Sensors and Materials, Vol. 18, No. 8 (2006) 445–456
MYU Tokyo

S & M 0662

A Novel Multichannel Immunosensor for Determination of Serum Hepatic Fibrosis Markers

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(Received November 17, 2006; accepted December 21, 2006)

***Key words*:** biosensor array, serum marker, hepatic fibrosis, MEMS, PPD

In this study, we develop a novel biosensor with multichannels to detect three hepatic fibrosis markers: hyaluronic acid (HA), laminin (LN) and type IV collagen (IV-C). By using micro mechanical system technology, a chip including eight gold electrodes is fabricated for detecting up to eight markers simultaneously. Different antibodies of serum markers are embedded in poly(o-phenylenediamine) by local electrochemical polymerization, forming semi-insulated membranes on different gold electrodes. The changes in peak currents caused by the immunoreactions are detected. The results show that the linear ranges for HA, LN and IV-C are 9.9–88.8, 2.0–107 and 1.5–6 ng/ml, with sensitivities of 0.2122, 0.6155 and 0.7742% / (ng/ml), respectively. The immunosensing technology is compared with conventional radioimmunoassay (RIA) technology. It is found that the two measures have similar sensitivities and consistency, while the immunosensor demonstrates the advantages of time saving and cost effectiveness over RIA.

1. Introduction

Miniaturization, integration and intelligentization are notable characteristics of modern analytical chemistry, where the micro-electro-mechanical system (MEMS) plays an important role in fabricating various miniaturized analytical devices. Among them, the MEMS-processed microelectrode array has been the most extensively explored. It has been employed in electrochemical biosensors, electronic tongues or electronic noses, and detectors of capillary electrophoresis or liquid chromatography because of the high sensitivity derived from its precisely controlled pattern.[1-6] Compared with the conventional enzyme-linked immunosorbent assay (ELISA),[7] radioimmunoassay (RIA)[8] or any other fluorescence-labeling methods,[9] miniaturized electrochemical immunoassay usually has the strong advantage of having no requirement for additional immunochemicals, which provides the basis for its enhanced sensitivity and specificity. Ag-Ab interactions can be detected via measuring changes in capacitance, potential, current or resistance, known as the label-free method.[10-14] The technology has been used for immunosensing

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tumor markers or tumor-associated antigens in human serum.[15] Several serum markers, such as hyaluronic acid (HA), laminin (LN) and type IV collagen (IV-C), have been reported to be useful for monitoring hepatic fibrosis,[16,17] which is of great practical significance, especially at an early stage of the illness.[18]

Meanwhile, both conductive and insulated polymers can provide an effective way of coimmobilizing biomolecules on conventional electrodes creating functionalized films for selectively probing desired analytes.[19,20] Among them, the material obtained by electropolymerizing o-phenylenediamine (oPD), commonly known as PPD, has received a great deal of attention for more than two decades. Photovoltaic cells, anticorrosion coatings, pH measurements, and, in particular, enzyme entrapping permselective membranes in biosensor design represent its main fields of application.[21] Recently, renewed interest in PPD has been generated for obtaining a biomimetic membrane,[22] which reveals the great potential of applying PPD as a biocompatible material.

In our previous work, poly pyrrol (PPy) was utilized as an immobilizing film for Ab, and conductivity change was measured.[23] The deficiency in the work was that the sensor was too sensitive to ions, so that the system worked with low resistance from environmental disturbances, making tests uncontrollable or even completely ineffective.[24] In this study, semi-insulated films made from copolymerized PPD with antibodies are developed on a MEMS multielectrode array. The impedance changes induced by the reaction of antigens with the embedded specific antibodies in the semi-insulated polymer membrane are detected. The concentrations of three fibrosis serum markers e.g., HA, LN and IV-C, can be determined simultaneously in the presence of the redox probe, i.e., ferrocenemethanol (FcM). The sensitivity and detection linear ranges are demonstrated. Thirty-one samples in PBS with known concentrations and approximately eighty samples of human serum are analyzed using the sensor array. The results are compared statistically with those obtained from RIA. The mechanisms of the impedance change, the optimization of the copolymerization conditions and the effects of nonspecific absorption and solution evaporation are then discussed.

