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Research Article

A Novel and Disposable Enzyme-Labeled Amperometric Immunosensor Based on MWCNT Fibers for *Listeria monocytogenes* Detection

Ying Lu,[1] Yongling Liu,[1] Yong Zhao,[1] Wenjiao Li,[1] Longbing Qiu,[2] and Li Li[1,2,3]

[1]*College of Food Science and Technology, Laboratory of Quality & Safety Risk Assessment for Aquatic Products on Storage and Preservation, Ministry of Agriculture, Shanghai Engineering Research Center of Aquatic-Product Processing & Preservation, Shanghai Ocean University, 999 Hucheng Huan Road, Shanghai 201306, China*
[2]*Laboratory of Advanced Materials, Fudan University, Shanghai 200438, China*
[3]*Research & Development Center of Food Thermal-Processing Technologies (Asia), Shanghai Ocean University, 999 Hucheng Huan Road, Shanghai 201306, China*

Correspondence should be addressed to Li Li; l-li@shou.edu.cn

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A sensitive and specific immunosensor was developed by immobilizing HRP-labeled antibody against *Listeria monocytogenes* onto the surface of the novel multiwalled carbon nanotube fibers. Firstly, the influence of immunoelectrode modification methods (chemical and physical method) on detection sensitivity and stability was studied. Next, immunosensor was developed, optimized, and applied for the detection of *L. monocytogenes*. The morphology was characterized by scanning electron microscopy (SEM), and electrochemical behaviors were by cyclic voltammetry. SEM image, relative response (%), and current data showed chemical modification for immunoelectrode was helpful to capture more target bacteria and obtain more stable current response, resulting in improving the detection sensitivity. The linear relationship between *L. monocytogenes* concentration and ΔI_{pc} was from 10^2 to 10^5 cfu/mL ($R^2 = 0.993$), and LOD was 1.07×10^2 cfu/mL. *L. monocytogenes* in mixed bacteria (1.51×10^3 cfu/mL) of milk sample (S/N > 14) were detected by developed immunosensor, showing good specificity. Good storage stability and reproducibility (RSD < 6.5%) also showed the potential application of immunosensor for the rapid detection of *L. monocytogenes*.

1. Introduction

Listeria monocytogenes (LM) is one of the most deadly foodborne pathogen. It can be found in not only the majority of unprocessed foods of animal origin [1], but also the ready-to-eat food products. So the attention should be strengthened to daily diet [2]. Meanwhile, in most countries, there is no tolerance about LM in foods. So it becomes very important to find effective methods to detect the LM.

Some traditional techniques of detecting the LM have been developed and applied [3]. These methods include typical plate colony counting [4, 5], polymerase chain reaction assay [6], loop-mediated isothermal amplification [7], and immunoassay-based methods such as enzyme-linked immune sorbent assay [3] and immunochromatographic lateral flow assay [8]. For these techniques, the grand challenges include complex pretreatment procedures; specialized or expensive laboratory facilities; skilled technical personnel [9, 10]; and false positive identification. So the rapid, simple, and low-cost methods are urgently needed, for example, sensors.

At the state of art the typical sensors are based on Surface Plasmon Resonance (SPR) [11], optics sensor [12], electrochemistry immunosensor [13], and so forth. Ascribed to the fast response, convenience, sensitivity, and high selectivity, the electrochemistry immunosensors have been applied in many fields, for example, hormones [14], toxins, pesticides, and pathogens [15].

For the immunosensor, based on the requirements of electrical conductivity, biocompatibility, and electron transfer rate, the selection of electrode material is very important. Carbon nanotubes, especially the MWCNT fibers, possess excellent mechanical and electrical properties and are

promising for many potential fields [16–19]. For MWCNT fibers, many CNT strands twisted along the axial direction of fiber, wind together with a uniform diameter, and have a high degree of local crystalline order. These CNT strands consist of highly aligned and multiwalled CNTs with the certain interlamellar distance [20, 21]. These enable the MWCNT fibers to have high tensile strengths and electrical conductivities [22]. Meanwhile, it has large specific surface area, stability, and biological compatibility. For the enzyme-labeled immunosensor, the selection of enzyme could affect the sensitivity analysis and accuracy. So the labeled enzyme should have the features of high purity, high conversion rate, and ease to label and save. For these features, the horseradish peroxidase (HRP) was selected to label the antibody [23].

In this paper, the immunosensor based on MWCNT fibers for detection of LM was fabricated. This study focuses on the application of new materials, immobilization of (HRP) labeled antibody and the chosen immobilization methods, and optimization of surface modification conditions. Thus, the conditions made a contribution to the improvement of sensitivity.

