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Immobilization of Firefly Luciferase on PVA-*co*-PE Nanofibers Membrane as Biosensor for Bioluminescent Detection of ATP

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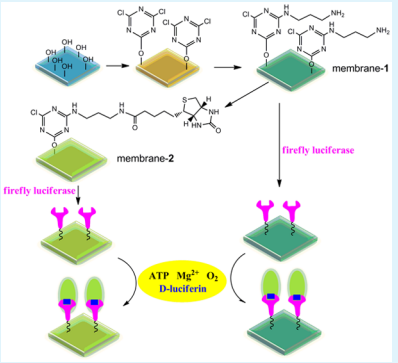
ABSTRACT: The bioluminescent reaction catalyzed by firefly luciferase has become widely established as an outstanding analytical system for assay of adenosine triphosphate (ATP). When in solution, the luciferase is unstable and cannot be reused. The problem can be partially solved by immobilizing the luciferase on solid substrates. The poly(vinyl alcohol-*co*-ethylene) (PVA-*co*-PE) nanofibers membrane has abundant active hydroxyl groups on the surface. The PVA-*co*-PE nanofibers membrane was first activated by cyanuric chloride with triazinyl group. Then the activated PVA-*co*-PE nanofibers membrane was subsequently reacted with 1,3-propanediamine and biotin. The firefly luciferase was immobilized onto the surface of 1,3-propanediamine- and biotin-functionalized membranes. The surface chemical structure and morphologies of nanofibers membranes were characterized by FTIR-ATR spectra and SEM. The hydrophilicity of membranes was tested by water contact angle measurements. The detection of fluorescence intensity displayed that the firefly-luciferase-immobilized PVA-*co*-PE nanofibers membranes indicated high catalytic activity and efficiency. Especially, the firefly-luciferase-immobilized nanofiber membrane which was functionalized by biotin can be a promising candidate as biosensor for bioluminescent detection of ATP because of its high detection sensitivity.



KEYWORDS: *firefly luciferase, adenosine triphosphate, poly(vinyl alcohol-co-ethylene), nanofibers, 1,3-propanediamine, biotin*

1. INTRODUCTION

Adenosine triphosphate (ATP) exists in all living organism, such as bacteria, cells, and viruses, and it functions for energy exchanges. Because of the direct source of all organisms, ATP has been widely used as an index for food safety and environmental monitoring.[1,2] Firefly luciferase is a bioluminescent enzymatic protein, and it has been extensively used to detect ATP in the presence of luciferin, Mg^{2+}, and molecular oxygen to produce fluorescence light, oxyluciferin, CO_2, and adenosine monophosphate (AMP).[3−5] The catalytic mechanism and the effect of stabilizers/activators such as coenzyme A (CoA), inorganic pyrophosphate (PPi), and tripolyphosphate (P3) on reaction process have been researched in detail in the last decades.[6−9] In solution, the detection limit of 10^{-15} g (ca. 1 amol) of ATP can be achieved, corresponding to a single bacterial cell.[10] However, when used in solution, luciferase is unstable and results in loss of biological activity and cannot be reused.

To solve the problem, some enzyme stabilizers,[11] gold−silver alloy nanoparticles,[12] and ionic liquid[13] were used to improve sensitivity, thermostability, and activity of firefly luciferase. Moreover, immobilization of luciferase has been developed to obtain the recyclability including the covalent attachment to a solid support, such as Langmuir−Blodgett behenic acid films,[14] cellulose films,[15] glass strips,[16] glass rods,[17] and polystyrene beads.[18] Furthermore, sugar-modified sol−gel-derived silica has also been used to entrap the firefly luciferase, and this work may push the detection limits of ATP to even lower levels with higher sensitivity and higher retention of enzyme activity.[19] However, regular and fibrous materials have quite limited surface features and active groups resulting in low loading capacity of the biomolecules and poor efficiency and sensitivity as biosensors.[20,21] And the nanofibers are wonderful candidates as a nanomaterial matrix because of their high intrinsic interfiber porosity, specific surface area, and ease of handling compared to nanoparticles and nanotubes.[22]

Poly(vinyl alcohol-*co*-ethylene) (PVA-*co*-PE) is commercial thermoplastic and possesses active hydroxyl groups, which can serve as reactive sites to triazinyl groups and carboxyl groups.[23] The PVA-*co*-PE nanofiber membranes have been used in wide range of applications, such as antibacterial and antifouling materials,[24,25] self-cleaning protective materials,[26] microfiltration,[27] heavy metal ion removal,[28] and lithium-ion battery separator.[29] However, it has not been reported that PVA-*co*-PE nanofiber is in the form of continuous yarns with controllable sizes and modified by cyanuric chloride to graft firefly luciferase as biosensors.

