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Electrospin-coating of nitrocellulose membrane enhances sensitivity in nucleic acid-based lateral flow assay

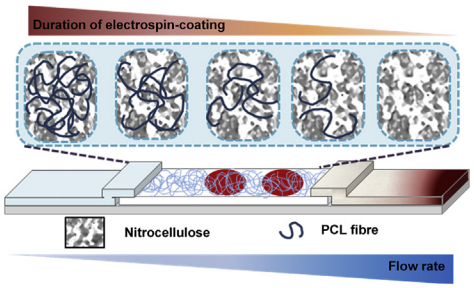
Chee-Hong Takahiro Yew [a, b], Pedram Azari [a, c], Jane Ru Choi [d], Fei Li [b, e, *], Belinda Pingguan-Murphy [a, f, **]

[a] Department of Biomedical Engineering, Faculty of Engineering, University of Malaya, 50603 Kuala Lumpur, Malaysia
[b] Bioinspired Engineering and Biomechanics Centre (BEBC), Xi'an Jiaotong University, Xi'an 710049, PR China
[c] Centre of Applied Biomechanics, Department of Biomedical Engineering, Faculty of Engineering, University of Malaya, 50603 Kuala Lumpur, Malaysia
[d] Department of Mechanical Engineering, University of British Columbia, 2054-6250 Applied Science Lane, Vancouver BC V6T 1Z4, Canada
[e] The Key Laboratory of Biomedical Information Engineering of Ministry of Education, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an 710049, PR China
[f] Centre for the Initiation of Talent and Industrial Training (CITra), University of Malaya 50603 Kuala Lumpur, Malaysia

HIGHLIGHTS

- Electrospin-coating PCL onto nitrocellulose membrane of LFA can control the flow rate.
- Electrospin-coating increases sensitivity of nucleic acid-based LFA by 10-fold.
- A proof of concept shows potential in highly sensitive detection of viral nucleic acids.

GRAPHICAL ABSTRACT



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ABSTRACT

Point-of-care biosensors are important tools developed to aid medical diagnosis and testing, food safety and environmental monitoring. Paper-based biosensors, especially nucleic acid-based lateral flow assays (LFA), are affordable, simple to produce and easy to use in remote settings. However, the sensitivity of such assays to infectious diseases has always been a restrictive challenge. Here, we have successfully electrospun polycaprolactone (PCL) on nitrocellulose (NC) membrane to form a hydrophobic coating to reduce the flow rate and increase the interaction rate between the targets and gold nanoparticles-detecting probes conjugates, resulting in the binding of more complexes to the capture probes. With this approach, the sensitivity of the PCL electrospin-coated test strip has been increased by approximately ten-fold as compared to the unmodified test strip. As a proof of concept, this approach holds great potential for sensitive detection of targets at point-of-care testing.

* Corresponding author. Bioinspired Engineering and Biomechanics Centre (BEBC), Xi'an Jiaotong University, Xi'an 710049, PR China.
** Corresponding author. Department of Biomedical Engineering, Faculty of Engineering, University of Malaya, 50603 Kuala Lumpur, Malaysia.
E-mail addresses: feili@mail.xjtu.edu.cn (F. Li), bpingguan@um.edu.my (B. Pingguan-Murphy).

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1. Introduction

Paper-based biosensors, as miniaturized, low-cost and portable platforms at point of care (POC), have found widespread applications in various fields, including medical diagnosis, food safety assurance, and environmental monitoring [1]. For instance, the lateral flow assays (LFAs) have shown great potential as a more affordable, simpler to use and portable diagnostic platform at POC compared to labour-intensive conventional analytical methods [2], providing a powerful tool for pre-clinical diagnosis to be applied by a non-clinician in resource-limited settings [3]. Compared to lateral flow immunoassays, nucleic acid-based LFAs employ nucleic acids, *i.e.*, aptamer and DNA, are more precise and accurate in detection due to their higher stability [4]. However, the sensitivity of these assays has been a perpetual challenge for low concentration targets in samples (*e.g.*, blood) and thus its use at the POC in critical situations has always been limited [5]. In recent years, similar early symptoms and serological cross-reactivity in pandemic diseases, *e.g.*, dengue and zika fevers, have led to many deaths and birth defects due to delayed diagnosis [6]. The number of zika virus infection cases in Brazil alone was reported as 1,673,272 in 2015 and 2016, which includes 1950 cases of infection-related microcephaly [7]. In this sense, there is still an immense demand for nucleic acid-based LFAs with improved sensitivity for early POC diagnosis and timely medical treatment.