2. Experimental Section

2.1. Materials and Instruments. Novel multiwalled carbon nanotube (MWCNT) fibers are prepared according to our previous study [20]. Thionine was purchased from Jiang Lai Biological. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and bovine serum albumin (BSA) were purchased from Sigma-Chemicals (St. Louis, MO, USA). Lightning-Link HRP conjugation kit was purchased from Innova Biosciences. The bacterial strains *L. monocytogenes* ATCC19115 were purchased from American Type Culture Collection, USA. The antibody against *L. monocytogenes* was prepared by our experiment. 30% H_2O_2 was purchased from Sinopharm Chemical Reagent Co., Ltd. (China), and all reagents were of analytical reagent grade.

Cyclic voltammetric measurements were characterized with a CHI 660C electrochemistry workstation (Shanghai CH Instruments, Shanghai, China). A three-electrode system was used for electrochemical studies: the modified immunoelectrode as the working electrode, platinum electrode as the auxiliary electrode, and Ag/AgCl electrode as the reference electrode.

2.2. Preparation of MWCNT Fibers Electrode. The MWCNT fibers were prepared according our early study [20, 22]. The preparation included growth of MWCNT arrays and spinning of fiber. Carbon nanotube arrays were synthesized by a chemical vapor deposition in a quartz tube furnace using Fe (1 nm)/Al_2O_3 (10 nm) on silicon wafer as the catalyst, ethylene (40 sccm) as carbon sources, and a mixture of Ar (560 sccm) and H_2 (40 sccm) gases as carrying gas. The reaction was typically performed at 750°C for 20 min. MWCNT fibers were spun from MWCNT arrays with controlled diameters from 2 to 30 μm and lengths up to 100 m. MWCNT fibers were spun with a spindle rotating at 2000 rpm and drawing at 10 cm/min. Then, the MWCNT fibers were immerged in a glass beaker with H_2SO_4 : HNO_3 mixture (3 : 1). The beaker was heated in water bath during 120 min; afterward, the MWCNT fibers were then washed with water and dried to air. The carboxylic MWCNT fibers electrode could be used directly to combine with biological molecules. It also could avoid the current decrease caused by embedding material. The morphologies of MWCNT arrays, MWCNT fibers, and MWCNT fibers electrode were shown in Figures 1(a), 1(b), and 1(d).

2.3. Fabrication of Immunoelectrode. The HRP-anti-LM antibodies were prepared according to the guideline of Lightning-Link HRP conjugation kit. The immunoelectrode was obtained by the following methods.

For the physical immobilized method, the antibodies were dropped onto the electrode surface directly and reacted for 3 h after washing with phosphate buffer sodium (PBS).

For the chemical immobilized method. First, the electrode was washed with 0.01 M pH 7.4 PBS for 3 times. After drying, it was mixed with 0.5 M EDC and 0.25 M NHS in PBS and shaked for 2 h. Secondly, after washing with PBS and drying, the HRP-anti-LM antibodies were dropped onto the surface of electrode and reacted for 3 h at room temperature. The electrode was rinsed with PBS for 3 times. Finally, 0.2% BSA in PBS was added to the surface of electrode to block the uncombined sites for 30 min at room temperature. The obtained immunoelectrode was stored at 4°C for further experiments.

The fabrication of immunoelectrode was depicted in Figure 1(c).

2.4. Electrochemical Measurements. Cyclic voltammetry (CV) and scanning electron microscopy (SEM) were selected as the methods of electrochemical measurements and characterization. When the steady current response of electrode was observed and recorded, a certain amount of H_2O_2 was added to the solution. After reacting with HRP, the change of current response occurred and steady current was recorded (I_1). Afterwards, the electrode was dipped into a certain concentration of bacteria suspension of *L. monocytogenes* and incubated for 30 min. The test current was recorded (I_2). The measurement was operated via a three-electrode system in an electrochemical cell containing 0.1 M PBS, 0.1 M NaCl, and 1 mM thionine as conductive liquid. The working potential was in the range of –1.0~0.5 V that was relative to Ag/AgCl reference electrode and the scan rate was 0.1 V/s. The detection of LM was based on the current changes (ΔI_{pc}) before and after the combination of antigen-antibody. In the text, $\Delta I_{pc} = I_1 - I_2$, and relative response (%) = $(I_1 - I_2)/I_1 \times 100\%$.

2.5. Optimization Operation. In order to get the best signal response, the amount of antibody (4.95, 14.85, and 24.75 ng), pH (6.2, 6.6, 7.0, 7.4, and 7.8), concentration of H_2O_2 (the volume of test liquid was same) (0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 mmol/L), and incubation temperature (20, 25, 30, 37, and 40°C) were selected to be optimized. The test process for optimization was according to Section 2.4.