In this work, the PVA-*co*-PE nanofibers membrane was first activated by cyanuric chloride with triazinyl groups. Then the activated PVA-*co*-PE nanofibers membrane was subsequently reacted with 1,3-propanediamine and biotin. The firefly luciferase was immobilized onto the surface of 1,3-propanedi-

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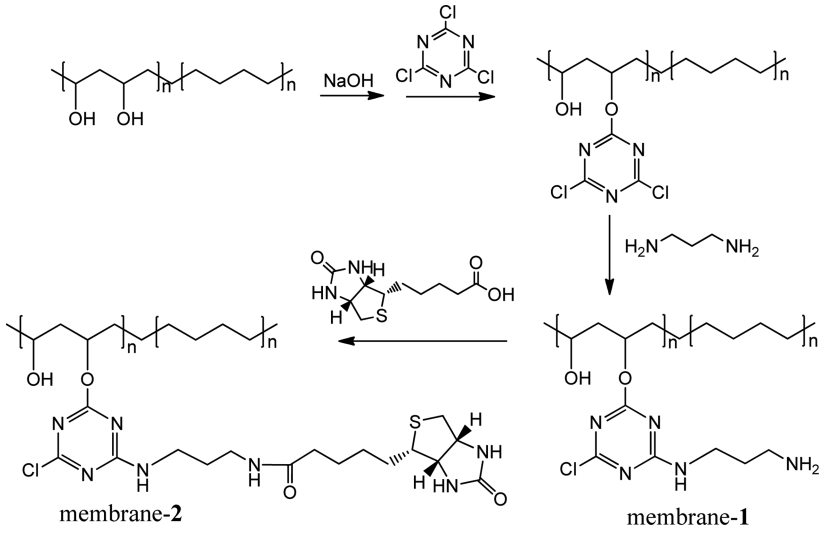
Scheme 1. Reaction Route for Grafting 1,3-Propanediamine and Biotin onto the Surface of Activated PVA-*co*-PE Nanofiber Membrane



amine- and biotin-functionalized membranes. The surface chemical structure and morphologies of nanofibers membranes were characterized by Fourier transform infrared attenuated total reflectance (FTIR-ATR) spectra and scanning electron microscopy (SEM). The hydrophilicity of membranes was tested by water contact angle. The fluorescence intensity from the reaction for ATP detection catalyzed by the firefly-luciferase-immobilized PVA-*co*-PE nanofibers membranes was measured by the fluorescence spectrophotometer.

2. EXPERIMENTAL PROCEDURE

2.1. Materials. Cellulose acetate butyrate (CAB; butyryl content 44−48%) was purchased from Eastman Chemical Company. Poly(vinyl alcohol-*co*-ethylene) (PVA-*co*-PE; ethylene content 44 mol %) with a melt flow index of 5 g/10 min (190 °C/2.16 kg), D-luciferin, adenosine triphosphate (ATP), sodium hydroxide, and cyanuric chloride were purchased from Aldrich Chemical Co. Inc. Firefly luciferase was supplied by ShenYang Zhongke Lianma Bioengineering CO., Ltd. All other organic solvents were purchased from Sinopharm Chemical Reagent Co., Ltd. Meltblown nonwoven fabric (18 g/m^2) was purchased from U.S. Pacific Nonwovens & Technical Texitile Technology Ltd.

