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Efficient protein immobilization on polyethersolfone electrospun nanofibrous membrane via covalent binding for biosensing applications

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ABSTRACT

In this paper we introduce novel strategy for antibody immobilization using high surface area electrospun nanofibrous membrane based on ethyl-3-(3-dimethylaminopropyl)-carbodiimide/N-hydroxysuccinimide (EDC/NHS) coupling chemistry. To present the high performance of proposed biosensors, anti-staphylococcus enterotoxin B (anti-SEB) was used as a model to demonstrate the utility of our proposed system. Polymer solution of polyethersolfone was used to fabricate fine nanofibrous membrane. Moreover, industrial polyvinylidene fluoride membrane and conventional microtiter plate were also used to compare the efficiency of antibody immobilization. Scanning electron microscopy images were taken to study the morphology of the membranes. The surface activation of nanofibrous membrane was done with the help of O_2 plasma. PES nanofibrous membrane with carboxyl functional groups for covalent attachment of antibodies were treated by EDC/NHS coupling agent. The quantity of antibody immobilization was measured by enzyme-linked immuno sorbent assay (ELISA) method. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) spectroscopy was performed to confirm the covalent immobilization of antibody on membrane. Atomic force microscopy, scanning electron microscopy and invert fluorescence microscopy were used to analyze the antibody distribution pattern on solid surfaces. Results show that oxygen plasma treatment effectively increased the amount of antibody immobilization through EDC/NHS coupling chemistry. It was found that the use of nanofibrous membrane causes the improved detection signal of ELISA based biosensors in comparison to the standard assay carried out in the 96-well microtiter plate. This method has the potential to improve the ELISA-based biosensor and we believe that this technique can be used in various biosensing methods.

1. Introduction

The accurate assessment of biomarkers likes proteins, hormones or other biomolecules that amount of them changes with state of disease or level of treatment and gives diagnostic information is a difficult duty. In these samples, pathogens exist in small quantity compared to other background molecules. Therefore, appropriate sampling process for concentrating and separating pathogens from the matrix should be developed in order to provide rapid and accurate pathogen detection test [1].

Electrospinning nowadays attract much more attention due to its facile ability to produce fine fibers which have specific surface area approximately one to two orders of magnitude larger than flat thin films. In this technique, a high voltage is applied to a polymeric droplet emanating from the tip of a nozzle. As soon as voltage passes threshold point, the deformation of a cone-like droplet occurs and continues jet stems from the nozzle tip and accelerates toward the collector to form nanofibrous membrane.

In most of cases, nozzle acts as cathode and collector plays the role of anode [2–3]. Electrospun nanofibrous membranes recently found versatile application in tissue engineering [4], wound dressing [5], drug delivery [6], filtration [7–9], sensor and sampling devices [10] and gas senor [11–12] and so on.

The combination of molecular biomarkers e.g. antibodies, aptamers, peptides, and liposccharides with high surface area electrospun

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nanofibrous membranes can provide new chance to create both novel sampling and biosensor devices [13]. In most recent studies some researchers have proposed using the perfect capability of high surface area electrospun nanofibrous membranes to improve the efficiency of immunoassays. They studied the capability of surface modified nanofibrous membranes containing primary amine group for covalent immobilization of biological elements likes chymotrypsin and lysozyme [14–16]. They found that nanofibrous membranes could immobilized greater amount of biological molecules compared to that of cast film.

In the most cases, the immunoassay employs the specificity of antibody–antigen interaction for the detection of relevant analytes. Immunoassay is widely used in pharmaceutical research, medical diagnostics and biological analysis. As before reports, wide range of new materials and techniques were proposed to improve the sensitivity and selectivity of traditional immunoassay [17].

ELISA is one of the widely used immunoassay techniques in the biological detection field all over the world and continuous efforts are being done to improve the sensitivity and selectivity of this technique [18–19]. It was accepted that the capacity of solid substrate for adsorbing protein has important effect on sensitivity of this assay [20]. Up to now, various techniques have been proposed to immobilize protein molecules to the different substrates such as magnetic microspheres [21–23], film [24–25], fiber [26–28], and silica microsphere [29]. However, recently electrospun nanofibrous membranes deal with increase interest toward immobilization of proteins on themselves [30–33]. In most of studies the immobilization of antibodies was done through physical adsorption. Physical adsorption on the solid surfaces through hydrophobic interactions is a commonly used antibody immobilization technique for immunoassay platforms [2,34–35]. However, this technique suffers from some lack including non-uniform distribution of antibody, loss of antibody functionality, poor signal to noise ratio, leaching of antibody and protein exchange [36–40]. An ideal antibody immobilization should follow some needs including selectivity and specificity, efficiency and fastness, activity retention and recovery rates [2].

In this study, nanofibrous membranes were produced from polyethersulfone (PES) solution using electrospinning technique. PES is a non-biodegradable polymer which is widely used as a membrane material in hemodialysis, filtration and bioreactor technology [41–42]. Oxygen plasma treatment was performed to create functional groups on the surface of produced nanofibrous membrane. Two different strategies were developed to immobilize anti-staphylococcus enterotoxin B (SEB), as an antibody model on the surface of nanofibrous membrane: covalent binding through 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)/N-hydroxysuccinimide (NHS) coupling strategy and the other through hydrophobic interactions. A simple ELISA assay procedure was performed to assess the antibody immobilization efficacy on PES electrospun nanofibrous membrane.

2. Experimental

2.1. Materials

PES was supplied by BASF, Ludwigshafen, Germany. Dimethylformamide, NHS and EDC were obtained from Sigma-Aldrich. Anti-SEB (X) antigen, monoclonal mouse IgG antibody was obtained in our laboratory by hybridoma technique (unpublished data). Secondary antibody (Goat pAb to MS IgG (HRP)) was purchased from Abcam. TMB substrate solution was obtained from Razi Biotech. Bovine serum albumin (BSA) and sulfuric acid (H_2SO_4) were supplied by Merck.