2. Experiments

2.1 *Apparatus*

Amperometric and cyclic voltammetric experiments are carried out using a CHI1030 electrochemistry workstation (CH Instruments Inc., USA), which provides 8-channel measurements simultaneously.

To fabricate the sensor array, an evaporation system, an oxidization furnace, lithographic equipment, a reactive ion etching (RIE) system, a wafer-dicing machine and a wire-bonding machine are utilized.

2.2 *Reagents*

HA, LN and IV-C and their respective antibodies HABP, α-LN and α-IV-C (all prepared in 0.01 M PBS, pH=7.4, with 1% BSA) and BSA are obtained from Sigma. oPD, FcM and phosphate are the products of Shanghai Reagent Factory (Shanghai, China). All solutions are prepared with doubly distilled water, and all other chemicals used are of

analytical reagent grade. The concentration of the FcM solution used in all the experiments is 1 mM.

2.3 *Immunosensor construction*

All experiments are conducted in a conventional three-electrode system. The electrodes on the MEMS-made chip with immobilized antibodies are set as the working electrodes, a platinum wire is used as the counterelectrode and Ag/AgCl is used as the reference electrode.

The MEMS-made chip is constructed starting from Si wafer furnace oxidation to form a 500-nm SiO_2 layer. Twenty-nanometer Ti and 400-nm Au thin films are evaporated and patterned in the next step (see Fig. 1(a)). The eight gold dots with diameters of 1 mm serve as the working electrodes. After wafer dicing, the single chips are dual in-line package (DIP) packaged for future use. The chip's dimensions are 4.5 mm by 5.5 mm. The ceramic walls of DIP and the top surface of the chip surround a small basin for the adoption of about 100 μl of solution in later experiments.

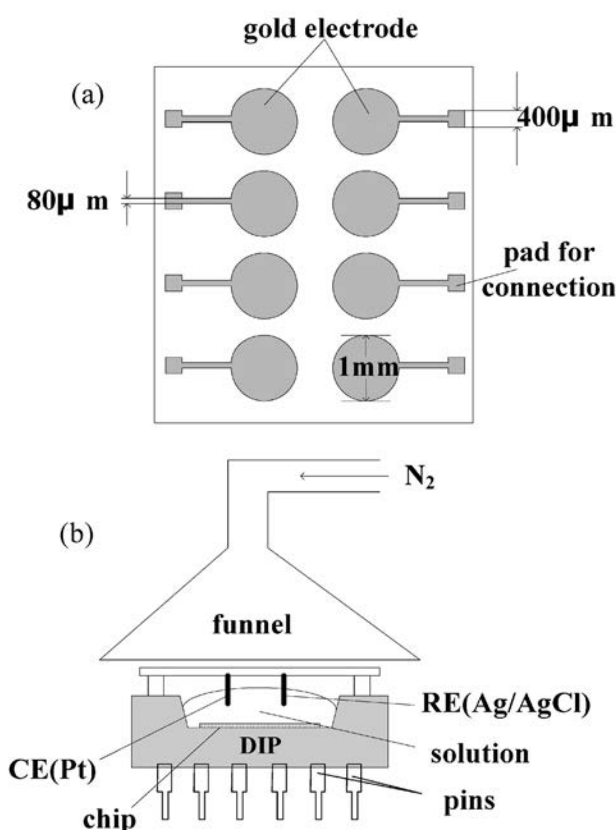

Fig. 1. (a) Schematic of sensor chip. (b) Experimental setup. All parts shown are not to scale and the wire connection is not demonstrated. The funnel hood provides a path for N_2 to protect the solution from oxygen and light, which is necessary for immobilizing antibodies but less crucial for other steps.