2.2. Preparation of PVA-*co*-PE Nanofiber Membrane. The PVA-*co*-PE nanofibers were prepared according to a previously published procedure.[30] Typically, the mixtures of CAB/PVA-*co*-PE powders with blend ratio of 80/20 were gravimetrically fed into a co-rotating twin-screw extruder (Chengrand Research Institute of Chemical Industry, China National BlueStar Co., Ltd.) with 18 mm screw diameter. The feed rate was 12 g/min, and the screw speed was 100 rpm. And the extruder barrel temperature profiles were 180, 190, 200, 210, 220, and 235 °C. The blends were squeezed out in a two strand (2 mm in diameter) rod die. The melt extrudates were hot-drawn at the die exit by a take-up device keeping a drawn ratio of 20−25 (the area of cross section of the die to the extrudate) and air cooled to room temperature. The PVA-*co*-PE nanofibers yarns were prepared by taking off the CAB matrix from the composite CAB/PVA-*co*-PE fibers via a Soxhlet extraction in acetone for 48 h. The technique of preparing PVA-*co*-PE nanofibrous membranes was reported elsewhere.[31] The typical procedure is described as below. PVA-*co*-PE nanofibers were dispersed in an aqueous solution with a high speed shear mixer to form a stable suspension. The suspension was then coated on surfaces of 18 g/m^2 meltblown nonwoven fabric to form nanofibrous membrane. The porosity of prepared PVA-*co*-PE nanofiber membranes was about 70%. The average pore diameter of nanofiber membranes was 190 nm, measured with a capillary flow porometer (CFP-1100A, PMI Inc.). Then the nanofibrous membrane was taken off from the matrix and stored for use.

2.3. Surface Activation of PVA-*co*-PE Nanofiber Membrane. The foursquare PVA-*co*-PE nanofiber membranes with sides of 3 cm were prepared, and then the nanofiber membranes were kept in 3 mol/L sodium hydroxide at 25 °C. After 30 min, the nanofiber membranes were taken out from the alkali liquor and were then air-dried. Afterward, the nanofiber membranes were immersed into 10 wt % cyanuric chloride solution using dioxane as solvent at 30 °C. Two hours later, the nanofiber membranes were rinsed thoroughly with dioxane and deionized water, and then they were air-dried at room temperature.

2.4. Preparation of 1,3-Propanediamine- and Biotin-Functionalized PVA-*co*-PE Nanofiber Membranes. 1,3-Propanediamine and biotin were grafted onto the surface of activated PVA-*co*-PE nanofiber membrane, respectively. The reaction route for grafting 1,3-propanediamine and biotin onto the surface of activated PVA-*co*-PE nanofiber membrane is shown in Scheme 1. A typical procedure is described as below. The solution of 1,3-propanediamine was obtained using ethanol as solvent, and the mass of 1,3-propanediamine and ethanol was 3.0 and 7.0 g, respectively. The activated PVA-*co*-PE nanofiber membrane was immersed into the above solution, and then the mixture was put into the thermostatic water bath shaker at 30 °C for 2 h. The 1,3-propanediamine-functionalized PVA-*co*-PE nanofiber membrane (membrane-1) was washed with ethanol twice and then was dried at room temperature.

The 0.5 wt % $NaHCO_3$ solution was obtained by using 0.5 g of $NaHCO_3$ dissolved in 99.5 g of deionized water. Then 0.05 g biotin was dissolved in 50.0 g of $NaHCO_3$ solution to prepare 0.1 wt % biotin solution. The 1,3-propanediamine-functionalized PVA-*co*-PE nanofiber membrane was immersed into the above 20 mL biotin solution for 2 h in a thermostatic water bath shaker at 30 °C. The biotin-functionalized nanofiber membrane (membrane-2) was washed three times using 0.5 wt % $NaHCO_3$ solution after the reaction, and then it was dried at room temperature.

2.5. Immobilization of Firefly Luciferase on Biotin- and 1,3-Propanediamine-Functionalized PVA-*co*-PE Nanofiber Membranes. The firefly luciferase was dissolved in buffer solution of 0.05 M tris-HCl (pH = 7.8) and 2 mmol/L dithiothreitol (DTT) to prevent enzyme inactivation. The firefly luciferase concentration was 0.5 mg/mL. Then the biotin- and 1,3-propanediamine-functionlized nanofiber membranes were immersed into the above solution, respectively. The time required for immobilizing the firefly luciferase was 24 h at 4 °C under gentle stirring. After being immobilized with firefly luciferase, the PVA-*co*-PE nanofiber membranes were washed with 0.02 M tris-HCl buffer solution (pH = 7.8) containing 2 mM EDTA and 6 mM