2.2. Nanofibrous membrane preparation and surface treatment

PES nanofibrous membranes were fabricated via electrospinning technique according to a method previously reported in our laboratory [43–44].

In brief, a 24 wt.% solution of PES in dimethylformamide (DMF) was poured into a 10 ml plastic syringe which was connected to a stillness needle with an extension tube. We used electrospinning apparatus (Electroris-NL, Fanavaran Nanomeghyas Co., Iran) to prepare nanofibrous membrane.

The distance between needle and collector was adjusted to 15 cm. The solution was ejected from needle by a syringe pump. High voltage of 18 kV was applied between the nozzle and collector to force the solution droplet from the needle and fabrication of fine fibers with the nanometer diameter on the collector. The flow rate of electrospinning was set to 0.3 ml/h.

In order to modify the surface chemistry of fabricated nanofibrous membrane oxygen plasma treatment was performed by a low frequency plasma generator of 40 kHz with a cylindrical quartz reactor. Pure

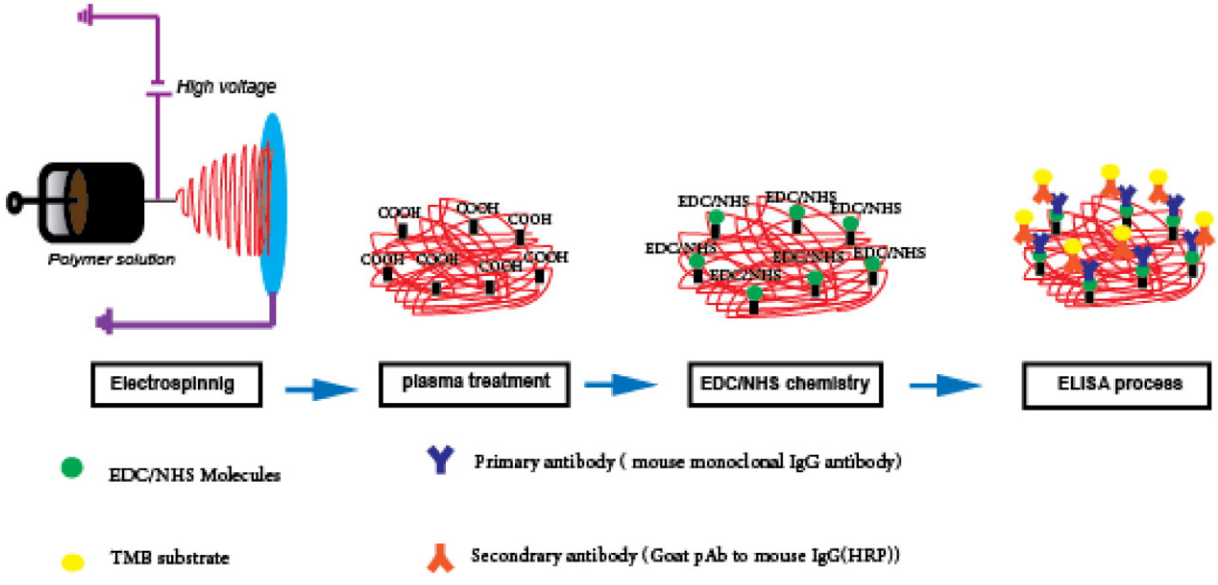

Fig. 1. Schematic of the nanofibrous membrane fabrication through electrospinning, plasma treatment and sandwich ELISA applied on nanofibrous membrane.

oxygen was introduced in to the reaction chamber at 0.4 mbar and glow discharge was flowed for 5 min.

2.3. Characterization study of prepared nanofibrous membrane

The morphology of electrospun membrane and PVDF industrial membrane (commonly used in Western blot process with the thickness of 140 μm) was observed by scanning electron microscopy (SEM) (Seron, AIS2300C, Korea) after gold spattering on membrane.

After plasma treatment and immobilization of anti-SEB, Attenuated Total Reflection Fourier Transform (ATR-FTIR Thermo Nicolet model: NEXUS 670, USA) was used to investigate the chemical characteristic of the membrane. Moreover, SEM was also used to further inspect how the morphology of non-plasma and plasma-treated nanofibrous membrane will be after incubation with EDC/NHS and antibody immobilization.

2.4. Evaluation of electrospun PES nanofibrous membrane as a substrate for immunoassay

Antibody capturing on different surfaces was measured by ELISA method to evaluate an immunoassay on nanofibrous scaffolds. For this purpose, monoclonal mouse IgG antibody was used for immobilization on different test surfaces and polyclonal gout anti-mouse IgG-HRP conjugated was used for detection of immobilized antibody. Three types of substrates, i.e. electrospun PES nanofibrous membranes, industrial PVDF membranes commonly used in Western blot assay and 96-well cell culture plates were used for our immunoassay procedure (the whole substrates were used as plasma and non-plasma types).