By connecting CE, RE and eight pins of DIP with the electrochemistry workstation, eight three-electrode cells for multichannel detection are established.

2.4 *Co-electropolymerization of o-phenylenediamine and antibodies*

N_2 flows into the hood at 200 ml/min for 3 min before the electropolymerization process. Fifty microliters of PBS (0.01M, pH=7.4) containing 10 mM oPD and 1 μg/ml HABP is added to the basin. Cyclic voltammetry is adopted to copolymerize oPD and antibodies at a voltage ranging from 0 to 0.8 V and a scanning rate of 100 mV/s for two cycles. The final step is to rinse the chip carefully five times with PBS. To locally copolymerize different antibodies at different working electrodes, repeating the aforementioned steps is necessary.

2.5 *Test procedure*

2.5.1 *Characterization of immunosensor*

Cyclic voltammetric experiments are performed for the characterization at a voltage ranging from +0.5 to –0.3 V and a scanning rate of 100 mV/s. Fifty microliters of 1 mM FcM in 0.01M PBS serves as the support electrolyte. Premade sample solutions with known concentrations of antigens in PBS are added dropwise to the basin. After hatching for 30 min at 25°C, cyclic voltammetry is carried out to characterize the solution. Note that the total volume of the sample solution added dropwise to FcM should be no more than 10% that of FcM, i.e., 5 μl. Otherwise, the reliability of the results is degraded.

Repeating these steps sequentially, a number of characterization curves can be obtained (see Fig. 2). The linear range, the sensitivity and the minimum detection limit of the immunosensor can be derived by analyzing the change in the peak current in the curves.

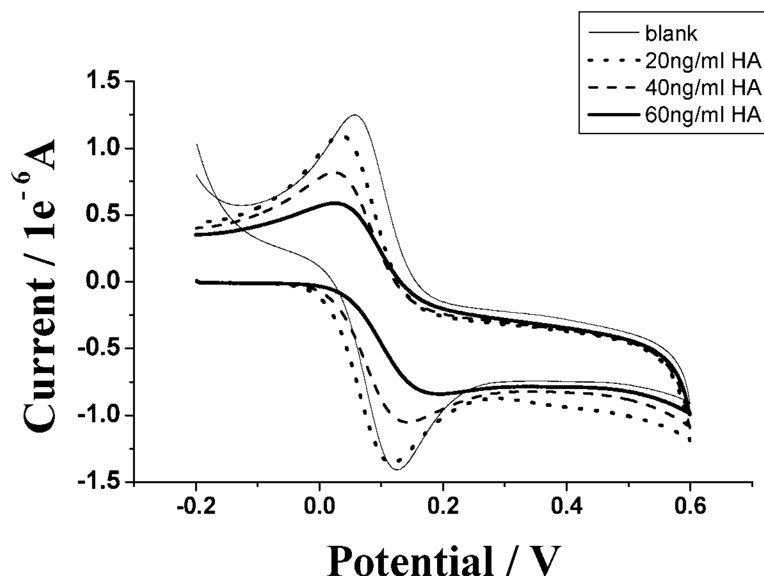

Fig. 2. Sensor responses to various concentrations of HA in PBS diluted 10–100 times with FcM solution. The values listed in the figure are the concentrations of HA in PBS and FcM solutions.

2.5.2 Detection of serum hepatic fibrosis markers

The detection of serum hepatic fibrosis markers is carried out using almost the same steps as those used for the characterization, but by means of standard addition.[24] Therefore, the detection of one serum sample needs four cycles of the above-mentioned steps: two cycles for serum samples and two cycles for premade samples. Assuming a linear relationship between the analytical signals and the analyte concentrations, the unknown concentrations of the serum markers can be obtained from the respective current peaks.