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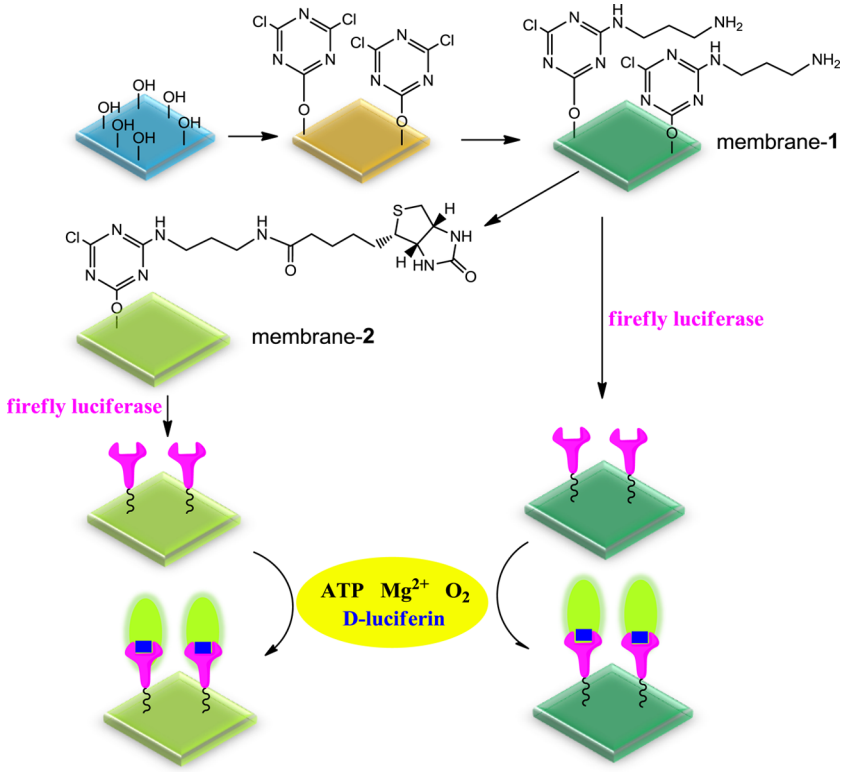
Scheme 2. Routes of Design and Mechanism of Luminescent Detection of ATP Using Functional PVA-*co*-PE Nanofiber Membranes



DTT, respectively. And then they were routinely stored at 4 °C. The routes for design and mechanism of luminescent detection of ATP using functional PVA-*co*-PE nanofiber membranes is shown in Scheme 2.

2.6. Characterization. *2.6.1. Morphology and Surface Structure of Pristine, Surface-Activated, and Luciferase-Immobilized PVA-co-PE Nanofiber Membranes.* The morphologies of PVA-*co*-PE nanofibers were examined using a SIRION scanning electron microscope. The chemical structure of pristine, surface-activated and luciferase-immobilized nanofibers were characterized by FTIR-ATR (Tensor 27, Bruker). The surface element contents of each membrane were detected by X-ray photoelectron spectroscopy (XPS; VG Multilab 2000, Thermo) with Al Kα as X-ray optical source (15 kV, 10 mA) and an initiation angle of 90 °C. The contact angle of PVA-*co*-PE nanofiber membranes was measured by using a Kruss DSA30S instrument.

In addition, the content of amine group on the surface of 1, 3-propanediamine- and biotin-functionalized nanofiber membranes was defined by titration method as below. The 1, 3-propanediamine- and biotin-functionalized nanofiber membranes was weighed and then immersed into HCl solution (V(HCl) = 10 mL, c(HCl) = 0.001 mol/L), respectively. The membranes were kept in HCl solution for 3 h to make the amine group on the membrane surface react with HCl adequately. Then the supernatant solution of 5 mL was taken out for titration with NaOH solution (c(NaOH) = 0.001 mol/L). The content of amine group was defined as $c(NH_2) = [c(HCl)V(HCl) - c(NaOH)V(NaOH) \times 2]/m(membrane)$, where c(HCl) and V(HCl) are the concentration and volume of HCl solution, c(NaOH) and V(NaOH) are the concentration and volume of NaOH solution, and m(membrane) is the mass of 1,3-propanediamine-functionalized or biotin-functionalized nanofiber membrane.