Plasma treated and untreated nanofibrous membranes with the thickness of 90 μm were punched to fit the wells of a 96-well of cell culture plates (diameter of 0.5 cm). Control membranes used in this study were plasma and non-plasma treated membranes which only exposed to the secondary antibody. First, plasma treated and non-treated PES nanofibrous membranes incorporated in 96-well cell culture plate and plates without membrane were incubated overnight by EDC/NHS solution (100 μl, 5 mg/ml). It should be noticed that instead of EDC/NHS, 100 μl deionized water was pipetted onto the wells to immobilize antibody hydrophobically. A schematic diagram of our proposed assay procedure is presented in Fig. 1. The reagents for our ELISA process were as follows: primary antibody 10 μg/ml monoclonal mouse IgG anti-SEB antibody in PBS buffer, pH: 7.2, BSA 1 wt.% in PBS buffer, pH: 7.2 as blocking agent, anti-mouse IgG antibody HRP-conjugate in PBS buffer as secondary antibody (simulate the antigen of interest, 1:8000, pH: 7.2), TMB substrate as signal generating agent and sulfuric acid 2 N as stop reagent. The ELISA procedure was conducted by pipetting 100 μl primary antibody (100 μl, 10 μg/ml) into each well and incubation for 1 h at RT. After incubation, the wells were wash 3 times with wash buffer (PBS/tween20 (0.05% v/v). In the second stage, blocking treatment was performed. To do this 300 μl BSA 1% was pipetted onto each well and incubated for 1 h in 37 °C then washed with wash buffer for 3 times. Secondary antibody immobilization was performed by the addition of 100 μl secondary antibody to each well and was incubated for 1 h at RT. After that the wells were washed for 5 times to remove antibodies which non-specifically adsorb to the membranes. Signal generating stage was performed by adding 100 μl TMB substrate solution and 15 min incubation in dark cabinet. To stop enzymatic reaction, 100 μl H_2SO_4 solution was added to each well then 100 μl of solution of each well was transferred to beneath well (because the presence of membrane in well cause error in signal reading by machine). The absorbance at 450 nm was recorded using a multi plate reader (Biotec, ELX800, USA).

2.5. Atomic force microscopy (AFM) study

In order to study the morphology of the immobilized antibody, AFM was employed as a discriminative method between different treating conditions including plasma and non-plasma situations with and without coupling agent, namely EDC/NHS. Also, Petri dishes were used as a smooth substrate for antibody immobilization in this examination. The surface morphology was investigated in air at ambient temperature, using the contact mode of AFM (DME, Dual Scope™ C-26, Denmark).

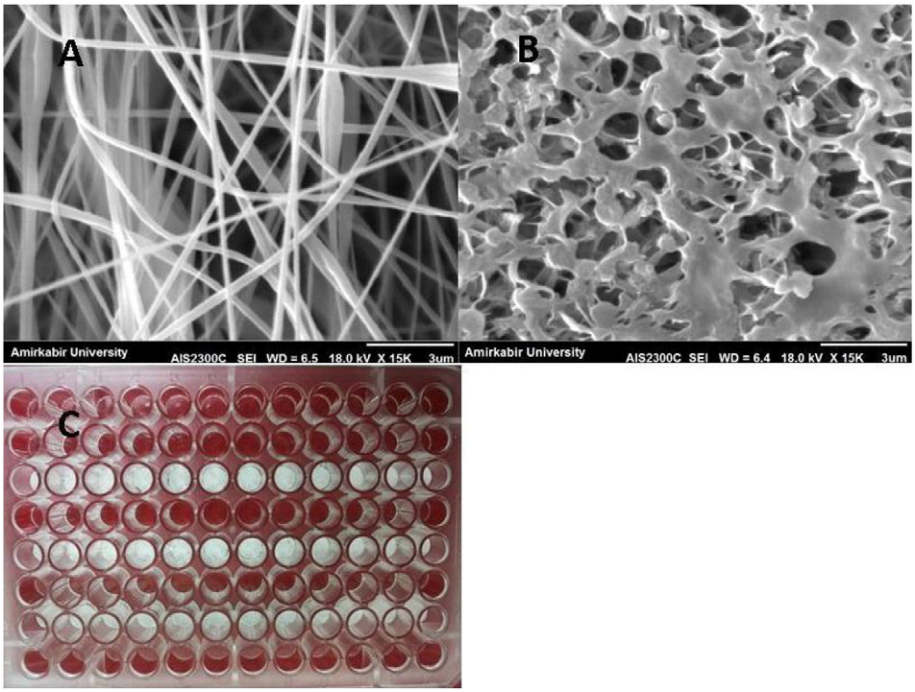

Fig. 2. Scanning electron microscopy (SEM) images of (A) PES nanofibrous membranes, (B) Western blot PVDF membrane and (C) digital image of 96-well plate contains membranes.

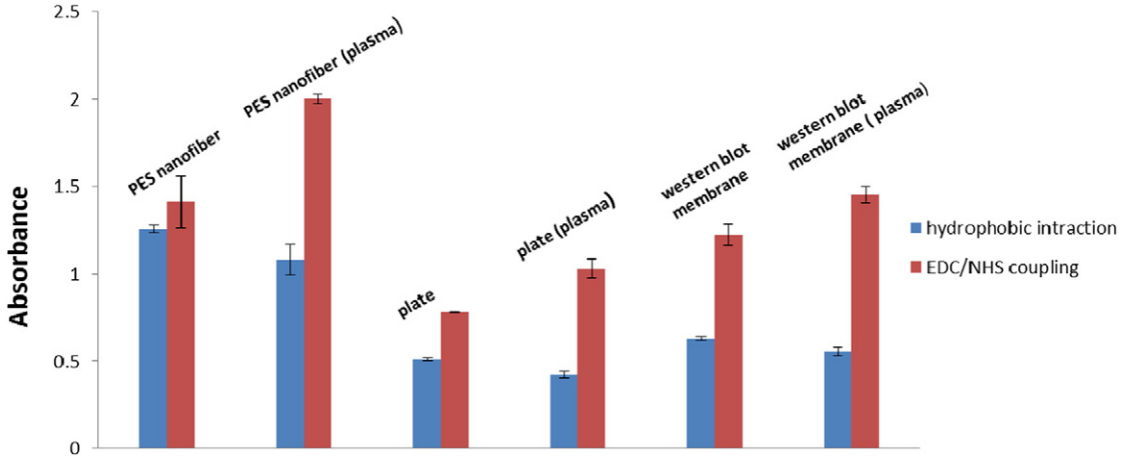

Fig. 3. ELISA results obtained from immobilization of antibody through hydrophobic interactions and EDC/NHS coupling agent on different substrates (plasma-treated and non-plasma treated).