Various methods have been developed to enhance sensitivity of LFAs. Some methods are based on physically concentrating the targets in sample solution before assay, including evaporation [8], isotachophoresis [9] and dialysis-based methods [10]. However, these methods may require sample pre-treatment, additional equipment, or a power supply for the assay. Although optimizing temperature and relative humidity could enhance sensitivity, it is still costly to design and build a portable instrument to serve the purpose [11]. Signal amplification of the assay is another alternative, and may be achieved by modifying the reporter particle or reacting probes [12]. However, this may involve additional amplifying probes or proteins to the signal particles, such as silver deposition on gold nanoparticles (AuNPs) [13], gold accumulation facilitated by oligonucleotides [14] or antibodies [15], enzymatic facilitation [16] or combinations of them. Another strategy for enhancing sensitivity is reducing the flow rate of solution in the assay by modifying the physical architecture of the strip [17], or by adding shunts to the LFA (*e.g.*, a cellulose pad [18], modified glass fibres [19] and sponge [20], wax [21] or polydimethylsiloxane (PDMS) [22] shunts). However, the shunts are generally either sensitive to temperature or require complex fabrication [20,23].

Nanofibrous materials (mostly fabricated by electrospinning) feature high surface-area-to-volume ratio, high porosity, interconnected porous network, and flexible functionality due to the modifiable nature of the polymeric materials [24], which have been widely utilized in biomedical applications [25,26]. Nanofibrous materials have also recently been integrated in biosensors for gaseous [27] and chemical detection [28]. For instance, chemicals and biomolecules have been incorporated in the electrospinning solution or electrospun membrane to produce functionalized fibres that can bind with target molecules like microorganisms, proteins and nucleotides [29–31]. These have been used in the development of biological optical sensors [32], electrochemical sensors [33], enzyme-linked immunosorbent assays (ELISA) [34], and other sensors [35]. Electrospun materials have also been integrated into LFAs to provide the functionalities of binding, absorption and wicking [31,36–38]. For instance, an electrospun polylactic acid (PLA)-biotin nanofiber membrane has been also used as a lateral flow sensing platform for *E. coli* DNA detection via biotin-streptavidin specific binding [36]. A co-polymer electrospinning material has been integrated into LFA, which provides a higher specificity of liposome binding as compared to nitrocellulose (NC) [37]. A bio-resorbable scaffold made of electrospun poly(glycolic acid) has been integrated as a part of a theranostic system combined with LFA to detect wound biomarkers [38]. However, the possibility of incorporating hydrophobic electrospun material into LFAs to control fluid flow for signal enhancement has not been explored yet.

The present study showcases the application of electrospun fibres to a nucleic acid-based LFA as a proof of concept to improve sensitivity. Polycaprolactone (PCL) nanofibers were directly electrospun onto the NC membrane at different duration of time to explore the conditions for improved sensitivity. The results showed that electrospin-coating of NC membrane for 60 s enhances the sensitivity of the nucleic acid based assay by approximately tenfold, compared to the unmodified test strip. This electrospin-coating strategy holds great promise for sensitive detection of various targets at point of care testing.

2. Experimental methods

2.1. Materials

PCL ($M_n = 80{,}000$), gold(III) chloride hydrate, trisodium citrate, sodium dodecyl sulphate (SDS), trisodium phosphate, phosphate buffer saline (PBS) and Tween-20 were all purchased from Sigma Aldrich (USA). N,N-dimethylformamide (DMF) and sodium chloride (NaCl) were procured from Merck, USA. Chloroform was purchased from Friedemann Schmidt. Sucrose and sodium acetate were obtained from Ajax Finechem Pty. Ltd, Australia. Bovine serum albumin (BSA) was acquired from AMRESCO, USA. Streptavidin was purchased from Promega, USA. Saline sodium citrate buffer (SSC × 20) was obtained from Roche Diagnostics, USA. 95% ethanol was acquired from Systerm, Classic Chemical Sdn Bhd, Malaysia. Glacial acetic acid was procured from Fisher Scientific, Malaysia. All solutions were prepared using ultrapure water from Aquinity P30 LS ultrapurification water system (membraPure GmbH, Germany). All the oligonucleotide probes and target were procured from SBS Genetech Co., Ltd (Beijing, PRC). Designation of the sequences for each oligonucleotide can be referred to Table S1 in Supplementary Material. Paper materials included HFB 13500 nitrocellulose (NC) membrane (Millipore, USA), H-1 cellulose pad (JNBio Co., Ltd, Shanghai, PRC) as absorbent pad, Pall 8964 glass fibre (Pall Corporation, Saint Germain-en-Laye) as sample pad and J-B6 backing card (JNBio Co., Ltd).

2.2. Far-field electrospin-coating of NC membrane

A 10% (w/v) PCL solution was prepared in a co-solvent system consisting of 9 parts chloroform and 1 part DMF (9:1 v/v) in a condensed flask under stirring conditions at room temperature to obtain the spinning dope. The electrospinning was carried out using an electric field of 12 kV (Gamma High Voltage Research, Ormond Beach, FL, USA), a blunt 20 G needle (Terumo), needle to aluminium collector distance of 18 cm, while the feeding rate was kept constant at 3 ml/h with a syringe pump (KD Scientific, Inc., Holliston, MA, USA). The NC membranes were taped on the aluminium collector using double-sided tape and electrospun fibres were directly deposited on them at different time duration. The electrospin-coated NC membrane was then left to dry at 37 °C at least overnight before assembly into the LFA test strip. The setup for the electrospinning and the resultant electrospin-coated NC membrane is depicted in Fig. 1A.

