An Electrochemical Sensor for Prothrombin-Time Test

XIN Lifei[1, a] and LIU Hong[2,b]

[1]State Key Laboratory of Bioelectronics, School of Biological Science and Medical Engineering, Southeast University, Nanjing 210096, China.

[2]State Key Laboratory of Bioelectronics, School of Biological Science and Medical Engineering, Southeast University, Nanjing 210096, China.

[a]elfiex@sina.com, [b]liuh@seu.edu.cn

Keywords: PT testing; microfluidic chip; POCT

Abstract. As a new interdisciplinary, blood coagulation and thrombosis, being involved in the development and progression of many diseases, have a prominent clinical significance. Prothrombin time (PT time) is the time when calcium and tissue factor is added into the blood sample until when the blood sample completely clots. Clinically, PT time is an extremely sensitive screening index in coagulation system, it has an irreplaceable influence to patients' daily medication and related treatments as it can primarily reflect the normality of the extrinsic coagulation. Until recently, most automated coagulation analyzers used in hospitals can detect a variety of samples and projects at the same time with high accuracy. However it could only be operated by the professionals, not to mention the high production and testing costs. For these reasons, the frequency and ease-of-operation of the detection have been limited. In order to develop a real-time and household detection method, paper-chips and POCT technology are mainly utilized in this work. Paper-chips possesses their own advantages, as it can easily reflect the blood clotting process by detecting a change in the conductance of blood during the blood coagulation process. After a series of design, tests and improvements, a chip for PT time testing is fabricated. Using this chip, the PT time of several blood samples are tested, and the testing results confirmed that the further improved chip can complete the measurement and correction of blood PT time test. Compared with conventional coagulation analyzer, paper-chips have several intrinsic advantages such as reduced cost, higher accuracy, and better application in POCT use. We believe that pushing the further experimental products to market could fill the vacancy in the domestic industry.

1. Introduction.

Cardiovascular disease, sharing various mechanism, is now the leading cause of death worldwide, mostly owing to disturbances of blood coagulation[1]. Coagulation, the process by which blood forms clots, involves a cascade of interactions among blood platelets, proteins and various coagulation factors, helping preventing blood flow resulting from the vascular damage[2,3,4]. Clinically, anti-coagulation medications, especially blood-thinning medications, such as warfarin[5] and heparin[6], are common therapies to cardiovascular patients aiming to prevent thrombosis, thus reduce the risk of stroke[7], heart attack and other life-threatening cardiovascular diseases. However, the dosage of drugs needs much more attention: if the anti-coagulant drug level is too high, patients will take on more risk of the inside hemorrhage[8]; oppositely, if the drug level is too low, blood clot will not be controlled as expected[9]. In order to keep the balance of blood dissolution and clot of patient, effective monitor for continuous anti-coagulation level is necessary. Prothrombin time (PT time), also called clotting time, is the simplest index of anti-coagulation therapy monitoring process in the hospitals[10]. During the PT time tests, tissue factor is added into plasma obtained from the whole blood centrifugation, which leads to the transformation from prothrombin to thrombin, and next the thrombin triggers blood clot[11].

Nowadays, the PT time tests are mainly operated in hospitals and central laboratories by automated analyzer[12]. The principles of these machines can be divided into three blocks: (1)immunity method.

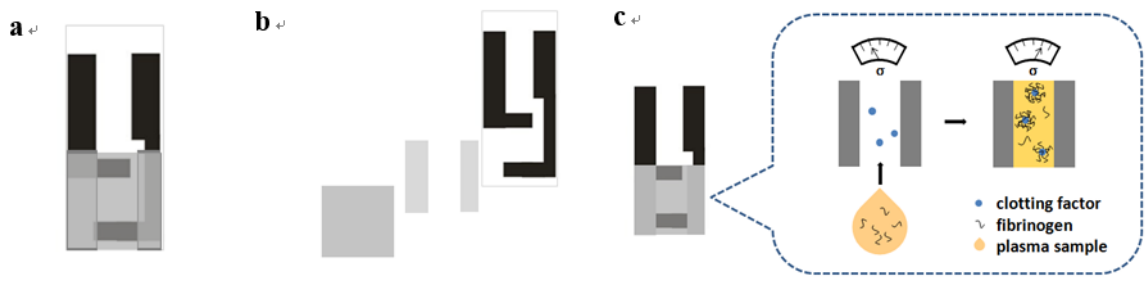

Figure 1. (a) Schematic illustration of the sensing chip. (b) The three layers of the sensing chip. The bottom layer (the right layer in the figure) is the substrate carrying the two L-shaped electrodes. The middle layer (the middle layer in the figure) is the double-sided adhesive tape which is patterned using a laser cutting machine. The top layer (the left layer in the figure) is a hydrophilic layer preloaded with TWEEN to facilitate the sample introduction by capillary action. (c) Schematic illustration of the PT test using a conductivity meter.