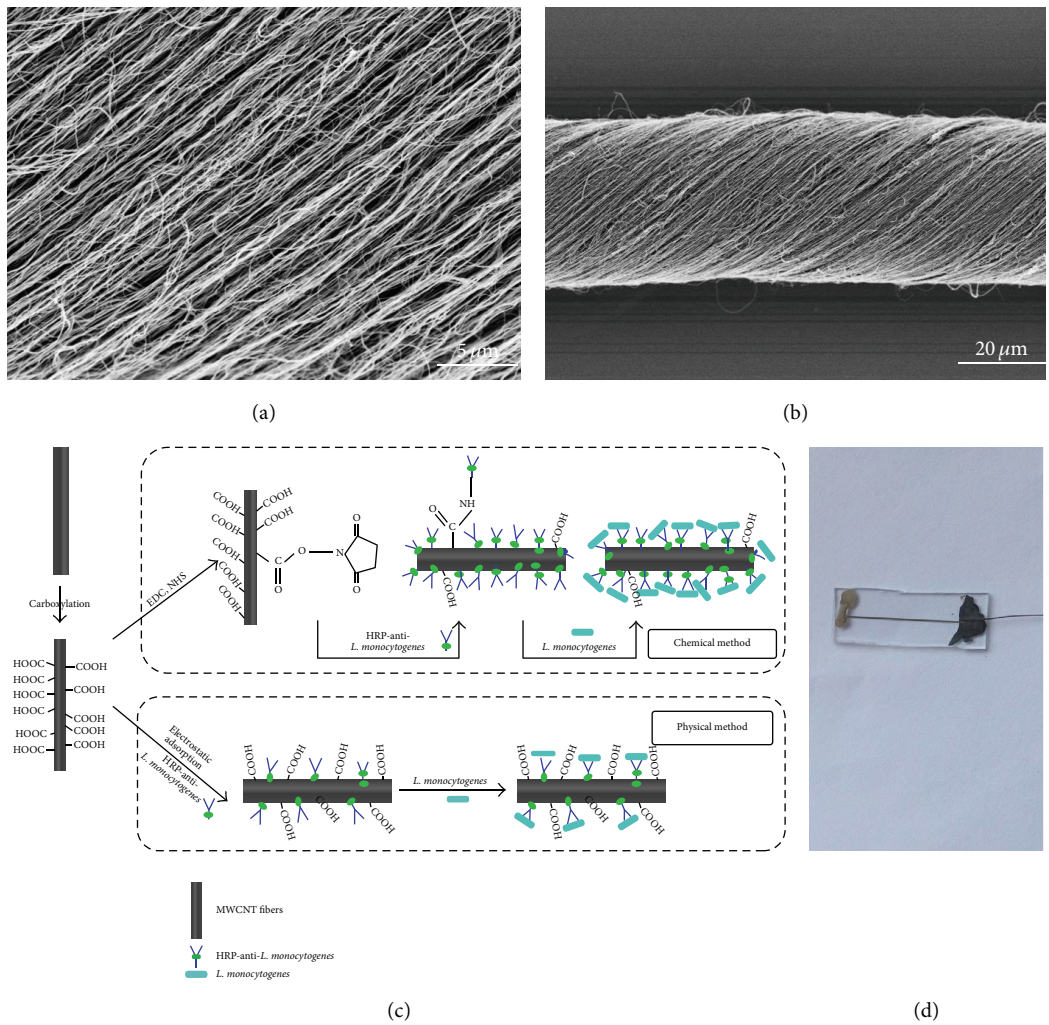

FIGURE 1: The images of MWCNT fibers and fabrication illustration of the immunoelectrode. (a) The SEM image of MWCNT arrays. (b) The SEM image of spun MWCNT fibers. (c) Illustration of the immunoelectrode fabrication by chemical and physical immobilization methods. (d) The fabricated immunoelectrode.

2.6. Actual Sample Processing. Milk sample was used to investigate the specificity of the immunosensor. 20 mL milk sample was added to the 180 mL 0.1 M pH 7.0 PBS and the 10-fold milk sample dilution solution was obtained. Two sample copies of dilution solution with and without 2 mL *L. monocytogenes* 19112, 19114, 19115, 19116, and 19118 addition were prepared. Afterwards, each 2 mL *Pseudomonas aeruginosa* (Pa), *Salmonella* (Sa), and *Vibrio parahaemolyticus* (Vp) were added into the above solution. The milk dilution without bacteria was regarded as control. All the experiments were operated at the sterile conditions.