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2.3. Preparation of gold nanoparticles (AuNPs) and AuNP-detecting probe (AuNP-DP) conjugates

Preparation of gold nanoparticles (AuNPs) of 13 nm diameter was based on the previous publications with some modifications [14]. To prepare the AuNP-detecting probe (AuNP-DP) conjugates, about 1 mL of the prepared AuNPs was conjugated with the addition of 6 μL of 100 μM detecting probe (DP). After 16 h, the solution was added with 1% SDS and 2M NaCl in an amount that achieved 0.01% SDS and 0.16M NaCl as the final concentration in the solution. The solution was then allowed to age for at least 24 h before being centrifuged at 14000 g for 25 min. Following centrifugation, the pellet was resuspended in 100 μL eluent buffer, comprised of 5% BSA, 0.25% Tween-20, 10% sucrose and 20 nM Na_3PO_4. Then the resulting AuNP-DP conjugate solution was kept at 4 °C before use. The DP was prepared by adding 42.2 μL of 500 mM acetate buffer (pH 4.68) to the lyophilized DP and topped up to 100 μM with ultrapure water.

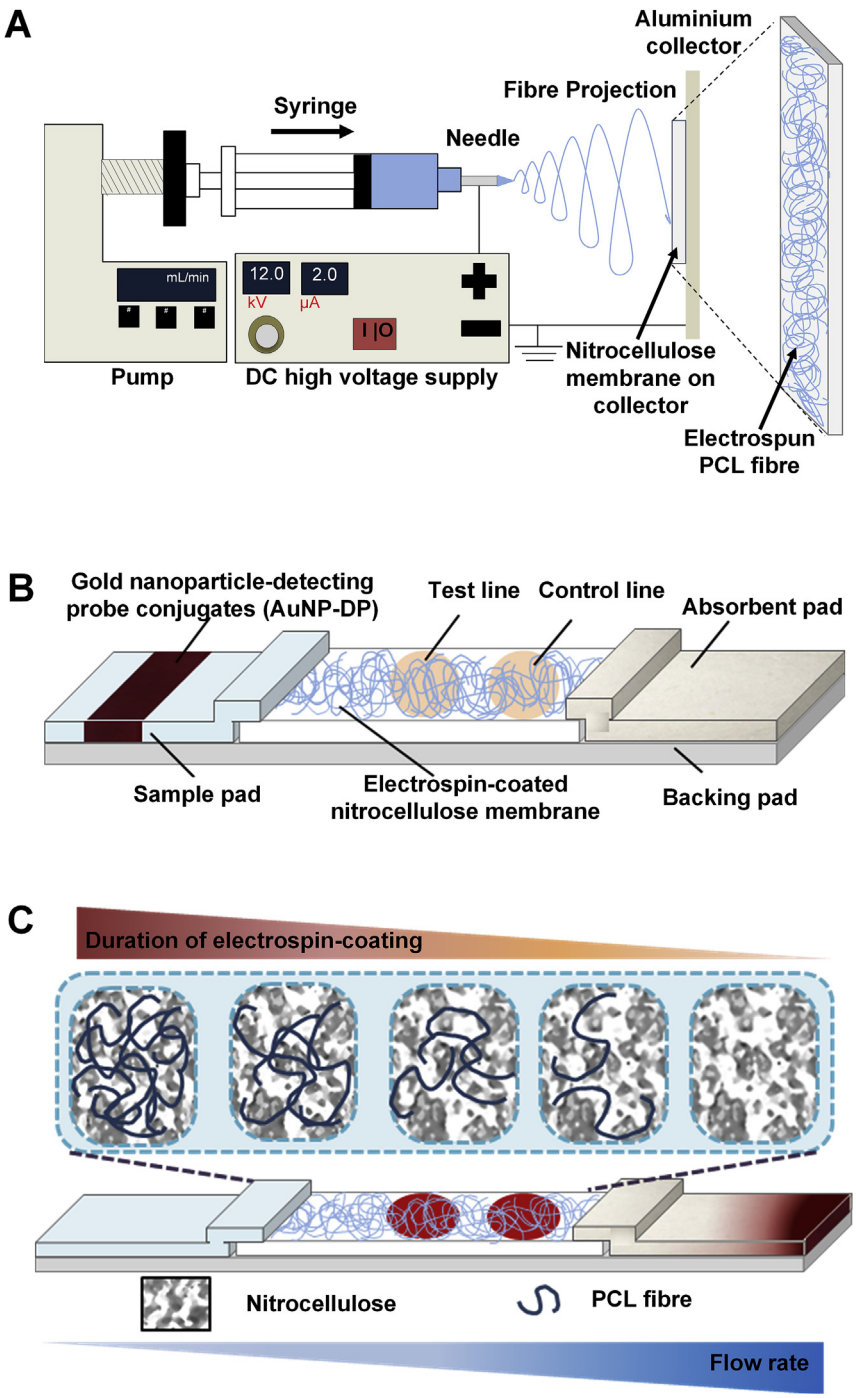

Fig. 1. Schematics of electrospin-coating nitrocellulose (NC) membrane with polycaprolactone (PCL). A) An electrospinning system electrospins PCL fibres that deposit directly onto the NC membrane. B) The PCL electrospin-coated NC membrane is assembled into a conventional lateral flow assay (LFA). C) As the duration of electrospin-coating increases, the density of PCL fibres on the NC membrane increases. This increase of fibre density reduces the flow rate in LFA because of PCL's hydrophobicity.

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2.4. Characterizations of the morphology

The surface morphology of the electrospin-coated NC membrane was examined with a Nikon Eclipse E100 upright microscope (Nikon, Japan) and a field emission scanning electron microscope (FESEM) (Carl Zeiss Auriga, Germany) after sputter-coating with gold. Fibre diameter and distribution were quantified using ImageJ software.

2.5. Characterizations of water contact angle

Due to swift absorption of water on the membrane, we measured water contact angle on electrospin-coated NC membrane by dispensing a 10 μL ultrapure water droplet from a micropipette tip. A video of the whole process of dispensing the water droplet was recorded using a Canon EOS 5D Mark III camera. The moment of the droplet contacting the electrospin-coated NC membrane and leaving the micropipette tip was selected for measurements using VLC video player. The contact angle of the water droplet was analysed using ImageJ software.

2.6. Flow rate measurement