Based on the antigen-antibody specific binding, the determinand in plasma, which is considered as the antigen, can be tested quantitatively or semi-quantitatively. The analytical method is based on the transmissivity and refractive index changes after blood clotting[13,14]. (2)optical method, which is regarded as one of the most reliable and reproducible methods. Optical method has been utilized to measure PT time in the central laboratories for years[15]. Blood clotting brings to a series of changes in physical characteristics, such as transmissivity intensity and scattering light intensity, and this method is exactly based on absorbancy increase. Optical method is widely used in modern automated analyzers, for the absorbancy test is reliable and easy to operate[16]. (3)biochemistry methods. The reagent used in blood-clotting apparatus is a chemical that can specifically react with active enzyme, which can trigger color change in the sample. As the color changes, the system can detect a change of absorbance(A), therefore the determinand corresponding to the active enzyme can be detected. To bench-top automated analyzers, those huge and sophisticated machines require trained personnel to operate and specific working environment,thus the testing time is prolonged and thereby is not beneficial for the therapeutic feedback from the patients[17]. To solve these problems, a detection device that can meet the requirements of point-of-care(POC) and self-monitoring test is urged. A large amount of researchers have worked on POC PT time test[18,19], some researchers designed new sensors, like piezo-electric quartz crystal sensors and acoustic sensors, to study the whole blood PT. Quartz Crystal(QC) microbalance biosensor was reported by Lothar Muller and others[20]. QCM, as an effective electromechanical transducer, has been widely explored by scientists. The 10 MHz sensors used in this work respond with a frequency shift to changes in viscosity during blood clot formation. G. Guhr and his colleagues made further tests based on surface acoustic wave sensor, like SAW and BAW device[21]. Their works had some special advantage on SAW and BAW device parameters (e. g. resonator frequency, quality factor, impedance). Surface plasmon resonance (SPR) , as a traditional analytical technique, can also be used for the analysis of PT analysis[22]. Microfluidic chip is a newly developing analytical device, drawing much attention from researchers and scientists. Several blood-related applications of microfluidics have been reported for separation and analysis of cells and plasma[23,24,25]. Compared to other POC methods, microfluidic chips, including silicon-, macromolecule- and paper-based chips, possess many advantages: (1)Reduced cost, including material cost and process cost. There exists several options to build 2-dimentional paper chip, e.g. ink-jet printing, drawing, photo-etching and so on. More conveniently, folding and cutting is useful for building 3-dimentional paper chip. (2)Ease of surface modification. Specifically, paper-based chip has better biocompatibility to be the reaction substrate for protein, nucleic acid and other biomolecules. (3)Lower background signal, which is necessary for colorimetry. (4)More environmental-friendly. After detection, disposal paper-based chips can be directly treated with burning, which is harmless to the environment and can kill the germs as well. On the other hand,

however, conventional microfluidic devices use flow pump and associated control units to transport fluidic, which obviously increases the size of system and the cost of whole device[25]. In order to simplify the operation of microfluidic device, capillary force[26,27] and pre-installed low pressure[28] are introduced into the micro-channels to provide the driving force of the flow.

Blood is usually liquid, and the fibrous protein solves in plasma as silky state. Once the blood accepts a stimulus, extrinsic coagulation pathway is initiated and the clotting cascade is turned on, which collapses the fibrous protein to solid phase, appearing as flocks in the plasma[29]. This kind of state change leads to nature change in blood covering resistance decreases, conductivity increases; light transmission decreases, refractive index increases; and so on. When the fibrous protein in plasma totally deposits, the change rate of conductivity decreases to zero, which can also be identified as the end of coagulation. So we designed an electrochemical device to test the PT time with only 3 μL samples in 20 seconds. Compared to other researches, our work has three main advantages:(1) Miniaturized device and reduced sample volume. A finger-stick can offer 10-20 μL blood which is enough for our test. (2)Shortened testing time. The faster patients get the results, the better for diagnosis and therapy. (3)Portability. With the help of paper's capillary driving force, fluidic pumping machine of traditional microfludic devices is discardable. (4)Ease of operation. Our device can make sure that the untrained people will be able to use it for self-monitor.