2.6. Binding capacity study of immobilized antibody

The binding capacity of antibody immobilization on nanofibrous membranes by different immobilization techniques were assessed and compared with each other. Plasma and non-plasma treated nanofibrous membrane with and without EDC/NHS coupling agent were incubated for 1 h with 1 μl/ml FITC-labeled MS IgG solution at room temperature. The nanofibrous membranes were then washed five times with wash buffer to remove excess FITC-labeled antibody and analyzed for fluorescence activity by fluorescent microscope (Nikon, Eclipse TE2000-S, Japan) to detect the intensity of immobilized antibody.

2.7. Stability test of immobilized antibodies on PES nanofibrous membrane

Different modifications of corresponding substrates were examined to study the stability of immobilized antibodies from denaturation as follows: First, plasma treated and non-plasma treated PES nanofibrous membranes were placed in 96-well cell culture plate and incubated overnight by EDC/NHS solution (100 μl, 5 mg/ml). It should be remembered that instead of EDC/NHS, 100 μl deionized water were pipetted onto the wells to immobilize antibody hydrophobically. Then whole wells were immobilized by primary antibody. After three times washing by wash buffer, plates were allowed to dry in RT. After drying, all chambers were placed in sealed plastic containing silica gel desiccant. Three identical sets of experiments were designed and preserved at 4 °C. After storing at controlled temperature for 5, 10 and 15 days, the rest of the ELISA procedure including blocking step and signal generating step were conducted as previously described.

3. Result and discussions

3.1. Morphology of fabricated PES nanofibrous membrane

The PES solution was electrospun onto the collector as a nanofibrous membrane. Fig. 2 shows the SEM images of electrospun PES nanofibrous membrane and PVDF membrane (as a model for comparison). Fig. 2C shows photographic image of conventional 96-well microtiter plate that contains the membranes used throughout this research.

Fine fibers with average diameter of approximately 587 ± 24 nm (calculated from Image J software) were obtained with the concentration of 24 wt.% of PES solution. Wettability of electrospun PES nanofibrous membrane is inherently low. Thus, this intrinsic feature inhibits the direct contact of used solutions with nanofiber surface and reduces the adsorption ability of PES nanofibrous membrane. We found that plasma treatment has significant effect on wettability of membrane. Moreover, it was reported [45] that oxygen plasma treatment creates functional groups on the surface of nanofibrous membrane to induce the side-selection reaction by covalent binding.

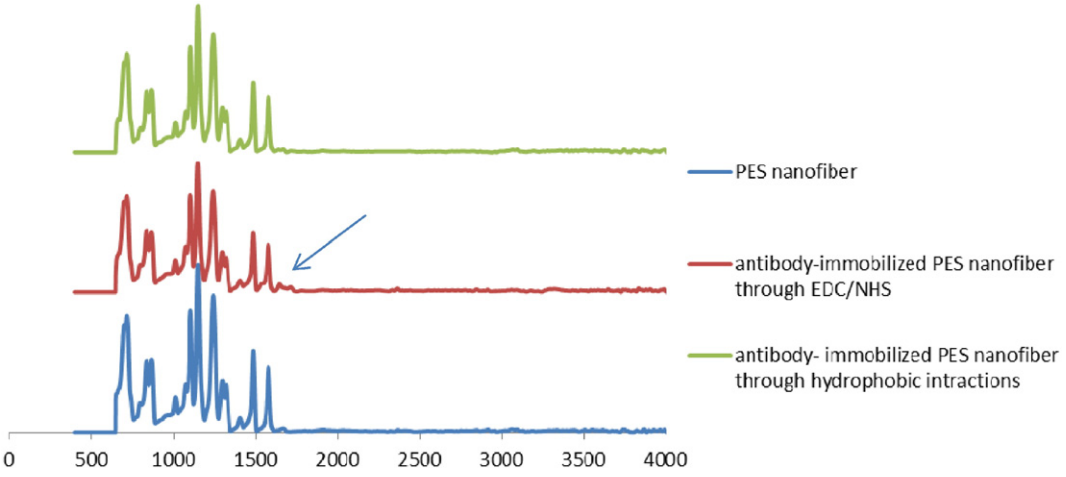

Fig. 4. Attenuated total reflection Fourier transform infrared spectra of antibody immobilized PES nanofibrous membranes and PES nanofibrous membrane.

3.2. Signal enhancement of immunoassay through EDC/NHS coupling

The ELISA results of the anti-SEB immobilization as primary antibody (concentration of 10 μg/ml) run on the conventional 96-well microtiter plate, PES nanofibrous membrane and industrial PVDF membrane (all of the substrates used as plasma treated and non-plasma treatment) are shown on Fig. 3.

As can be seen, immobilization of the primary antibody through EDC/NHS coupling chemistry has significant effect on ELISA signal. Moreover, sharp enhancement is seen on ELISA result of nanofibrous membrane compared to native microtiter plate and PVDF membrane. We believe that this phenomenon relates to the merit of larger specific surface area due to nanostructure of electrospun membranes and accordingly provides higher possibility interaction of antibody to substrate. Therefore, the ELISA signal from electrospun PES was higher than flat conventional microtiter and PVDF membrane. When comparing plasma treated and non-plasma treated samples, we found that plasma treatment has significant role on signal resulted from sample which antibody immobilized by EDC/NHS coupling chemistry. In our best knowledge, the creation of functional groups and side-selection effect on the surface could be the prominent parameters on this occurrence. On the other hand, plasma treatment of membranes reduces the hydrophobic immobilization of antibody due to the increase in hydrophilicity.