3. Results and Discussion

3.1. Electrochemical Characterizations of Immunoelectrode. In order to verify whether the immunosensor was built successfully, the CV was used to characterize the current change of electrode before and after modification. The results of current changes were displayed in Figure 2. Compared to the redox peak of bare-MWCNT fibers electrode, the peak current decreased after HRP-anti-LM absorbing onto the surface of MWCNT fibers (curve (b)). This is because the immobilization of antibody causes a barrier for the electron transmission on the space. After addition of H_2O_2, the peak current increased greatly (curve (c)). It was due to the release of electrons when H_2O_2 was catalyzed by the HRP. But after incubating with *L. monocytogenes*, the peak current reduced once again (curve (d)). For that the transmission of electron was hindered by the formation of immunocomplexes. According to the above phenomenon, we can judge that the immunosensor was built successfully.

3.2. Influence of Immobilization Method on Performance of Immunosensor

3.2.1. Influence of Immobilization Method on SEM Images of Electrode. Scanning electron microscopy (SEM) images (Figure 3) showed the surface morphology of the immunoelectrode prepared by different coating method. As shown in Figures 3(a) and 3(b), HRP crystallizations were both

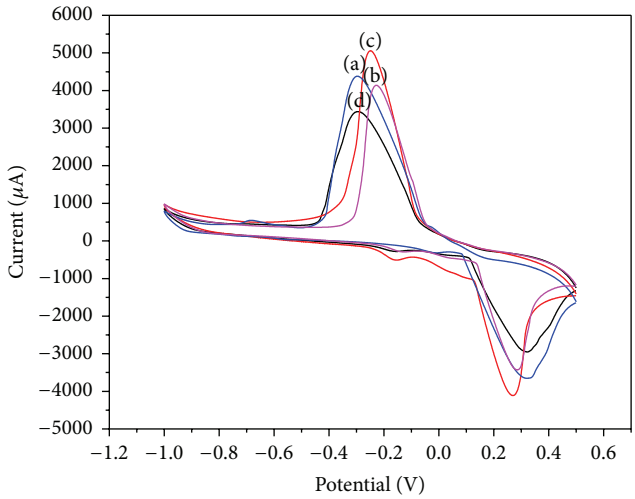

Figure 2: Cyclic voltammograms of the different modified electrodes in 0.1 M pH 7.4 PBS containing 1.0 mM thionine, 0.5 mM H_2O_2, and 0.1 M NaCl at room temperature; scan rate, 100 mv/s. (a) Bare-MWCNT fibers electrode. (b) HRP-anti-*L. monocytogenes* labeled immunoelectrode. (c) H_2O_2 combined with immunoelectrode. (d) Immunoelectrode incubated with *L. monocytogenes* at 37°C, 30 min.

observed on the surface of immunoelectrode fabricated in physical and chemical immobilization method. Clearly, the amounts of HRP-anti-*L. monocytogenes* antibodies were almost equal for the physical immunoelectrode and the surface of chemical immunoelectrode. However, for the immunoelectrode fabricated prepared by chemical method, many small and dense HRP crystallizations were observed not only on the surface, but also in the internal parts. In addition, after incubating with target bacteria, biological molecules captured by the immunoelectrode modified by physical method were less than those by chemical method (Figures 3(c) and 3(d)). Figures 3(a1), 3(b1), 3(c1), and 3(d1) were the corresponding magnified images of Figures 3(a), 3(b), 3(c), and 3(d). According to the compared data, obviously, the immobilization rate and capture rate for target bacteria of immunoelectrode modified by chemical method were higher than those by physical method. Maybe for that the crosslinking agent of EDC and NHS could activate the groups and expose a part of groups from inner layer to surface, and the antibodies could better combine with activated groups. For physical adsorption, the antibodies only combined with surface groups and easily formed big crystallizations for randomness. Therefore, the chemical immobilization method could improve the sensitivity.

3.2.2. Influence of Immobilization Method on Current Response and Stability of Electrode. In order to further verify the influences of immobilized methods on the performance of immunosensor, the change of relative response (%) before and after antigen-antibody reaction and test operational stability were discussed in Figure 4. In Figure 4(a), for the change of relative response (%), it is significantly higher in chemical method based electrode (19.96%) than physical method (6.8%). Therefore, we can see that the chemical method may influence the sensitivity of immunosensor.

In addition, the test operational stability of every five minutes during the test time was compared to that in Figure 4(b). The current after six times was 96.63% of its original current in the test for the chemical method. However, the current after four-time test decreased to 61.5% of its original current for the physical method. Thus, we can obtain that the chemical method could have better stability and accuracy than physical method.