2.5.3 *Comparison between immunosensor and RIA*

To further evaluate the developed immunosensor, RIA is chosen as the reference technique for detecting the same samples used in immunosensors. A comparison between the two groups of detected data is made by statistical analysis.

The RIA tests are carried out in Shanghai Rui Jin Hospital. All the RIA kits used in the experiment are made in Shanghai, China. It is claimed that the coefficient of variation for the intraassay is less than 10% and that for the interassay is less than 15%.

3. Results

3.1 *Sensitivity and linear response of immunosensor*

Figure 3(a) shows typical relationships between the relative rates of change in the peak current and concentrations of the markers. The slopes of the linear fittings to the data give the sensitivities of the immunosensor. The sensitivities to HA, LN and IV-C are 0.2122, 0.6155 and 0.7742%/(ng/ml), respectively.

In addition, Fig. 3(a) shows that the linear ranges of HA, LN and IV-C are 9.9–88.8, 2–107 and 1.5–26 ng/ml, respectively. Note that these are the concentration ranges of the markers in FcM solution. The linear response concentration ranges in serum should be 10–100 times that large, because the serum markers were diluted 10–100 times after being addition to the FcM solution for testing.

The different sensitivities (slopes) of the three markers may have a close relationship with the molecular weight and size of both the antigens and antibodies, of which the former is the predominant factor. The molecular weights of HA, LN and IV-C are approximately 5000, 1000 and 180 kDa, respectively. The number of molecules of the three markers per unit volume of solution at the same concentration should differ from one another. For example, a 1-ml solution of 1 ng/ml antigens may contain about 3.3×10^{8} IV-C molecules but only 6×10^{7} and 1.2×10^{7} LN and HA molecules, respectively. Therefore, because the active sites in the copolymerized membrane are not saturated by antigens, the linear response curve of HA has the smallest slope, whereas IV-C saturates α-IVC in the membrane rapidly and shows the highest sensitivity (see § 4.1 and § 4.2).

The minimum detection limits of HA, LN and IV-C are all found to be lower than 1 ng/ml. These detection limits are sufficiently small, since even the concentrations of HA, LN and IV-C in the serum of a healthy person are much higher. The responses to 1 ng/ml HA, LN and IV-C result in peak current changes of 1.2×10^{-7}, 1.7×10^{-7} and 2.1×10^{-7} A, respectively.

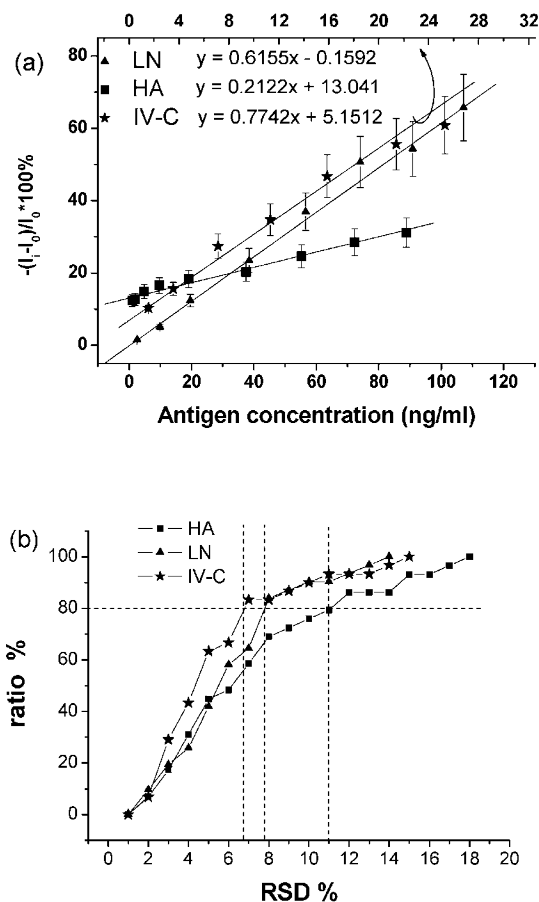

Fig. 3. (a) Sensitivities and linear responses of immunosensor to HA, LN and IV-C. I_0 and I_i are the currents before and after adding the markers to the FcM solution and hatching for 30 min at 25°C. The top x-coordinate only indicates the concentration of IV-C. (b) RSD distribution of 30 samples. The ratio marked on the y-axis is the percentage of samples in which RSD is less than or equal to the corresponding value on the x-axis.