ATR-FTIR spectroscopy was done to confirm the covalent immobilization of antibody onto the surface of PES nanofibers through EDC/NHS coupling agent. In Fig. 4, new peaks were observed in IR spectra of antibody-immobilized PES nanofibrous membranes under different conditions including with and without EDC/NHS coupling agent. This typical characteristic peak relates to C═O (~1700 cm^{-1}) stretching for amide I and evidences the presence of amide bonds between amine groups on FC part of the antibody with carboxyl groups on the surface of PES nanofibrous membrane.

3.3. Scanning electron microscopy studies

SEM images of non-plasma and plasma treated nanofibrous membrane with different treatments are depicted in Fig. 5A–I. No significant morphological changes were observed after plasma treatment (Fig. 5A). However, when plasma-treated nanofibrous membrane was incubated with EDC/NHS coupling agent, a uniform coating of EDC/NHS is settled out on the membrane (Fig. 5B). On the contrary, most EDC/NHS is washed out from non-plasma nanofibrous membrane (Fig. 5C) because there are few carboxyl and hydroxyl sites on the membrane to react with EDC/NHS molecules. It is observed from Fig. 5D and 5E that antibody immobilization on plasma treated nanofibrous membrane through EDC/NHS coupling strategy is much more uniform than antibody immobilization through hydrophobic interaction. We hypothesize

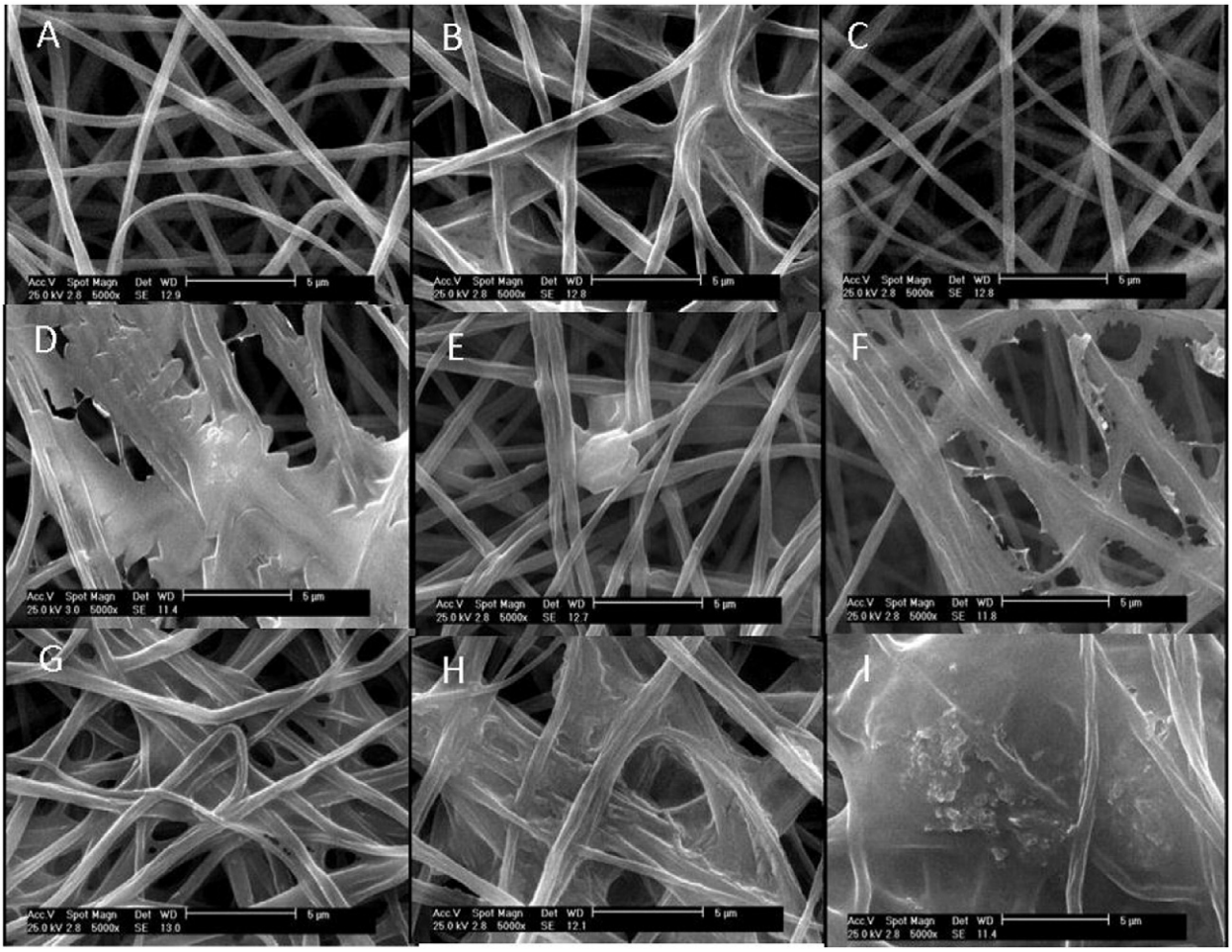

Fig. 5. Scanning electron microscopy images of (A) plasma-treated PES electrospun membrane, (B) EDC/NHS coating on plasma-treated PES electrospun nanofiber, (C) EDC/NHS coating on non-plasma treated PES electrospun membrane, (D) 10 μg/ml antibody immobilization through EDC/NHS coupling chemistry on plasma-treated PES electrospun membrane, (E)10 μg/ml antibody immobilization through hydrophobic interaction on plasma- treated PES electrospun membrane, (F) 10 μg/ml antibody immobilization through EDC/NHS coupling chemistry on non-plasma treated PES electrospun membrane, (G) 10 μg/ml antibody immobilization through hydrophobic interactions on non-plasma PES electrospun nanofiber, (H) antibody immobilized through EDC/NHS on plasma-treated PES membrane with blocking agent (BSA) and (I) antibody immobilized through EDC/NHS on non-plasma PES membrane with blocking agent (BSA).