According to the comparison, the chemical immobilization method could improve the accuracy, sensitivity, and stability of immunosensor. Therefore the chemical method is more suitable for immobilizing antibody than physical method.

3.3. Optimization of Test Conditions. Many studies have shown that the amount of antibody, pH, H_2O_2 concentration, and incubation temperature have great influences on the performance of immunosensor [24–26]. The influence of the amount of HRP-antibody on the current of immunosensor was shown in Figure 5(a). It was observed that the relative response (%) before and after incubation with LM changed greatly when antibody amount is 14.85 ng.

In general, the electrochemical properties of immunosensor and activity of enzyme are sensitive to the pH of test buffer. In order to verify the influence, the electrochemical measurement was performed in the pH from 6.2 to 7.8 PBS. As shown in Figure 5(b), the relative response (%) firstly increased with the increase of pH (6.2–7.4) but decreased with further increase of pH (7.4–7.8). The maximum change of current response occurred at pH 7.4. It was reported that acidic environment could affect the activity of HRP and antibody which caused the change of current response [27]. Thus pH 7.4 was selected for further experiment.

The H_2O_2 concentration is very important to sensitivity and could cause the inactivation of the enzyme by high concentration of H_2O_2 [28]. The effect of H_2O_2 concentration on the current response was shown in Figure 5(c). The maximum relative response (%) was at the 0.6 mM of H_2O_2, and the value decreased with the further increase of H_2O_2 concentration. Thus, 0.6 mM H_2O_2 was used for the detection of LM.

Figure 5(d) displayed the effect of incubation temperature on the current change of the immunosensor from 20 to 40°C. The current change was the highest at 37°C but decreased sharply when the temperature was over 37°C. Temperature has a large influence on the binding of antigen-antibody, the sensitivity of enzyme, and immunoreaction rate; however, the high temperature could cause the denaturation of protein to a certain extent [29]. According to our data, the current change response was similar between 25°C and 37°C. Thus, 25°C was selected as the working temperature considering the convenience of actual operation.

3.4. Electrochemical Immunoassay and Performance Assessment. The detection of *L. monocytogenes* was according to the change of direct electrochemical signal caused by

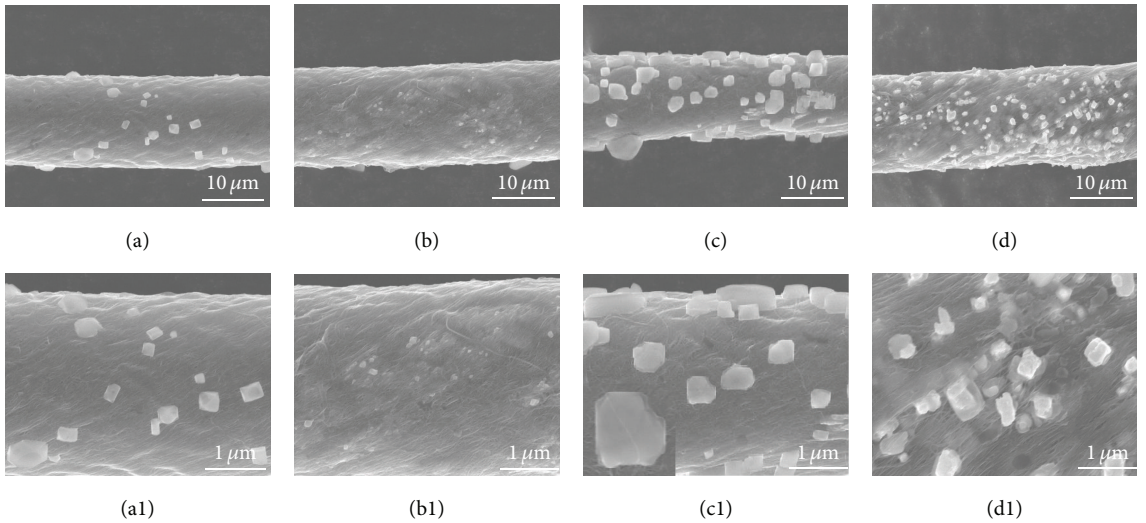

Figure 3: SEM images of different immunoelectrode. (a) The image of immunoelectrode fabricated by physical method. (b) The image of immunoelectrode fabricated by chemical method. (c) The image of immunoelectrode in physical method incubated with *L. monocytogenes*. (d) The image of immunoelectrode in chemical method incubated with *L. monocytogenes*. (a1), (b1), (c1), and (d1) were the corresponding magnified images of (a), (b), (c), and (d).