3.2 *Coefficient of variation of immunosensor*

Note that the linear responses in Fig. 3(a) should not serve as standard references for all detections. Because of small changes in the distances between the electrodes, the volume of liquid added and the detection circumstances, slopes and intercepts could vary by as much as 20% from test to test. That is one of the reasons why we have to use a standard addition method for concentration calculation.

To obtain the coefficient of variation of the immunosensor, 30 serum samples with various concentrations of HA, LN and IV-C were each tested six times. The relative standard deviations (RSDs) of the results derived from the 30 samples are less than 18, 14

and 15% for HA, LN and IV-C, respectively. Figure 3(b) shows the distribution of RSD of these samples. The ratio on the y-axis indicates the percentage of test results with RSD less than or equal to the corresponding value on the x-axis. For example, as illustrated by the dashed lines in Fig. 4(b), 80% of the detection results have RSDs within 6.8, 7.7 and 11% for IV-C, LN and HA, respectively. These coefficients of variation and the RSD distributions of the immunosensor make it statistically reliable for serum marker determination compared with the commercial RIA technology (see § 2.5.3).

3.3 *Comparison of detection results between immunosensor and RLA*

To verify the feasibility of the immunosensor, 31 premade samples and about 80 serum samples are tested using the immunosensor and by RIA. The detection results are compared statistically.

Figure 5 shows a linear relationship between the results from immunosensor and RIA tests for serum samples. There is a strong correlation between the results from the two methods (HA, 74 samples, r=0.959, p<0.0001, slope K=1.016; LN, 48 samples, r=0.877, p<0.0001, K=0.9665; IV-C, 50 samples, r=0.845, p<0.0001, K=1.0831). Among them, HA shows the best results, probably because HA and HABP have a stronger binding force than the other two pairs. Such significant correlations indicate that the immunosensor, to some extent, could partially replace RIA for serum hepatic fibrosis marker determination.

Apart from the correlation of the detection results, the advantages of the immunosensor over RIA include the simultaneous detection of the three markers, thus time saving. If needed, more antibodies of serum markers could be immobilized on other electrodes and tested without additional time or sample volume required. The time for one-sample detection using the immunosensor is approximately 2.5 h, whereas it is at least 24 h for RIA.

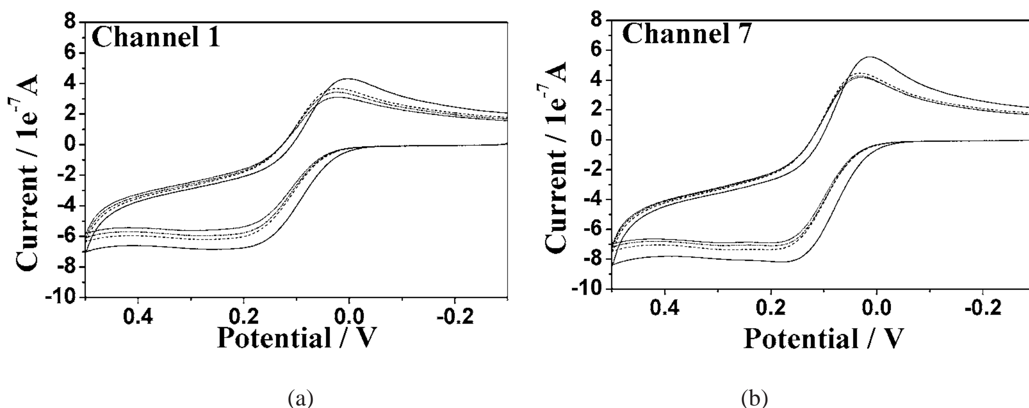

Fig. 4. CV detection curves of IV-C in serum using multichannel immunosensor. Antibodies immobilized on channels 1 (a) and 7 (b) are diluted with PBS to 1:500 and 1:1000, respectively.