2.6.2. Catalytic Activity of Firefly-Luciferase-Immobilized PVA-co-PE Nanofiber Membranes. Two kinds of foursquare firefly-luciferase-immobilized PVA-*co*-PE nanofiber membranes with the sides of 1 cm were immersed into standard transparent quartz cuvettes with the solution consisting of 5 mM $MgCl_2$, 4 mM DTT, 5 mM tris-HCl buffer solution (pH = 7.4), and 0.2 mM luciferin. And the total volume of above solution mixture was 1.5 mL. Then the mixture containing 0.2 M tris-HCl buffer solution (pH = 7.4) and 2×10^{-7} M ATP was injected into the quartz cuvettes, respectively, and was mixed with the luciferin solution rapidly. After these steps, the cuvettes were placed in the test chamber of the fluorescence spectrophotometer (Hitachi F4600) to detect the fluorescence intensity.

3. RESULTS AND DISCUSSION

3.1. The Surface Chemical Structure of PVA-*co*-PE Nanofiber Membranes. The hydroxyl groups in PVA-*co*-PE nanofibers survived in the extrusion process and were still able to react with cyanuric chloride after the treatment with 3 M sodium hydroxide solution. The FTIR-ATR spectra of surface-activated, 1,3-propanediamine-functionalized, and biotin-functionalized PVA-*co*-PE nanofiber membrane are shown in Figure 1, and the FTIR spectrum of pristine PVA-*co*-PE nanofiber membrane is also shown for comparison. From Figure 1b, it can be seen that two new and sharp absorption peaks at 1504 and 1550 cm^{-1} appeared and they were assigned to the planar triazine rings in the cyanuric chloride.[25] In Figure 1c, 1640 and 1577 cm^{-1} can be assigned to the absorption peaks of amine group from 1,3-propanediamine.[32] Moreover, the characteristic features of the spectrum of biotin-functionalized PVA-*co*-PE nanofiber membrane are peaks at 1666 cm^{-1} (amide I band, C═O stretching vibration), and 1565 cm^{-1} (amide II band, N−H bending vibration).[33]

The chemical structure and element contents of membranes surface were also investigated by XPS, and the results are shown in Figure 2 and Table 1, respectively. From Figure 2, it can be seen that the peaks for nitrogen (N 1s) at 402 eV appeared after the activation by cyanuric chloride. Moreover, the content of nitrogen was different in the different functionalized membranes, and the peak for sulfur of the

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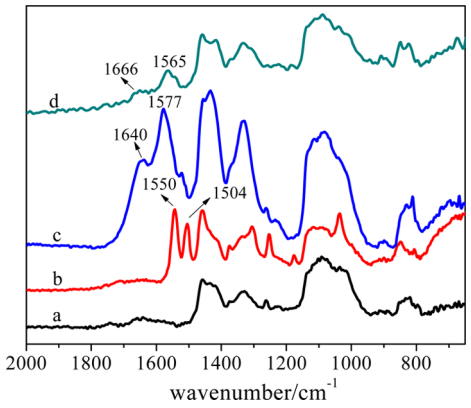

Figure 1. FTIR-ATR spectra of (a) pristine PVA-*co*-PE nanofiber membrane, (b) surface-activated PVA-*co*-PE nanofiber membrane by cyanuric chloride, (c) membrane-1, and (d) membrane-2.

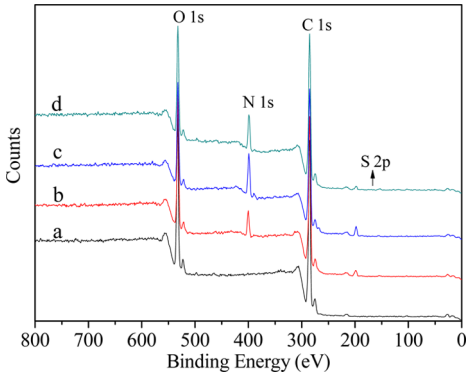

Figure 2. XPS spectra of (a) the pristine PVA-*co*-PE nanofiber membrane, (b) the surface-activated PVA-*co*-PE nanofiber membrane, (c) membrane-1, and (d) membrane-2.