that this difference is based on the hydrophilic nature of plasma treated nanofibrous membrane and hydrophobic feature of antibody causes relative repellency between substrate surface and antibody molecules. On plasma treated nanofibrous membrane, antibodies tend to shrink to bundles (Fig. 5E) compared to antibody immobilization through hydrophobic interactions on non-plasma treated nanofibrous membrane. Herein, the antibodies make a uniform coating on the substrate surface (Fig. 5F). In addition, weak interaction of EDC/NHS on non-plasma treated nanofibrous membrane causes some rupture to occur on the antibody coating (Fig. 5G). We also investigated coating of BSA as a blocking agent after antibody immobilization on plasma treated and non-plasma treated nanofibrous membrane. As is obvious from Fig. 5H and 5I, BSA can create a uniform coating on antibody-immobilized membrane and blocks free sites of membrane to prevent nonspecific reactions.

3.4. Morphologies of anti-SEB immobilized with different techniques

Fig. 6 shows AFM images of the plasma and non-plasma treated surfaces of Petri dish modified by anti-SEB in the presence and without EDC/NHS linker. As can be seen (from Fig. 6A) the surface of plasma treated Petri dish after EDC/NHS coating displays homogeneous small pyramid forms. But in non-plasma surface (Fig. 6B), EDC/NHS linker forms non-uniform pyramidic form. New Y-shape protruding feature with similar orientation were observed after antibody immobilization (Fig. 6C). In contrast, Fig. 6F is related to hydrophobic immobilization of antibody on surface without EDC/NHS linker. This figure indicates the non-uniform structure of anti-SEB on the substrate surface. The related observation is discussed in detail in the following section.

3.5. Binding capability of nanofibrous membrane to immobilize antibody

The binding capacity of anti-SEB was compared on plasma and non-plasma treated nanofibrous membranes with different modifications. To illustrate the effect of oxygen plasma treatment and EDC/NHS linkage chemistry, we used FITC-conjugated anti-mouse IgG to show the difference in binding capacity of nanofibrous membrane with different modifications. In the first step, FITC-conjugated anti-mouse IgG was hydrophobically immobilized on plasma treated and non-treated nanofibrous membrane (electrospun on a piece of glass). In the second set of experiment, FITC-conjugated anti-mouse IgG was immobilized on oxygen plasma activated nanofibrous membrane hydrophobically. As the results shown in Fig. 7, fluorescence intensity of FITC-conjugated anti-mouse IgG molecules was significantly increased with plasma treated nanofibrous membranes in the presence of EDC/NHS chemistry. Our results are completely in consistence with the previous reports who found that plasma treatment has significant influence on side selective immobilization of antibody [46].