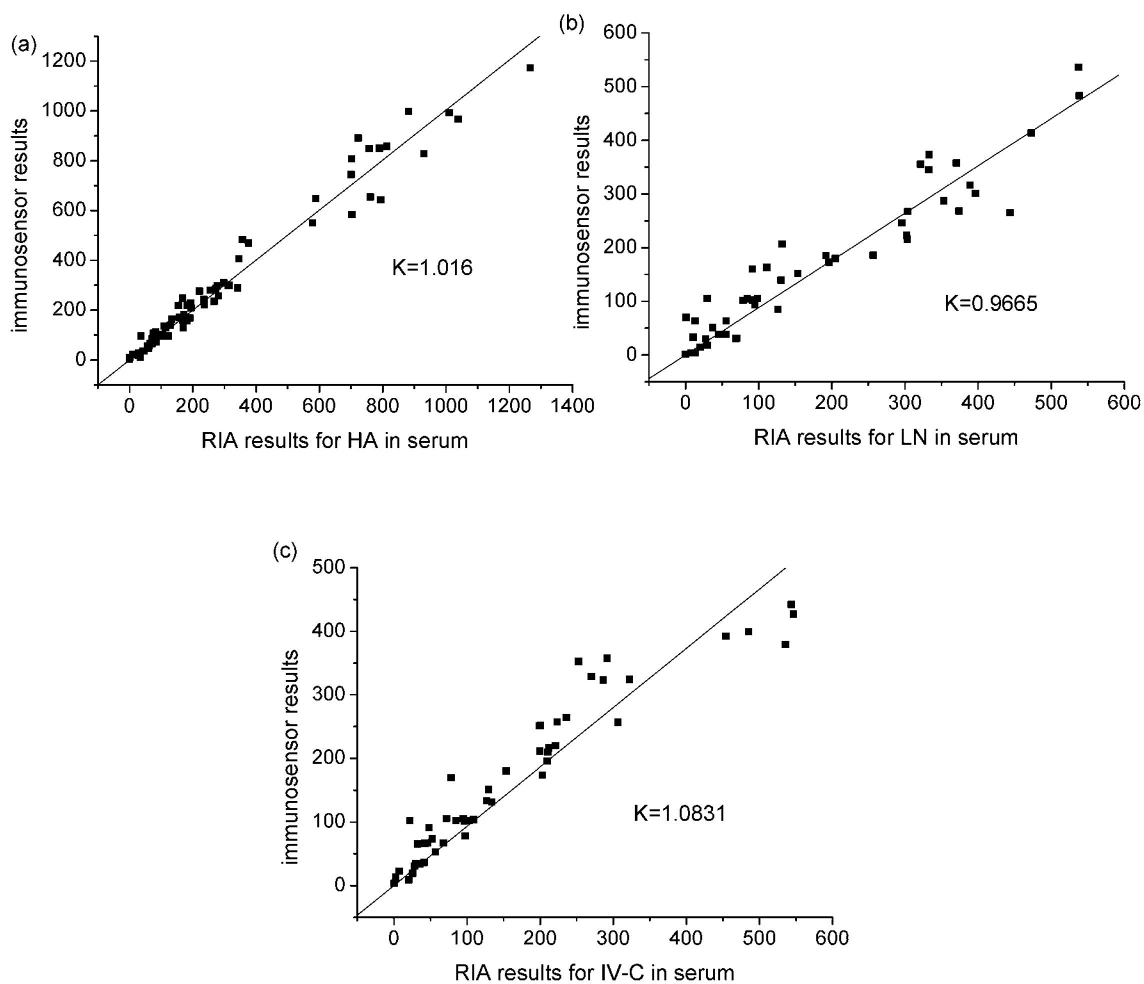

Fig. 5. Linear regression analysis of detection results and correlations between immunosensor and RIA method results for (a) HA (74 samples, r=0.959, p<0.0001, slope K=1.016), (b) LN (48 samples, r=0.877, p<0.0001, K=0.9665) and (c) IV-C (50 samples, r=0.845, p<0.0001, K=1.0831) in serum samples.