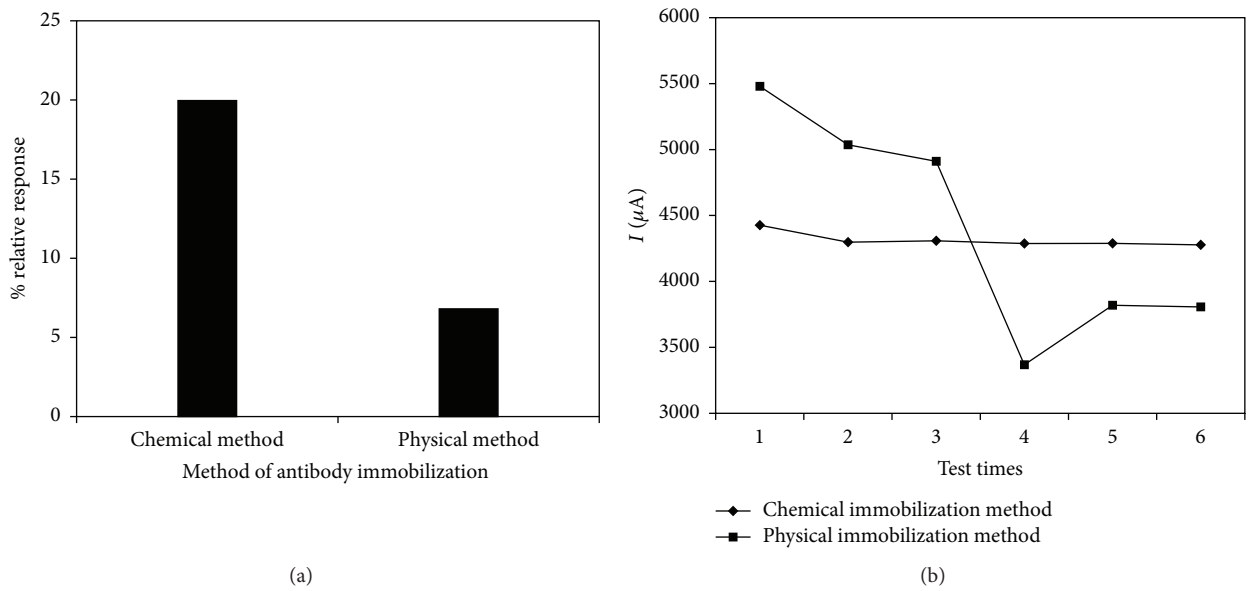

Figure 4: The influence comparison of different immobilization methods on the performance of immunosensor. (a) The change of current response before and after antigen-antibody combination for different methods. (b) Test operational stability for different methods.

the binding of antigen and antibody. There is the access of the active center of HRP to thionine. The access would be hindered after the binding of the antigen and HRP-antibody in the incubation solution [30]. Therefore, the CV response decreased with the binding of *L. monocytogenes* antigen and its specific antibody. Figure 6(a) showed the change of current (ΔI_{pc}) before and after incubation under optimal conditions. ΔI_{pc} has a linear relationship with the logarithm of *L. monocytogenes* concentration ($\log_{10}C$) ranging from 10^2 to 10^5 cfu/mL with a high correlation coefficient of 0.993. Thus, the immunosensor of detecting LM has been successfully developed.

3.5. Performance Assessment of Immunosensor. Specificity, storage stability, and reproducibility are very important for immunosensor. In order to evaluate the specificity of the immunosensor for *L. monocytogenes* detection, mixture bacteria of Pa, Sa, and Vp including and excluding *L. monocytogenes* 19112, 19114, 19115, 19116, and 19118 in milk were tested by the developed immunosensor. As shown in