2. Experiment.

Materials and reagents. Thrombin kit was provided by Jiangsu Province Hospital. Conductive carbon ink was obtained from Creative Materials, Inc. (Tyngsboro, USA). Transparency film (No.2190) was obtained from Minnesota Mining and Manufacturing Company (Minnesota, USA). Double-faced adhesive tape was purchased from Soken Chemical & Engineering Co., Ltd (Tokyo, Japan). Laser cutting machine from Huatai Laser Engraving Co., LTD (Shenzhen, China) was used for film patterning. Rex DDS-11A conductivity meter was purchased from Shanghai Instrument Electric Ltd. Co.. 5.0% TWEEN-20 (v/v) was prepared to increase the hydrophilicity. All solutions were prepared with deionized water(18.0 MΩ cm, Milli-Q Gradient System, Millipore). All reagents were used as received without further purification.

Sensing chip fabrication and test. For the fabrication of the sensing chip, the conductive carbon ink was screen-printed on an A4-size transparency film. When the ink was dried in a drying oven, two L-shaped carbon electrodes were obtained on the transparency film. After complete drying at 65℃, the bottom layer was adhered to the middle layer (A4-size) which is patterned by laser cutting. The aqueous solution containing 5.0% TWEEN was dropcast onto the top layer (A4-size), and next the top layer was adhered to the middle layer. In this way, an A4-size 3-layer complex containing 108 identical sensing chips is prepared. The sensing chips were cut off to work as electrochemical sensing units. After the fabrication of the sensing chip, 3.0 μL aliquot of thrombin was introduced into the capillary channel and completely dried in a dark 37℃ environment for 2 hours. The frozen-dried thrombin was renatured by water bath at 37℃ for 2 hours.

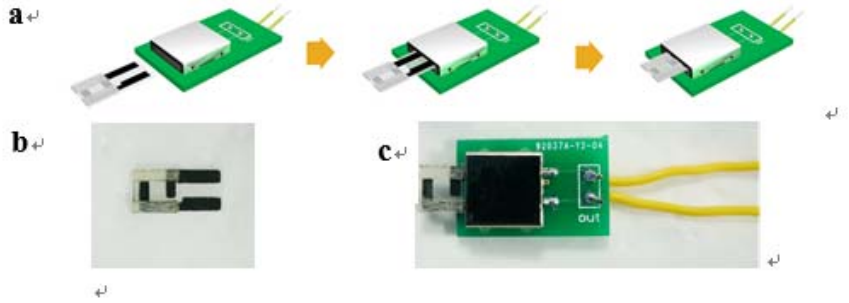

Figure 2. (a) Illustration of the system. Sensing chip was easily insert into chip carrier socket for conductivity measure.
(b)Photograph of the sensing chip and (c) photograph of the chip carrier socket inserted with a sensing chip

Human plasma was offered by Jiangsu Province Hospital of Traditional Chinese Medicine, which had been measured by automated analyzer. For PT time test, the sensing chip was inserted into the chip carrier socket, and then connected to the chip carrier socket with conductivity meter. 10 μL Plasma sample was introduced into capillary channel of the sensing chip. The conductivity of the sample was recorded every 5 second, and after 30 second, the record is stopped. Because the human PT time is about 12-15 second, our testing samples are limited in the general range.

3. Results and Discussion.

As shown in Figure 1a, the sensing chip (6 mm × 12 mm) contains three layers (Figure 1b). The bottom layer is a transparency film carrying a working electrode and a reference electrode. The electrodes are prepared by screen-printing of the conductive carbon ink on the transparency film. The middle layer was a comparably thicker double-sided adhesive tape which is patterned by laser cutting to form a channel between top and bottom layer. Before the sensing chip is ultimately fabricated, the aqueous solution containing 5.0% TWEEN was dropcast and dried on the top layer to increase the hydrophilicity. By assembling the three layers together, the capillary channel was formed in the sensing chip and can provide enough capillary force for the sample introduction. Thrombin from the purchased thrombin kit was introduced into the channel and dried at 37℃ for later detection.

