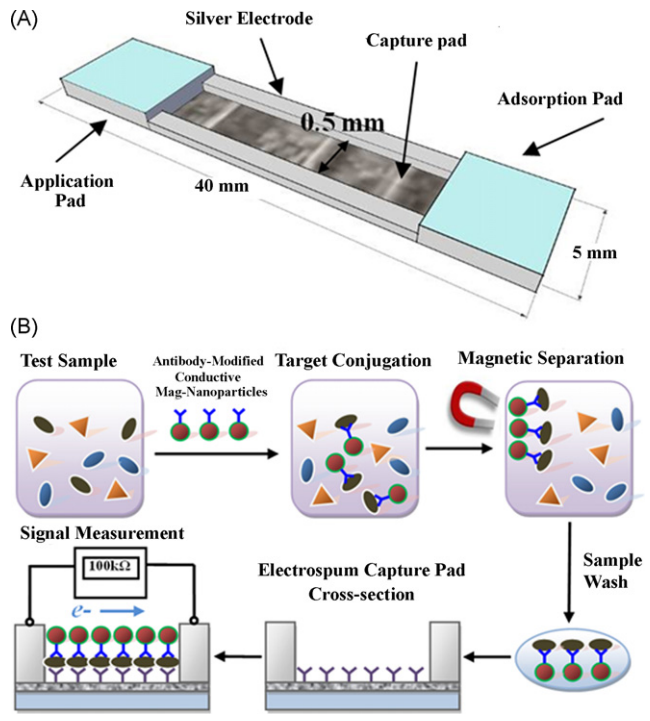

Fig. 3. (A) Schematic of the biosensor structure and membrane assembly consisting of cellulose application and absorption pads and electrospun cellulose nitrate capture pad. (B) Detection scheme of the lateral flow immunosensor based on the antibody functionalized electrospun capture membrane.

2.3. Test pathogens and antibodies

A pure culture of *Escherichia coli* O157:H7 was obtained from the collection of the Nano-Biosensors Laboratory (Department of Biosystems and Agricultural Engineering, Michigan State University). *E. coli* O157:H7 cells were inoculated using sterile loop into 10 mL of Tryptic soy nutrient broth from Difco Laboratories (Detroit, MI) and incubated for 24 h at 37 °C to make a stock culture. The 24-h culture was serially diluted in 0.1% peptone water in logarithmic scale to obtain different concentrations (10^1 to 10^7) in colony forming units per milliliter (CFU/mL). Each dilution was used as a sample during the succeeding experiments. Samples of bovine viral diarrhea virus (BVDV) in serum were collected from BVDV-infected cattle maintained by the MSU Large Animal Clinical Sciences. These BVDV samples were stored at −80 °C before use. A BVDV test solution was thawed and serially diluted four times to get 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions just prior to use. The antibodies used for BVDV biosensor were affinity purified mouse anti-BVDV 15c5 (gp48) monoclonal antibody (Ed DuBovi, Cornell University, Ithaca, NY) and purified swine anti-BVDV polyclonal antibody (USDA: NADL, Ames, IA). The *E. coli* O157:H7 biosensor applied affinity purified goat anti-*E. coli* O157:H7 polyclonal antibody (KPL, Inc., Gaithersburg, MD), and purified mouse anti-*E. coli* O157:H7 monoclonal antibody (Meridian Life Science, Inc., Saco, ME). All of the experiments were performed in a certified Biological Safety Level II laboratory.