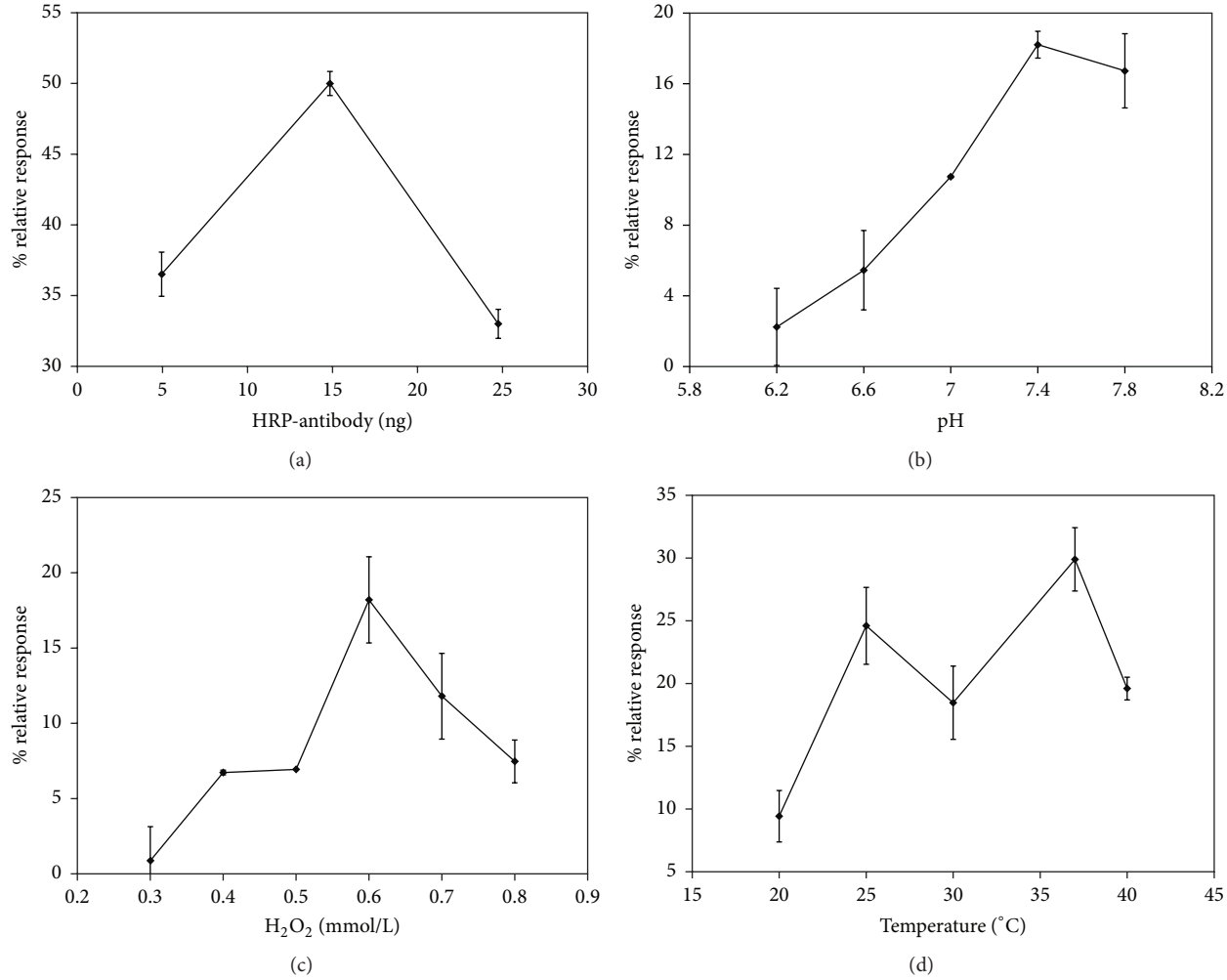

Figure 5: Influence of experiment conditions on the performance of immunosensor. (a) Influence of the immobilization amount of HRP-antibodies on the response of immunosensor. (b) Effect of buffer solution pH on the current response of immunosensor. (c) Influence of H_2O_2 concentration on the immunosensor current response. (d) The effect of incubation temperature to the properties of immunosensor.

Figure 6(b), the current change of mixture bacteria with LM was greatly larger than that negative sample without LM (S/N > 14). The result was higher than reported previously [31], indicating that the immunosensor was highly specific for *L. monocytogenes* detection and its potential in the development of the high sensitive immunosensor.

The long-time storage stability as a parameter to evaluate the performance of immunosensor was shown in Figure 6(c). The storage stability for 25 days at 4°C was measured by the CV using the same electrode. Clearly, in the first 20 days, the current was retained 97.1% of its original current. For further test, the current reduced substantially. At the end of 25 days, the current has reduced to 80% of its original current. It may be for the reduction of enzyme activity and the shedding of immobilized antibodies [24]. Compared with the previous report [24], the value was larger. It was due to the chemical immobilization method of antibody and good biocompatibility of electrode.

The reproducibility of the immunosensor was measured by choosing different batches of the electrode to detect the same samples under the same operating conditions. The relative standard deviation (RSD) of *L. monocytogenes* (10^6 cfu/mL) was calculated to be 6.18%. This result indicated that the immunosensor has good fabrication reproducibility.