Comparing the detection results for the 31 premade samples obtained from the immunosensor and by RIA with the already known concentrations, it is found that all errors are within 20%, except for one obtained by RIA. The error distribution of the immunosensor and the RIA results are randomly distributed.

4. Discussion

4.1 *Mechanism of impedence increase caused by immunoreaction*

As shown in Fig. 2, the peak current decreases with an increasing concentration of the antigen. That means that the impedance of the system increases with the immunoreaction. The mechanism can be explained as follows.

Under certain conditions of copolymerization, the embedding of very large molecules in the antibodies (approximately 60, 400 and 80 kDa for the molecular weights of HABP, α-LN and α-IVC, respectively) embedded causes many defects in the polymer film. The smaller molecules or ions of FcM can penetrate into the network of PPD. Along with the immunoreaction, the path consisting of those successive defects is blocked to some extent by the much larger binding molecules (5000, 1000 and 180 kDa for HA, LN and IV-C, respectively). Therefore, the charge exchange of FcM with the gold electrodes is greatly hindered. As a result, the membrane demonstrates higher impedance after the immunoreaction.

4.2 *Optimized antibody copolymerizing dilution rate*

The dilution rate of the antibody using PBS in the copolymerization process is an important factor that affects the sensitivity and linear response range of the sensor. The larger the dilution rate is, the higher the sensitivity, but the narrower the linear response range.

Figure 4 shows typical CV curves for IV-C detection with two different dilution rates of embedded α-IVC. Curves shown in the same line style represent detection after immunoreaction with the same concentration of IV-C. Antibodies immobilized on channels 1 and 7 are diluted with PBS to 1:500 and 1:1000, respectively. It is clear that compared with the results of channel 1, the CV curves of channel 7 are more difficult to identify. That means that channel 7 has already been saturated by IV-C. In contrast, the sensitivity, i.e., $-(I_0-I_1) / I_0\%$ with the same definition as the y-axis of in Fig. 4(a), demonstrates a higher value for channel 7 than for channel 1.

In order to cover the full range of concentrations of human serum hepatic fibrosis markers while possessing an acceptable sensitivity, the optimized dilution rate for HABP and α-LN is 1:1000 and that for α-IVC is 1:500 after considering the trade-off between detection range and sensitivity.

4.3 *Effects of nonspecific adsorption and solution evaporation*

Because the immunosensor may be very sensitive to nonspecific adsorption, it is necessary to evaluate the effect of other large proteins in serum on this sensor. Experiments are separately carried out in two groups. In the control group, the immunosensor is

used to test serum directly; however, in the contrast group, serum is pretreated with 1% BSA to eliminate nonspecific adsorption before being examined. It is found that treatment with BSA only slightly improves the immunosensor's performance by 0–3%. It is possible that the 1% BSA contained in the commercial antibodies had already dispelled the nonspecific adsorption. As a result, additional BSA is not considered to be the key factor affecting the detection accuracy of the sensor.

The effect of solution evaporation, however, is an important issue that we have to discuss, since evaporation can be very fast in dry air at 25°C. The condensation of solute, particularly FcM, could cause severe errors during detection.