A strip of the electrospin-coated NC membrane (3.0 cm × 0.25 cm) was dipped vertically into a well of 96 well plates containing 20 μL AuNP solution. The time taken for the lateral flow in the strip was observed and measured every 0.5 cm travelled using a CT-20 digital timer (Canon, Japan). A ruler was set at the side as a reference to the travelled distance. The AuNP solution used for this measurement was produced by centrifuging 1 mL as-prepared AuNPs, discarding the supernatant and resuspending the pellet in 100 μL eluent buffer. 500 μL SSC × 4 solution was then mixed with the AuNPs for the flow rate test.

2.7. Fabrication of LFA test strip with electrospin-coated NC membrane

Electrospin-coated NC membrane (2.0 cm × 0.25 cm), sample pad (2.0 cm × 0.25 cm × 0.05 cm) and absorbent pad conjugate pad (2.5 cm × 0.25 cm × 0.1 cm) were tailored using a guillotine paper cutter and assembled together by mounting on a piece of PVC backing card (6.0 cm × 0.25 cm × 0.02 cm), as shown in Fig. 1B. The sample pad was soaked wet in 0.1% of Tween-20 and dried in the oven before assembly. On the test strip, 4 μL of AuNP-DPs were added to the middle of the conjugate/sample pad while 0.5 μL of control probes and capture probes were dispensed onto the NC membrane using a micropipette to produce a control line and a test line, respectively. The test strip was then dried under 37 °C for at least 30 min before using. The control probe was prepared by dissolving 2 mg streptavidin in 500 μL of 10 mM PBS. The capture probe was prepared by adding 269.5 μL of the 2 mg/mL streptavidin-PBS solution and 47.1 μL of 10 mM to the capture probe. The solution was then left for 1 h before 36.2 μL 95% ethanol was added.

2.8. Colorimetric analysis of LFA

The synthetic target oligonucleotide used was designed according to zika viral cDNA. Target DNA was diluted using sodium citrate saline (SSC) buffer (pH 7.4, 4 ×). The prepared LFA test strips were dipped into the 96 wells plate pre-dispensed with the target DNA solution. Comparison between different durations of electrospin-coating was tested with 5 nM target solution. The sensitivity of assay was tested at the target's concentration range of 5 nM−0.1 nM. When the signals at the control line and the test line were stable, the resultant LFA test strips were scanned at 600 dpi using a Canon LiDE 110 scanner. A fixed area of test line was selected when the mean density was determined using Image Pro Plus 6.0 software (Media Cybernetics. Inc. Bethesda, MD). The signal's optical density (OD) was the difference of mean densities between the test line and the strip's background.

2.9. Statistical analysis

Data are tested using IBM SPSS Statistics 24 software. All tests are based on $n = 3$ and results of $p < 0.05$ are considered significant or otherwise stated. Data are represented as mean ± standard error of the mean.