2.4. Surface functionalization

To enhance protein binding and reduce pH effect, glutaraldehyde ($C_5H_8O_2$) was used as a cross-linking agent to attach the antibody to the electrospun fiber mat. The concentration was optimized to be 20 μL of 0.1% glutaraldehyde solution per 1.0 in^2 of fiber mat. After soaking with PBS buffer, 0.5 mg/mL polyclonal antibodies (affinity purified goat anti-*E. coli* O157:H7 polyclonal antibody or purified swine anti-BVDV polyclonal antibody) were dispensed on the electrospun membrane with concentration of 10 μg/in^2 and incubated at 25 °C for 30 min. The process was completed after the wash step using Tris buffer with 0.1% Tween-20 to remove excess antibody. The functionalized nanofiber mat was stored at 4 °C in a refrigerator until needed. The antibody attachment protocol was verified using confocal laser scanning microscopy (CLSM) to measure the amount of emission of fluorescent antibodies. The significant emission at 530 nm confirmed the efficient antibody attachment on the electrospun mat. And the novel nanostructure by electrospinning method was retained after the surface functionalization.

2.5. Immunomagnetic concentration

The electrically conductive magnetic nanoparticle was prepared using 100 μg of magnetic iron oxide (Fe_2O_3) and polyaniline (PANi) conjugate, and dispersed in 1 mL of phosphate buffer solution (PBS) after sonication for 10 min (Pal and Alocilja, 2009). A volume of 100 μL of 0.15 mg/mL monoclonal antibodies (purified mouse anti-*E. coli* O157:H7 monoclonal antibody and affinity purified mouse anti-BVDV 15c5 (gp48)) in PBS was applied and incubated for 1 h in a hybridization oven at 30 rpm at 25 °C. The obtained antibody functionalized magnetic nanoparticle (AFMN) was vortex mixed and placed in a magnetic separator for 2 min. The supernatant was discarded using a micropipette. Then 1 mL of pathogen solution obtained by serial dilution was added and incubated in a hybridization oven at 30 rpm at 25 °C for 30 min. After three magnetic separation rinses (by adding 1 mL of Tris buffer solution with 0.1% casein and removing the supernatant), the magnetic material which captured the target pathogen was re-suspended in 1 mL PBS. All the test solutions with different concentrations were processed by magnetic separation for purification and conductance labeling, and stored at 4 °C until needed.

2.6. Detection and data analysis

The biosensor signal was measured via a data acquisition (DAQ) system (Model NI USB-6281, National Instrument, Austin, TX) which was connected to the biosensor strip. The DAQ system was linked to a computer with USB interface and controlled by LabVIEW software. For each sample, a volume of 100 μL of the test solution (200 μL for BVDV) was applied to the biosensor and the resistance signal across the silver electrodes was measured by the DAQ system. When capillary flow equilibrium was achieved at around 8 min, the biosensor resistance was recorded to determine pathogen concentration. For data analysis, a minimum of three replications were performed for each experiment. All biosensors were calibrated using a control sample which consisted of the AFMN nanoparticles suspended in sterile DI water. Standard deviations and mean values for the data of each experiment were calculated. Statistical analysis was performed based on a single factor analysis of variance using ANOVA.

3. Results and discussion

The effect of surface functionalization method was verified using a confocal laser scanning microscopy (CLSM). Fluorescein isothiocyanate (FITC) conjugated antibody was functionalized on the electrospun nanofibers using a bio-modification process described above. After several wash steps to remove unbound antibodies, the CLSM was used to observe the antibody attachment by measuring the laser excited fluorescein emission at 530 nm (Fig. 4A). The untreated nanofiber was also analyzed to compensate background noise generated by the substrate and fibers (Fig. 4B).