4. Conclusion

In this paper, we have successfully developed an HRP-labeled, sensitive, stable immunosensor based on the novel MWCNT fibers electrode. Chemical and physical immobilization methods were used to fabricate the immunosensor by immobilizing HRP-anti-*L. monocytogenes* antibodies. Our results showed that chemical method for immobilizing has better performance on the accuracy, sensitivity, and stability of immunosensor. The amount of antibody, pH, incubation temperature, and H_2O_2 concentration were optimized under different gradient. Our results showed that optimized conditions could significantly enhance the performance of immunosensor. Under optimal conditions, the immunosensor array gave a corrected linear type response from 10^2

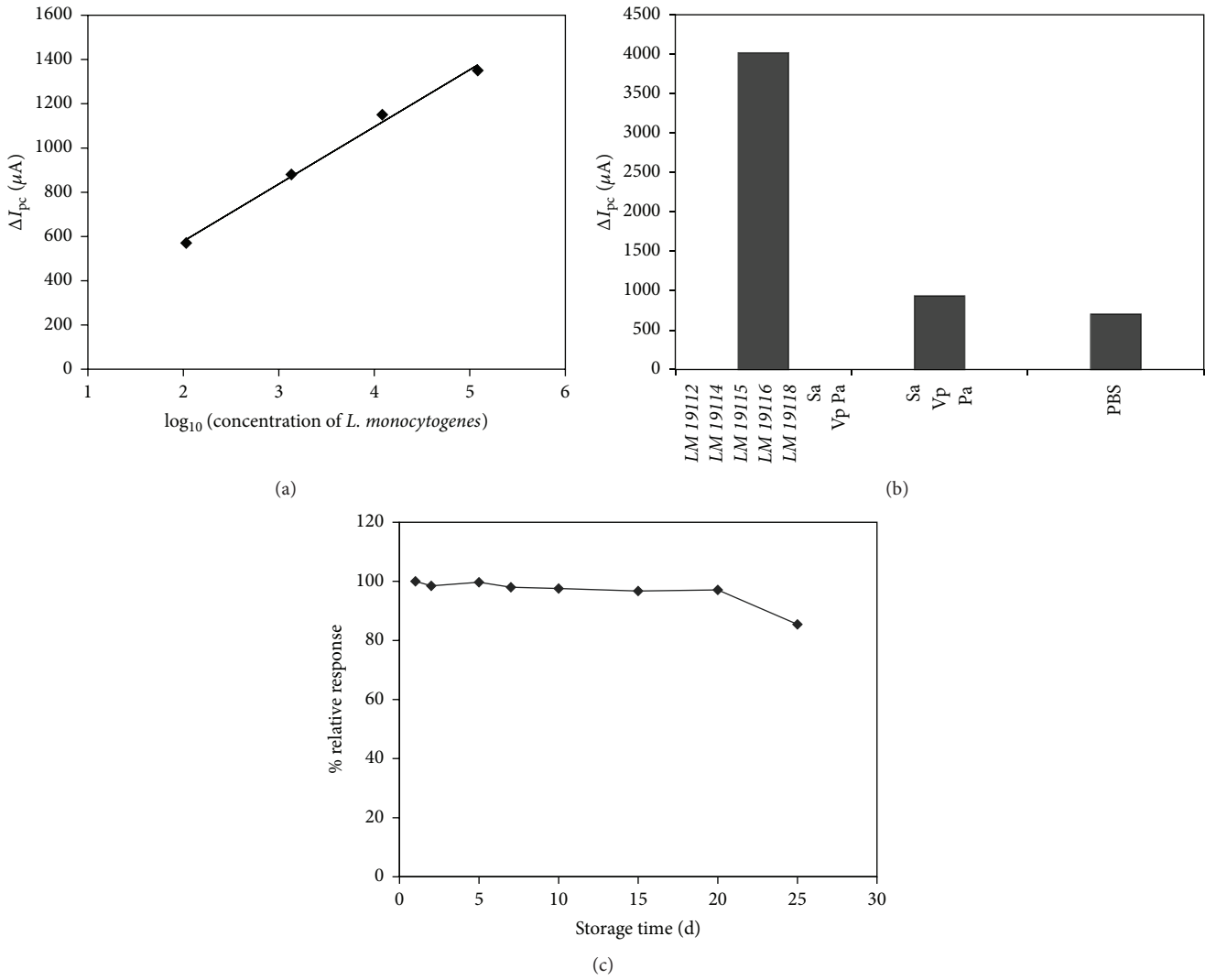

Figure 6: Standard curve of immunosensor and performance evaluation. (a) Standard curve of immunosensor for *L. monocytogenes* detection. (b) Specificity of immunosensor for *L. monocytogenes* detection in milk sample. (c) Storage stability of the immunosensor (10 mM pH 7.4 PBS, 4°C).

to 10^5 cfu/mL. Moreover, the fabricated biosensor exhibited good reproducibility, specificity, and stability, implying its potential application in the detection of cells for public and environmental health protection.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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