3. Results and discussion

3.1. Working principle of electrospin-coated LFA

In this study, we coated electrospun fibres on NC membrane to improve the sensitivity of nucleic acid-based LFAs. We showcased the incorporation of electrospun fibres by electrospin-coating PCL into LFA to achieve flow delays for sensitivity enhancement (Fig. 1A,B). We chose PCL as our candidate for electrospin-coat material due to its hydrophobicity, good mechanical properties and slow degradation rate that make it an excellent material for controlling fluid flow in LFA. In the working principle of LFA, the target particles bound to the gold-labelled detecting probes (AuNP-DP) are captured by the capture probes at the test lines in the NC membrane, resulting in a visual signal [2]. As the number of bound targets on the test line determines the signal intensity, the flow rates of the targets and the AuNP-DP affect their interaction rate before being captured by the capture probe at the test line, which ultimately influences the intensity of the test line [3]. As shown in Fig. 1C, when the amount of electrospun PCL fibres on the NC membrane increases, this renders a change in hydrophobicity at the coated region that will reduce the flow rate, which in turn will enhance the biomolecule interaction and improve the signal intensity of the test line. As compared to the existing publications [3–5,18–22], this platform does not require additional installation of shunts or equipment, improving its simplicity of both fabrication and operation.

3.2. Morphological analysis of electrospin-coated NC membrane

To evaluate the interaction of the electrospun PCL nanofibres and the NC membrane, we characterized the microstructures of pre- and post-electrospin-coating PCL on NC membrane using optical microscopy and FESEM (Fig. 2). The porous structure of NC membrane can be clearly observed in Fig. 2A, C and E, which provides capillary action for fluid to travel in the membrane. A 60 s period of electrospinning covers the NC membrane with a thin layer of PCL nanofibres that are bead-free with some fibre bending as shown in Fig. 2B, 2D and 2F, in which the diameter of fibers ranges from 250 nm to 2.85 μm (799 ± 363 nm, $n = 180$) (Fig. 2G). The top view of the electrospin-coated NC membrane (Fig. 2B, D and F) shows that the layer of PCL nanofibres does not obscure the NC membrane completely, as the interfibrillar pores provide spaces for the fluid to reach the bottom layer of NC membrane. From the side view on the edge of electrospin-coated NC membrane (Fig. 2H), the nanofibers are shown only deposited on the top of NC membrane without any penetration.

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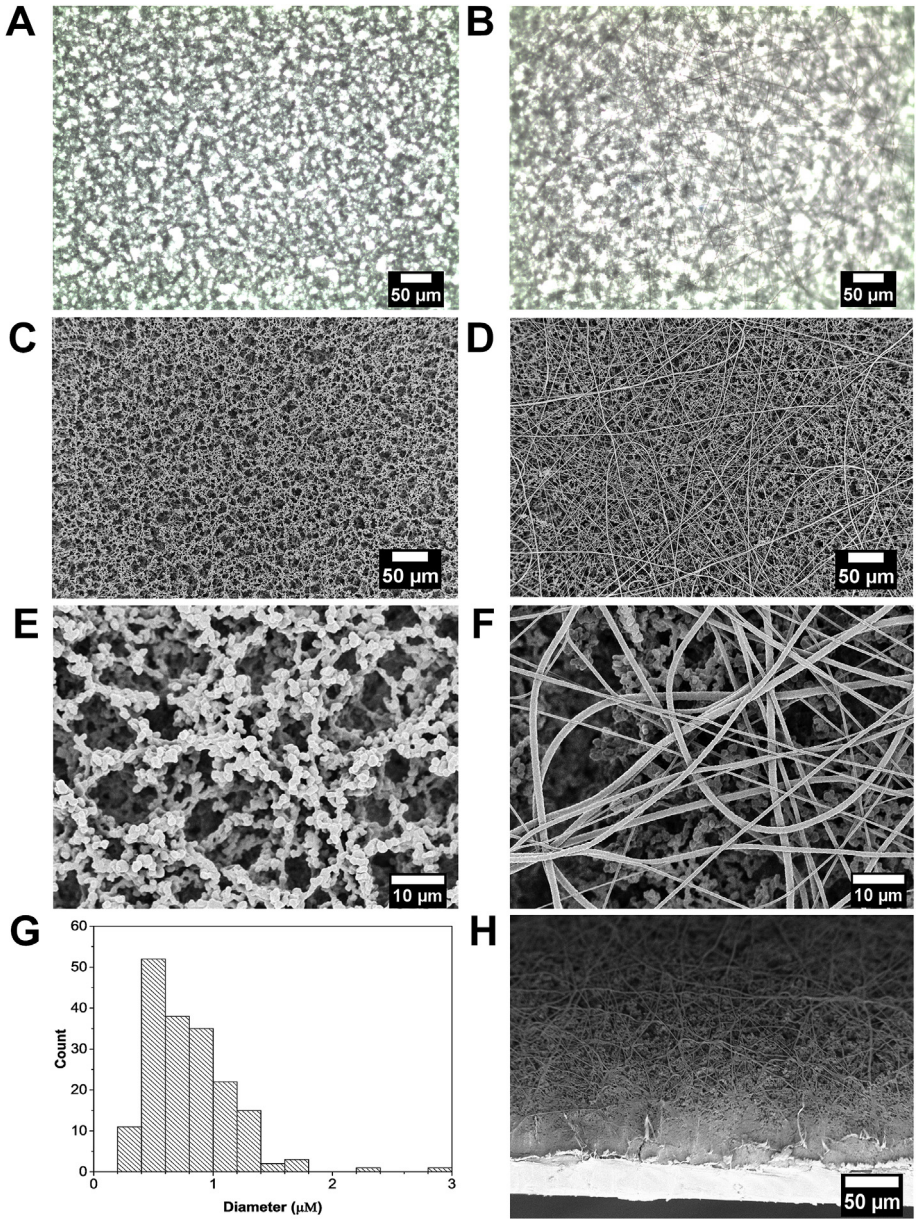