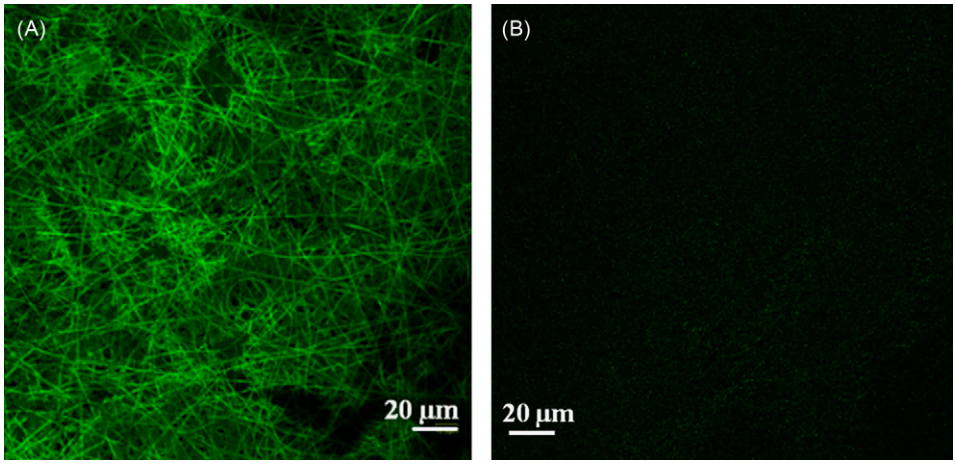

Fig. 4. The CLSM image of functionalized membrane using FITC antibody: (A) CLSM image of nanofibers with FITC Ab functionalization and (B) CLSM image of nitrocellulose nanofibrous membrane without antibody. Significant fluorescence emission at 530 nm verifies the antibody immobilization.

The sensing condition and process parameters were optimized to improve sensitivity. The solution pH affects protein solubility, which affects antibody attachment and pathogen conjugation (Pal and Alocilja, 2009). The chemical composition of the assay was optimized using Tris buffer to sustain a neutral environment to facilitate biochemical reaction and reduce background noise (Tahir and Alocilja, 2003; Kim et al., 2000). Due to high surface area and low cross-section, the electrospun mat significantly improved the impurity separation and pathogen binding event. The applied antibodies for electrospun membranes were optimized to be 10 $\mu g/in^2$, which is more cost effective compared to conventional planar material which uses around 50–500 $\mu g/in^2$.

The bacteria binding of the bio-modified electrospun nanofibrous membrane was confirmed using the SEM. Lateral flow tests of *E. coli* O157:H7 bacteria were performed on the electrospun membrane samples with and without bio-modification. Fig. 2C shows that the target pathogen could be effectively immobilized on the bio-modified membrane. The untreated membrane does not contain any organism after rinsing (Fig. 2D). Furthermore, the SEM image verified that the morphology of nanofiber networks was retained after surface functionalization and capillary action.

The sensitivity and performance of the electrospun biosensor was verified by experiments using *E. coli* O157:H7 and BVDV in varying concentrations. Fig. 5 demonstrates the time response of sensor conductance signal for different concentrations of *E. coli* O157:H7 samples. During the initial time around 50 s, the conductance signal increases with fluctuations as the antigen–polyaniline complex flows along the capture pad. The immunoreactions tether the polyaniline coated nanoparticles to form conducting bridges between the silver electrodes. After the flow reaches the absorption pad, the sensor conductance gradually decreases as the excess reagent are separated and absorbed by capillary action. When absorption achieves equilibrium after 8 min, the conductance signal becomes stable and is suitable for sensor reading. The conducting bridges formed by captured sandwich complex are proportional to the pathogen concentration which is confirmed by the conductance measurement shown in the figure below.

The pathogen detection results are illustrated in Fig. 6. The electrospun biosensor demonstrated linear sensing response for different *E. coli* O157:H7 concentrations from 0 to 10^4 CFU/mL. Its sensitivity (detection limit) is down to 61 CFU/mL. The biosensor was also tested using BVDV sample. The results are shown in Fig. 6b. The estimated original viral concentration is 10^6 CCID/mL. The detection limit is its 10^{-3} dilution, which is estimated at 10^3 CCID/mL, which is equivalent to 1/1000 of virus in the blood serum of infected bovine (Brock and Chase, 2000).