Table 1. Chemical Element Contents of the Pristine, Surface-Activated, Membrane-1, and Membrane-2

nanofiber membranes	element composition (%)			
	C	O	N	S
pristine	77.97	22.03		
surface-activated	72.92	20.2	6.88	
1,3-propanediamine-functionalized	70.59	16.78	12.63	
biotin-functionalized	73.18	17.07	9.68	0.07

biotin at 167 eV can be seen in Figure 2d.[34] It meant that the surface activation and functionalization with cyanuric chloride, 1,3-propanediamine, and biotin were successfully carried out and 1,3-propanediamine-functionalized and biotin-functionalized membranes were obtained. Furthermore, according to the titration measurement, the content of amine groups (NH_2) on the surface of 1,3-propanediamine-functionalized nanofiber membrane was 0.63 mmol/g, and the content of amine groups (NH_2) on the surface of biotin-functionalized nanofiber membrane decreased to 0.59 mmol/g. It was ascribed to that some amine groups (NH_2) on the 1, 3-propanediamine-functionalized nanofiber membrane reacted with biotin,[35] which can be seen from Scheme 1. Therefore, the content of biotin on the membrane surface was 0.04 mmol/g.

3.2. Morphologies of PVA-*co*-PE Nanofiber Membranes. The morphologies of pristine and surface-functionalized nanofiber membranes were characterized by SEM, and the images are shown in Figure 3. From Figure 3, it can be found that the average diameter of the nanofibers was in the range of 200−300 nm and all the nanofibers maintained well-defined nanofibrous but randomly distributed morphologies. Especially, the maintenance of nanofibrous morphology of the luciferase-immobilized nanofiber membrane is beneficial for keeping the catalytic activity in the process of detecting ATP.

3.3. Surface Hydrophilicity. As we know, the hydrophilicity of the membrane surface directly influences the accessibility of surface active sites on the nanofibers.[36] Moreover, compared to the hydrophobic surface, the electrostatic interaction between luciferase and the polar groups on the hydrophilic surface was stronger.[37] The water contact angle test was used to monitor the hydrophilicity of the membrane surfaces, and the results are shown in Figure 4. From Figure 4, it can be seen that the contact angle of the pristine PVA-*co*-PE nanofiber membrane was 106° while the contact angles of 1,3-propanediamine functionalized and biotin-functionalized PVA-*co*-PE nanofiber membranes were 86° and 65°, respectively. Therefore, the hydrophilicity of the nanofiber membrane's surface was improved after functionalization with 1,3-propanediamine and biotin, and the hydrophilicity of the biotin-functionalized nanofiber membrane was stronger.

3.4. The Catalytic Property of Firefly-Luciferase-Immobilized Nanofiber Membrane. The catalytic property of firefly-luciferase-immobilized nanofiber membrane was evaluated by detecting ATP in the solution mixture including $MgCl_2$, DTT, tris-HCl buffer solution, and luciferin. The photos for solution mixture and detecting ATP using firefly-luciferase-immobilized nanofiber membrane are shown in Figure 5. Furthermore, the fluorescence intensity for different samples was measured by using a fluorescence spectrophotometer, and the effect of ATP concentration on relative light unit (RLU) of different samples is demonstrated in Figures 6 and 7. And the fluorescence intensity from the reaction with catalysis of free luciferase versus time at the ATP concentration of negative exponent (mol/L) of 7 was also investigated for comparison, which is shown in Figure 8.

In Figure 6, it is shown that the RLU from the catalysis reaction decreased with the decreasing of ATP concentration from 1×10^{-5} to 1×10^{-11} mol/L. The RLU was about 4500 when the ATP concentration was 1×10^{-5} mol/L, but the RLU was about 150 when the ATP concentration was decreased to 1×10^{-11} mol/L. Moreover, when the ATP concentration was in the range of 1×10^{-9}−1×10^{-11} mol/L, the RLU varied rarely, which implied the detection sensitivity of firefly-luciferase-immobilized membrane-**1** decreased obviously. In Figure 7, it can be seen that the RLU also decreased with the decrease of ATP concentration from 1×10^{-5} to 1×10^{-11} mol/L. However, when the ATP concentration was 1×10^{-5} mol/L, the RLU was about 6.76×10^6 which was much more than that resulted from the catalysis of firefly-luciferase-immobilized membrane-**1**. While the ATP concentration was decreased to 1×10^{-11} mol/L, the RLU was about 660. In addition, the change rate of RLU as ATP concentration varying was larger than that in Figure 6. Therefore, it can be concluded that the firefly-luciferase-immobilized membrane-**2** possessed better catalytic property and the detecting sensitivity was stronger. It is ascribed to that the binding activity between luciferase and more hydrophilic surface modified by biotin was stronger and more luciferase was adsorbed onto the surface of nanofiber membrane.[37] The catalytic activity and detecting sensitivity of nanofiber membrane functionalized by biotin was improved. Comparing with the RLU in Figure 8, we can clearly see that