Fig. 2. Morphological characterization of PCL electrospin-coated NC membrane. The optical microscopic images of A) the uncoated NC membrane and B) the electrospin-coated NC membrane were taken at 100 × magnification, respectively. FESEM images of C) the uncoated NC membrane and D) the electrospin-coated NC membrane were taken at 200 × magnifications (1 kV), respectively. FESEM images of E) the uncoated NC membrane and F) the electrospin-coated NC membrane were taken at 1000 × magnifications (1 kV), respectively. G) The diameter distribution of the PCL fibres on the electrospin-coated NC membrane shows the highest count at the range of 0.2–0.4 ($n = 180$). H) FESEM image is showing the side view of the edge of the electrospin-coated NC membrane at 600 × magnification (1 kV).

3.3. Wettability and flow analysis of electrospin-coated NC membrane

To study the effect of nanofiber coating on the hydrophobicity of NC membrane, we measured the contact angle of the water droplet on electrospin-coated PCL on NC membrane for durations of 15 s, 30 s, 60 s, 180 s, 300 s, 420 s, 540 s, respectively (Fig. 3A). We observed that the water droplet was immediately absorbed by the membrane without electrospin-coating (0 s), and the longer duration of electrospin-coating is, the larger the water contact angle (*i.e.*, change from hydrophilic to hydrophobic) is. The contact angles of water droplet for electrospin-coating duration of 0 s, 15 s, 30 s, 60 s, 180 s, 300 s, 420 s and 540 s were 0°, 44.8 ± 8.4°, 44.0 ± 7.3°, 52.4 ± 3.1°, 126.8 ± 8.7°, 121.8 ± 10.9°, 130.1 ± 3.8° and 140.7 ± 1.7° ($n = 3$), respectively. The rising contact angle indicates the reduced wettability of the surface and the surface becomes more hydrophobic.

To evaluate the effect of nanofiber coating on flow rate, the time of flow in the electrospin-coated and the uncoated (0 s) NC membranes were recorded (Fig. 3B). Since sample solution flows laterally in the NC membrane, the rate of the flow affects the binding rate between the targets and the AuNP-DP, which in turn determines the number of target-AuNP-DP complexes captured by the capture probe and the signal intensity of the test zone. In the present study, we observed that the overall time taken by the lateral flow of solution on NC membrane strip increases with the travelled distance, which is presumed according to the relationship in Washburn's equation [39] (Eq. (1)).

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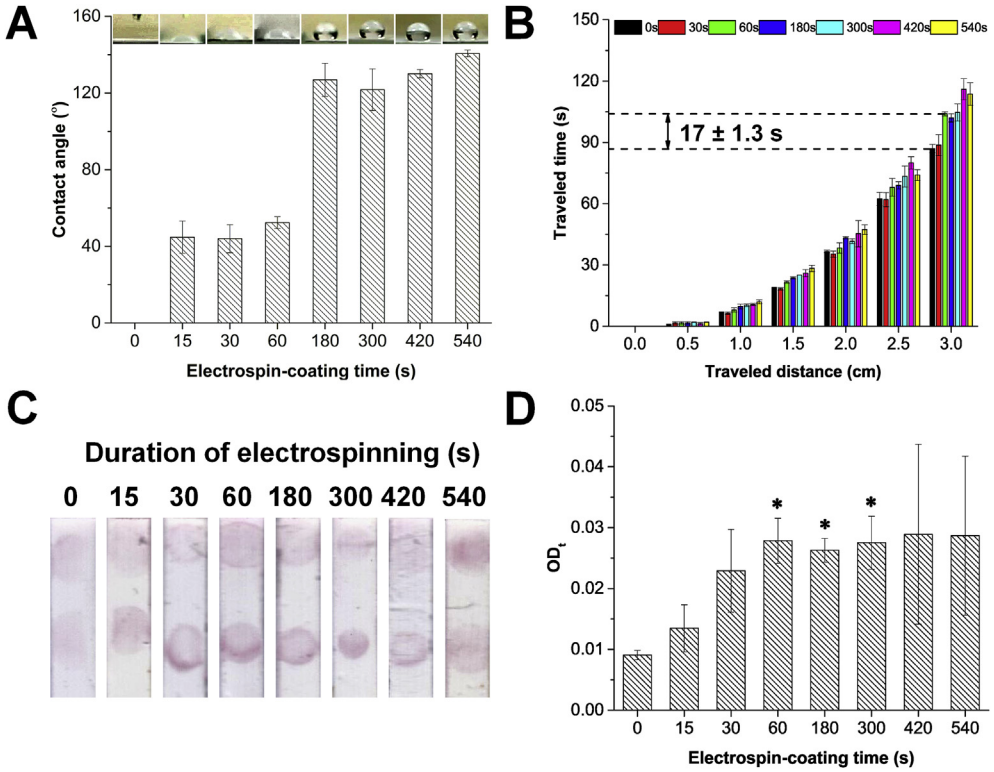