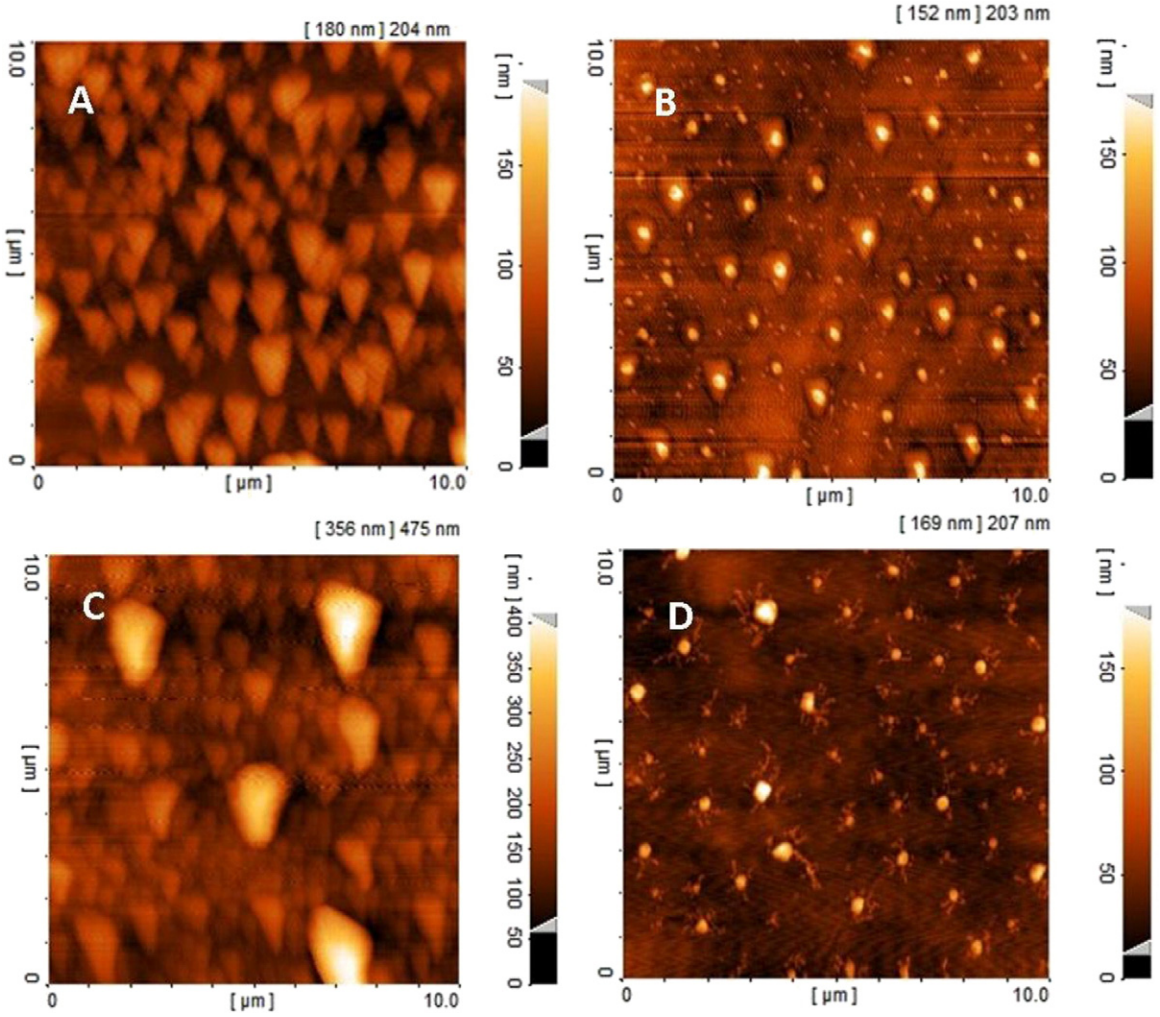

Fig. 6. 2D AFM images of Petri dish surface after different modifications (A) EDC/NHS coating on plasma treated surface, (B) EDC/NHS coating on non-plasma treated surface, (C) 10 µg/ml antibody immobilization through EDC/NHS coupling chemistry and (D) 10 µg/ml antibody immobilization through hydrophobic interactions.

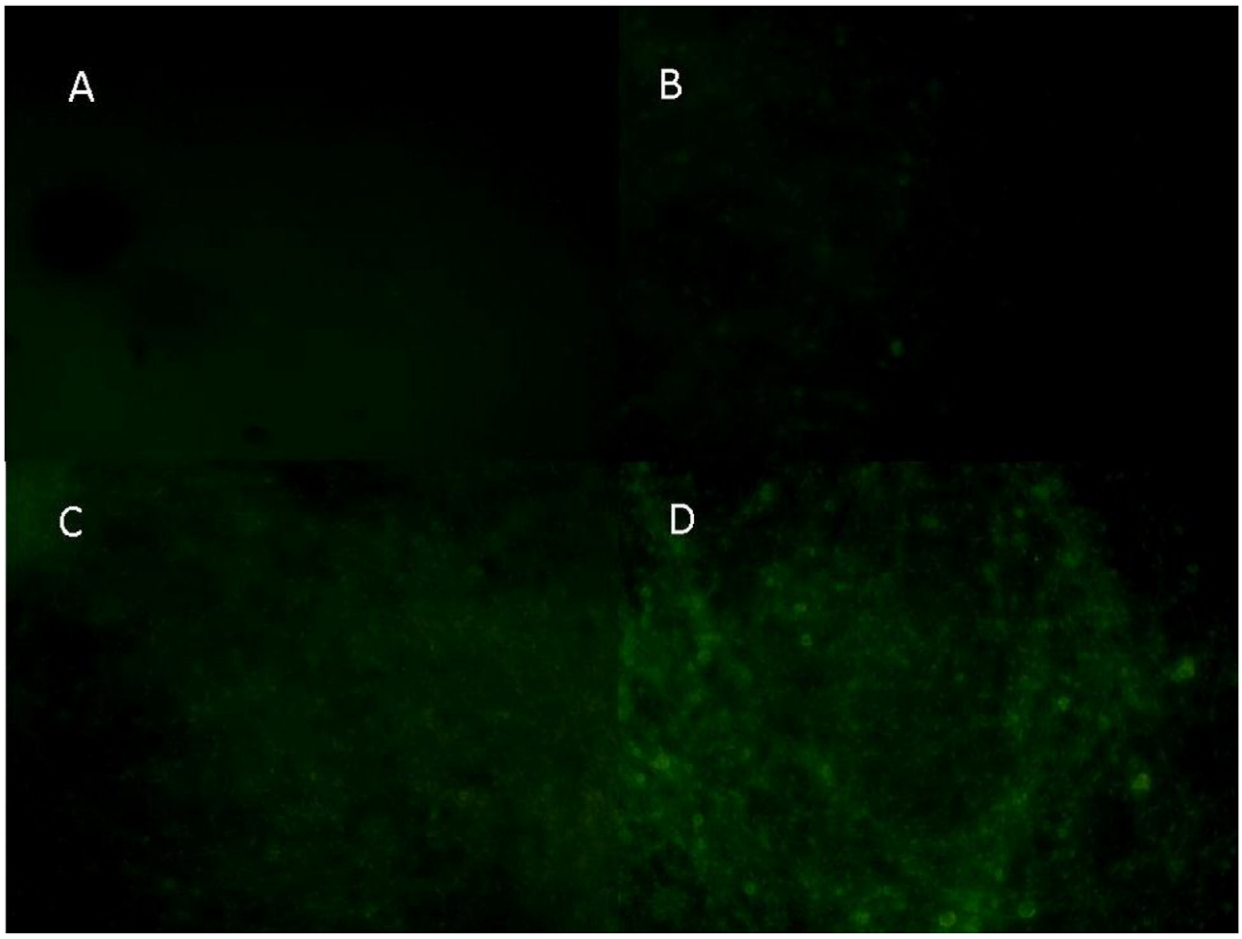

Fig. 7. Fluorescence images of immobilization of FITC-labeled anti-rabbit IgG antibody on the PES nanofibrous membrane through (A) hydrophobic interactions on plasma-treated membrane, (B) hydrophobic interactions on non-plasma membrane, (C) through EDC/NHS coupling agent on non-plasma treated membrane and (D) through EDC/NHS coupling agent on plasma treated membrane.

3.6. Stability test of antibody immobilization through EDC/NHS coupling

In most cases, immobilization of antibody to a solid substrate was done with the help of hydrophobic interaction. It is acceptable that hydrophobic interactions are unstable and protein especially antibodies deform through hydrophobic interaction leading loss of their functionality.