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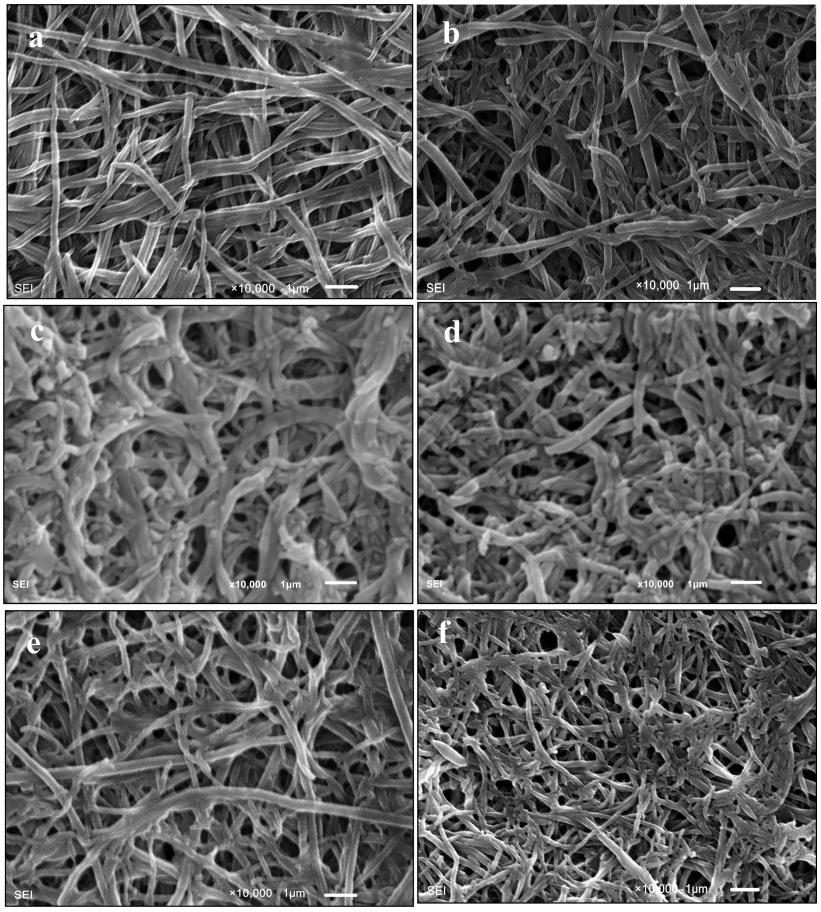

Figure 3. SEM images of PVA-*co*-PE nanofiber membranes: (a) pristine membrane, (b) surface-activated membrane, (c) membrane-1, (d) membrane-2, (e) firefly-luciferase-immobilized membrane-1, and (f) firefly-luciferase-immobilized membrane-2.

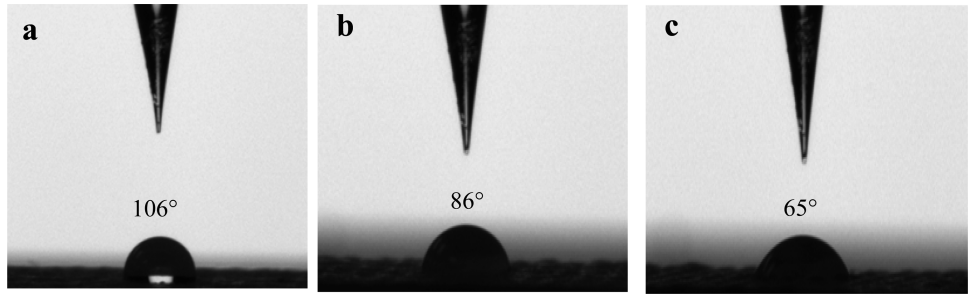

Figure 4. Contact angle of (a) pristine PVA-*co*-PE nanofiber membrane, (b) membrane-1, and (c) membrane-2.