Fig. 3. Wettability of electrospin-coated NC membrane on signal intensity. A) The electrospin-coating time durations of 15 s, 30 s, 60 s, 180 s, 300 s, 420 s, 540 s show increased contact angle of 10 μL water droplet on the surface of NC membrane compared to the non-coated (0 s) NC membrane ($n = 3$). Insets show the water droplets on each NC membrane. B) The traveling time has a quadratic increase with the increase of travelled distance. The increasing electrospin-coating time shows increased traveling time at every 0.5 cm travelled distance on NC membrane. The time delay in 60 s electrospin-coated NC membrane was 17 ± 1.3 s compared to the uncoated one (0 s) ($n = 3$). C) Duration of the electrospin-coating NC membrane on LFA shows increases in signal intensity based on 5 nM of target concentration. The upper signal represents control line and bottom signal represents test line. D) The optical density (OD_t) of the test line on the LFA was enhanced as the duration of electrospin-coating on the NC membrane reaches 60–300 s ($^*p < 0.05$, relative to 0 s, $n = 3$).

$$L^2 = \frac{\gamma D \cos\theta}{4\eta}.t \quad (1)$$

where L is the length of the liquid has advanced, γ and η are the surface tension and the viscosity of the liquid respectively, D is the pore diameter, θ is the contact angle between liquid and solid, and t is the time taken for the liquid to advance. Due to the capillary forces in NC membrane, the solution travels faster at the beginning (0 cm) and slows down when it reaches the end (3.0 cm). The time of travel gradually increases as the solution reaches the end of the strip. However, the time for wicking the whole strip increases with increasing coating thickness of nanofibers on the NC membrane. For instance, the NC membrane that is electrospin-coated for at least 60 s takes 17 ± 1.3 s more than an uncoated NC membrane to travel a 3.0 cm distance ($p < 0.05$). According to Washburn's equation (Eq. (1)), the penetrability of a fluid is derived from $\gamma/\eta \cdot (\cos\theta)/2$ and it equals to the velocity or the penetrated distance of a fluid per unit time. By assuming no variation in the liquid properties, the distance travelled by the liquid, L under given time span, t decreases when the liquid-membrane contact angle, θ increases toward 90° due to reducing value of $\cos\theta$. In other words, the increase in liquid-membrane contact angle indicates the reduction of flow rate (Fig. 3A). Taken together, the hydrophobic PCL nanofibers that are electrospin-coated on the NC membrane causes flow resistance.

To examine the effect of flow rate on the signal intensity of LFAs, we tested the optical density of test line on the LFA strips coated with different nanofiber thickness by detecting 5 nM synthetic Zika virus DNA (Fig. 3C–D). The results show that the OD of the test line for strips with 60 s, 180 s and 300 s electrospin-coating indicate a significant difference from the strip without electrospin-coating (Fig. 3D). The OD results for the test strips beyond 60 s electrospin-coating do not show significant difference with 60 s. The results indicate that the enhanced signal OD is associated with the delayed flow rate as driven by the presence of PCL nanofiber coating on the NC membrane. Because of the hampered water absorption in highly hydrophobic surface of electrospin-coated membrane, we proceed to sensitivity test with 60 s as our optimum electrospin-coating duration for the LFA.

3.4. Enhanced sensitivity of electrospin-coated NC membrane in LFA

To assess the effect of nanofiber coating on LFA detection sensitivity, the uncoated and electrospin-coated NC membrane were examined by LFA for synthetic zika virus oligonucleotide at 0 nM (negative control), 0.1 nM, 0.25 nM, 0.5 nM, 1.0 nM and 5 nM, respectively (Fig. 4). We visually observed that the signal intensity increases with increasing target concentration as more target particles are captured at the test lines (Fig. 4A,B). The limit of detection (LOD) for uncoated and coated NC membrane is 5 nM and 0.5 nM, respectively, indicating a tenfold signal enhancement. The enhancement was further confirmed by quantitative analysis of the signal intensity using software (Fig. 4C).