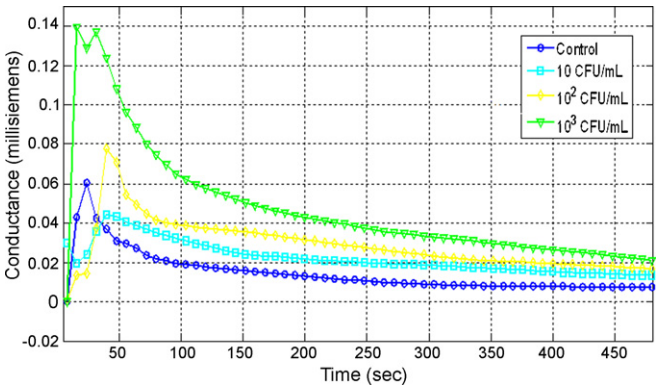

Fig. 5. Biosensor conductance versus test time for different target concentrations of *E. coli* O157:H7 bacteria samples.

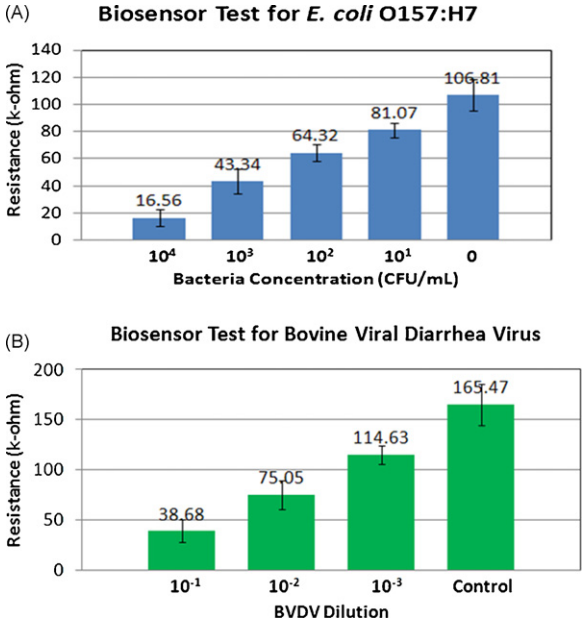

Fig. 6. (A) Biosensor test results for *E. coli* O157:H7 bacteria demonstrate the linear sensor response and lower detection limit of 61 CFU/mL. (B) Biosensor test results for BVDV virus demonstrate the linear sensor response.

The functionalized electrospun biosensor exhibited higher sensitivity and wider linear response for pathogen detection. To compare the detection performance, biosensors using nitrocellulose nano-porous membrane (Millipore, MA, USA) were fabricated following the functionalization and assembly process. The linear detection range of *E. coli* O157:H7 using the conventional biosensor was from 0 to 10^2 CFU/mL. Due to the unique porous nanostructure and high surface area, the nitrocellulose electrospun mat of the electrospun biosensor provided faster mass transfer rate and more capillary channel, which enhanced the immunoreaction rate and separation effect. Compared to the conventional mesoporous membranes, the contact area of the active mass transfer region of the nanofibrous membrane and the underlying substrate was greatly reduced. This substantially reduced the background noise in direct-charge measurement caused by stability problems related to electronic charge interactions between substrate and sensor material.

4. Conclusion

An electrospun biosensor based on immunochromatography, nanoparticle based magnetic separation, and immunoassay was designed and optimized for rapid and sensitive detection of microbial and viral pathogens. Due to the unique nanostructure and biocompatibility of electrospun nitrocellulose membranes, the biosensor had linear detection response for *E. coli* O157:H7 and BVDV virus samples. In an 8 min detection process, sensitivity of the portable and low cost biosensor was 61 CFU/mL and 10^3 CCID/mL for *E. coli* O157:H7 and BVDV, respectively. The functionalization process can be extended to other microbial or viral organisms by appropriately changing the antibodies. The electrospinning method and functionalization protocol can be implemented in other biosensor detection mechanisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.08.028.

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