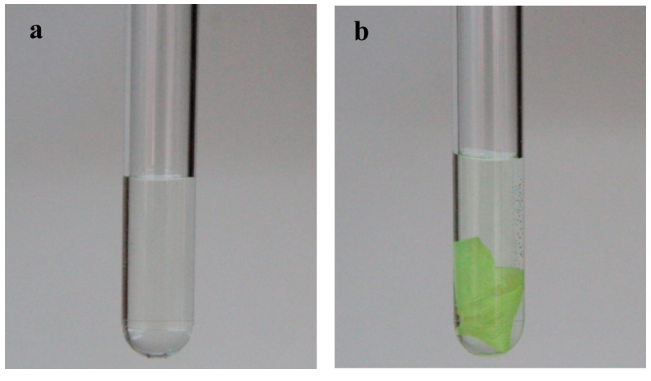

Figure 5. Photos for (a) solution mixture and (b) detecting ATP using firefly-luciferase-immobilized nanofiber membrane.

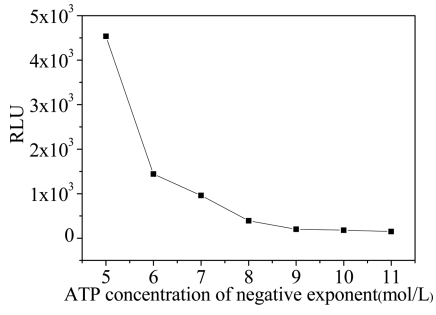

Figure 6. Effect of ATP concentration on RLU for firefly-luciferase-immobilized membrane-1.

the RLU from the catalysis of free luciferase was much higher than that of immobilized luciferase at the same ATP

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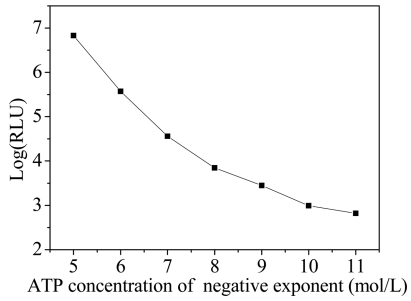

Figure 7. Effect of ATP concentration on RLU for firefly-luciferase-immobilized membrane-2.

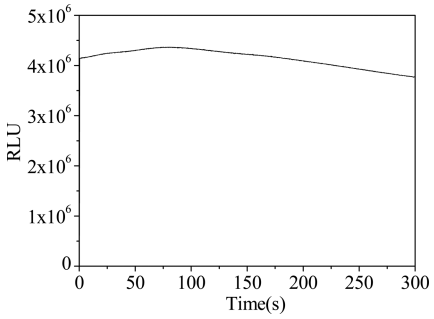

Figure 8. RLU from the reaction with catalysis of free luciferase versus time when ATP concentration of the negative exponent (mol/L) was 7.

concentration. Therefore, the immobilization of firefly luciferase could decrease its catalysis activity in the reaction, which is in agreement with the reference.[14,18] However, the catalysis activity of firefly luciferase immobilized on the biotin-functionalized nanofiber membrane in this work was higher than that of firefly luciferase immobilized on Langmuir–Blodgett films and PS beads at the similar concentration of ATP.[14,18]

4. CONCLUSION

The PVA-*co*-PE nanofiber membrane was first activated by cyanuric chloride, and then 1,3-propanediamine- and biotin-functionalized nanofiber membranes with different surface hydrophilicity were prepared for immobilizing firefly luciferase. The surface chemical structures of PVA-*co*-PE nanofiber membranes were characterized by FTIR-ATR spectra, and the surface morphologies of nanofiber membranes were investigated by SEM. The contact angle test demonstrated that the surface of biotin-functionalized nanofiber membrane possessed better hydrophilicity, and that the binding activity between firefly luciferase and more hydrophilic surface was stronger to be beneficial for the catalytic reaction in the process of detecting ATP. Therefore, the firefly-luciferase-immobilized nanofibers membrane which was functionalized by biotin can be a promising candidate as a biosensor for bioluminescent detection of ATP because of its high detection sensitivity. Furthermore, the detailed research about the reusability and other characterization of firefly-luciferase-immobilized nanofiber membrane will be reported in the future work.

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Author Contributions

†W.W. and Q.Z. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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