In short, we have demonstrated the presence of electrospun PCL nanofibers on NC membrane as a proof of improving sensitivity of LFA detection for nucleic acid. By electrospin-coating PCL nanofibres, we have exhibited electrospinning hydrophobic materials as

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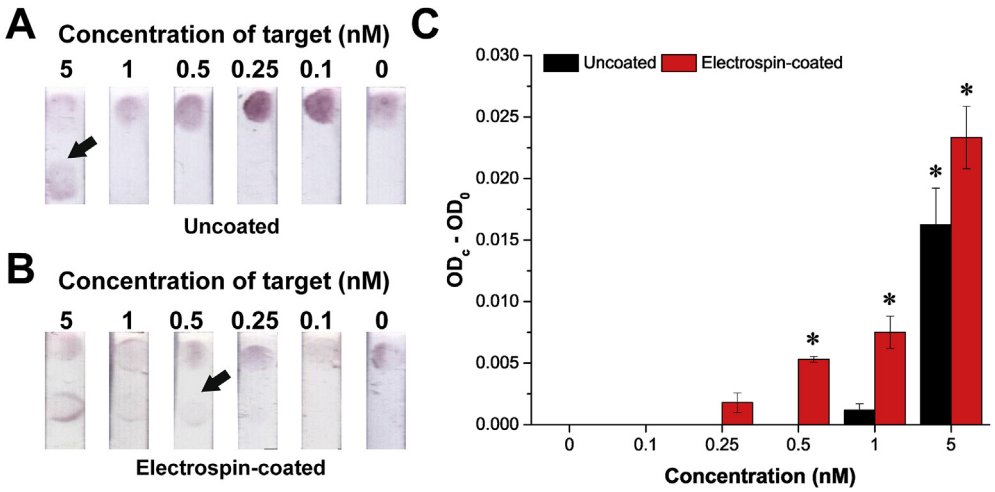

Fig. 4. Enhanced sensitivity of LFA with the electrospin-coated NC membrane. Signal intensity of LFA strips with A) the uncoated and B) the electrospin-coated NC membrane at different concentration of target. The signal intensity of test lines reduces as the target concentration reduces. Naked eye detection on the LFA is limited at 5 nM with the uncoated NC membrane while 0.5 nM with the electrospin-coated NC membrane. C) The difference of OD ($OD_c - OD_0$) at each target concentration with negative control (0 nM) for the uncoated and the electrospin-coated NC membrane of LFA is measured using software analysis. The LFA without coating shows lower OD difference compared to LFA with electrospin-coating. The limit of detection (LOD) of LFA with electrospin-coated NC membrane (0.5 nM) shows ten-fold improvement compared to the LFA with uncoated NC membrane (5 nM) ($^*p < 0.05$, relative to 0 nM, $n = 3$).

a novel method to modify NC membrane for detection purpose, which holds a number of advantages: (i) being low-cost compared to the conventional platform (*e.g.*, enzyme linked immunosorbent assay (ELISA) and quantitative polymerase chain reaction (qPCR)), (ii) easy to fabricate and operate (*e.g.*, eliminate the use of additional shunt presented by other studies [18–22]), (iii) being more rapid (*i.e.*, ≈1.5–15 min) as compared to the conventional method (>1hr) [3,9,14]. Moreover, the electrospin-coated NC membrane could be integrated with the sample-to-answer biosensor as a part to further improve the signal intensity and sensitivity [40]. Future work should include the optimization of the electrospin-coating conditions, selection of materials and functionalization for enhanced detection of different targets in LFA. Due to the low degradability of polymeric materials, we will also focus on the assessment of long-term storage towards functionality using real clinical samples. We anticipate great progress to be brought by electrospinning polymeric materials to enhance sensitivity for a broad range of targets in LFA or other point-of-care testing platforms.

4. Conclusion

In conclusion, we have successfully demonstrated a new approach of manipulating fluid flow by incorporating electrospin-coating hydrophobic PCL onto NC membrane of LFA to improve biomolecule interactions and enhance the detection sensitivity. Electrospin-coated NC for 60 s achieves a delay of flow as long as 17 ± 1.3 s. The delayed flow increases the interaction rate between the target molecules and the gold nanoparticle-detecting probe conjugates, resulting in the binding of more complexes to the capture probes and hence improving the detection sensitivity. Compared to the uncoated NC membrane, the sensitivity of the electrospin-coated NC membrane has increased up to tenfold. The electrospin-coating integrated device has the potential to greatly improve the sensitivity of detection of various targets in point-of-care platforms in the future.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2018.01.016.

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