Figure 6 shows the test results for the effect of solution evaporation. In this test, CV characterization is performed every 30 min after adding 5 μl of PBS (without antigen). It is shown in Fig. 6 that the peak currents are almost constant (the variation is smaller than 4%) in an ambient temperature of 25°C and 62% RH, which thereby indicates that the water evaporation rate is close to 5 μl/30 min. A feasible solution to the problem is to keep a bare channel as a reference, i.e., no polymerization is conducted on that electrode, and save the peak current of the bare channel at first scanning as the reference data. The evaporation effect can be eliminated when all the test results of the other channels will be normalized referring to the data of the bare channel. For instance, at any scanning cycle later on, if the peak current of the bare channel has increased 10% comparing to its saved data of first scanning, all the peak currents of other channels for the same cycle should be divided by 1.1.

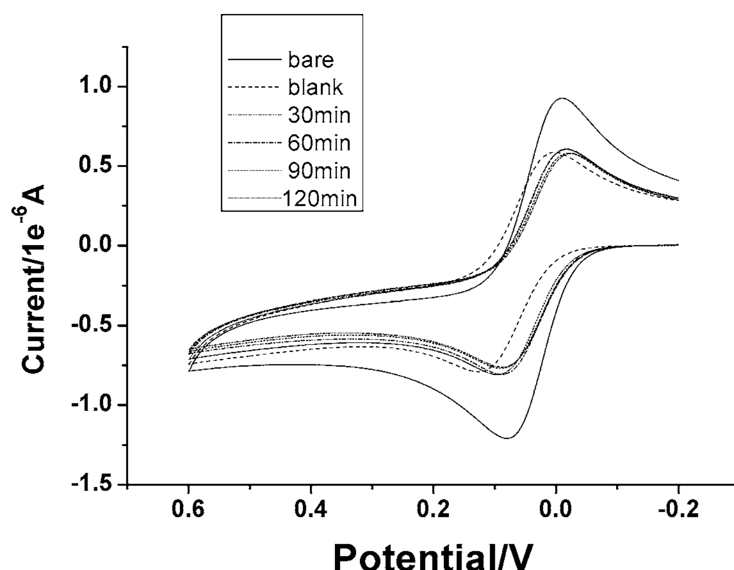

Fig. 6. Performance of immunosensor during periodical tests (at 25°C, 62%). 'Bare' indicates the response of the sensor in 50 μl of FcM solution before copolymerization. 'Blank' represents its response in 50 μl of FcM solution after copolymerizing oPD and α-LN at a dilute rate of 1:1000. '30 min' stands for the sensor performance after 5 μl of PBS is added to the previous 50 μl of FcM solution and left for 30 min. '60 min' indicates the result of adding another 5 μl of PBS and waiting for another 30 min. The following '90 min' and '120 min' can be explained likewise.

4. Conclusion

We have developed a multichannel immunosensor for the determination of serum hepatic fibrosis markers. It is based on an electrochemistry method and utilizes a new kind of semi-insulated membrane made by immobilizing specific antibodies of serum markers in PPD on gold electrodes. The MEMS manufacture ensures that such sensor is affordable for one-time use and free of intercontamination. Up to eight markers can be determined simultaneously. One test can be finished within 2.5 h. The error of the immunosensor is within 20% and coefficients of variation for both inter- and intraassays are less than 20%. The linear ranges for HA, LN and IV-C determinations are 9.9–88.8, 2–107 and 1.5–26 ng/ml, with sensitivities of 0.2122, 0.6155 and 0.7742%/(ng/ml), respectively. The minimum detection limits for the three markers are all lower than 1 ng/ml. A strong correlation is found between the immunosensor and RIA method results for the determination of serum hepatic fibrosis markers.

Considering its time saving ability and cost effectiveness, the developed immunosensor has great potential for partially replacing the RIA technology. Furthermore, it may have many applications for other serum markers or the determination of large protein molecules if respective specific binding molecules are available.

Acknowledgements

This work is supported by the Shanghai Natural Science Foundation under Grant 05ZR14015 and the National High Technology Research and Development Program of China (863 Program) under Grant 2004AA404253.

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