Moreover, rinsing and ambient condition can reduce the efficiency of biosensor and this lead to loss of biosensor functionality over time. To test the advantages of using EDC/NHS linker to immobilize antibody covalently to the substrate, compared to conventional hydrophobic technique, a stability test was examined to study the capability of different substrates to preserve the functionality of immobilized antibody. Fig. 8 shows the changes in ELISA signal in different substrates with different modification after storage in seal condition at 4 °C. As it is obvious from this figure, after storage over a period of time at 4 °C, ELISA signal is significantly higher with samples that antibody was immobilized using EDC/NHS coupling chemistry, compared to samples with antibody immobilization hydrophobically. This phenomenon indicates powerful effect of covalent bonds to preserve the functionality of proteins over time.

4. Conclusion

The combination of nanofibrous membrane in ELISA based biosensors is an effective solution to overcome the drawbacks related to the low signal strength of conventional ELISA immunoassay performed in microtiter plates.

Moreover, in the most of ELISA protocols, primary antibody immobilized through hydrophobic interactions causes easy denaturing of antibody and losing their functionality, while covalent immobilization of the antibody helps to preserve their structure. Therefore sensitivity and stability of designed biosensor will be increased.

In this study, a side-selective, covalent immobilization of antibody trough EDC/NHS coupling chemistry on PES nanofibrous membrane was successfully developed to enhance the sensitivity and stability of ELISA based immunoassay. Compared to conventional microtiter plate and PVDF industrial membrane, the electrospun PES nanofibrous membrane increased the intensity of ELISA signal due to higher surface area of this substrate. We found that oxygen plasma treatment has significant influence on antibody immobilization through EDC/NHS coupling chemistry. This novel method has extreme potential to improve the performance of conventional ELISA immunoassay. We hope that this novel technique of antibody immobilization can open new window toward highly sensitive and stable biosensors and helps to recognize hard diseases such as cancer types in the early stages.

Acknowledgements

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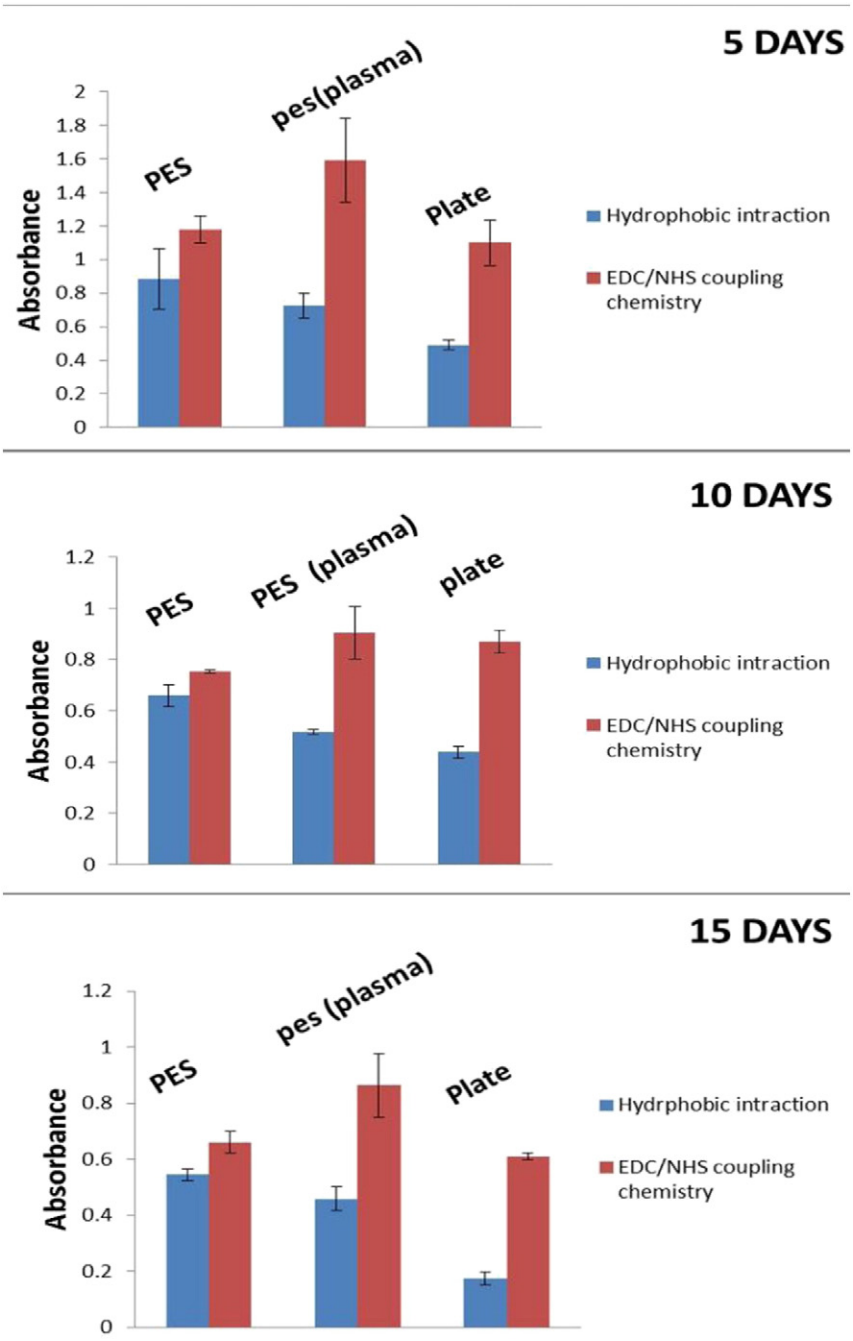

Fig. 8. Long-term stability test of antibody immobilization on various substrates through hydrophobic interactions and EDC/NHS coupling agent. All of samples were stored under dry condition in the presence of silica gel at 4 °C.

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