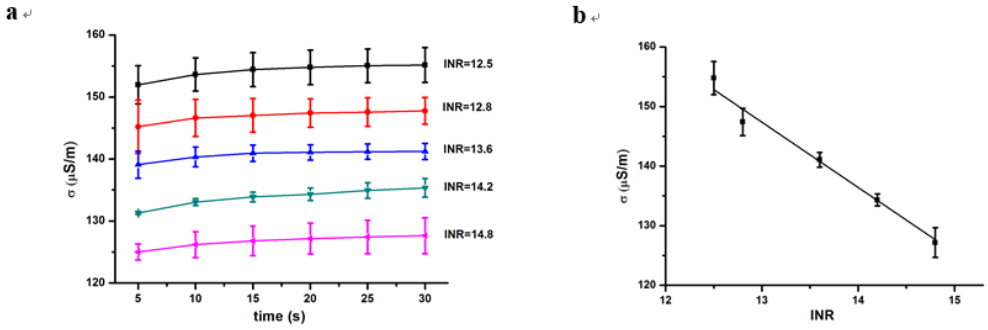

Figure 3. (a) Conductivity value as a function of the reaction time measured after introduction of plasma sample into the sensing chip.
The chip was preloaded with 3 μL thrombin solution and was completely dried before the test. Each detection curve represents samples
with a specific INR. INR, International Normalized Ratio, is established to standardize results from various detection systems, which
is the ratio of a patient's PT to a normal (control) sample, raised to the power of the ISI value for the analytical system being used.
(b) The plot of conductivity value as a function of the INR.

Because of the hydrophilicity of the capillary channel, the plasma sample can be introduced into the channel by capillary force. This design freed the sensing chip from other liquid driving units such as syringe pump, which clearly reduced the size and cost of the sensing chip. According to the forementioned fabrication method, 108 sensing chips can be simultaneously prepared on a 4A-size film, which is a simple and efficient operation that can reach mass production.

After the fabrication, the extinct clotting factor was immobilized in the capillary channel when the thrombin from human placenta was dried in the capillary channel. When plasma sample was introduced into the sensing chip, fibrinogen in the plasma started to fold into fibrous protein upon receiving the stimulation from the extinct clotting factor. In the meantime, many detectable changes in the blood will be triggered, such as viscosity, refractive index and conductivity of the plasma. In order to accomplish the POC test, we adopted conductivity as a function of the PT time, since the conductivity of the plasma sample can be directly read by a conductivity meter in a single step. Stronger coagulation ability the sample possesses, shorter the PT time is, which leads to a higher conductivity value detected by the sensing chip. In this work, the sensing chip is connected to the conductivity meter through a chip carrier socket (Figure 2c). The conductivity was recorded every 5 seconds in a 30-seconds test period. As shown in Figure 3a, for each individual measurement, the conductivity rose sharply at the beginning of the reaction, and shortly after the conductivity was observed to reach a relatively stable value, which is called platform value. The curve of conductivity showed high consistency among different samples, however, samples with different PT time had different platform values and platform times. For each plasma sample the platform value was recorded. A linear correlation was observed between the PT time of the plasma sample and the platform value of the conductivity. The obtained standard curve was showed in Figure 3b.

The experiment, however, still have space for improvement. The data in the standard curve needs to be enriched to improve the accuracy and reliability of the result. Owing to the limited sample source, only the samples with PT time between 12 and 15 (represents the normal range in human body) are tested in this work. To reach the realization of mobile health, an APP for cell-phone that can convey the results from testing module to user's phone and the cloud database will be helpful for the following applications in real life.

4. Summary

To summarize, we have reported a sensing chip based on electrochemical method to test the PT time in our work. PT time test has great significance not only for clinical diagnosis but also for the patients' self-evaluation[1]. Besides the help in the drug usage control and therapeutic condition assessment, PT time test can also reduce the death rate caused by cardiovascular disease[7]. With more and more people began to have the awareness of self health, a simple and point-of-care testing method for disease alert had become a new popularity. In our experiments, a PT time sensing chip based on electrochemical method was developed. Thrombin is immobilized in the channel, so when plasma flow into the chip, thrombin will be resolved and initiate a series of clotting reaction, finally cause the coagulation. Blood coagulation leads to the conductivity change, therefore the PT time is measured by a conductivity meter. According to the collected data, a standard curve has been established for self-monitoring. We believe that the sensing chip we report here can fill the vacancy of the PT test market, which is a promising progress for PT test.

Acknowledgements

This work was financially supported by the Chinese Recruitment Program of Global Experts, Innovative and Entrepreneurial Talent Recruitment Program of Jiangsu Province, the National Natural Science Foundation of China (21635001), State Key Project of Research and Development (2016YFF0100802), the Science and Technology Development Program of Suzhou (ZXY201439), the Project of Special Funds of Jiangsu Province for the Transformation of Scientific and

Technological Achievements (BA2015067). the Fundamental Research Funds for the Central Universities (2242017K41015)

References

[1] Kelly, B. B., & Fuster, V. (Eds.): National Academies Press(2010), in press.

[2] Dahlbäck, B.: The Lancet Vol. 355 (2000), p. 1627-1632.

[3] Furie, B., &Furie, B. C.: Cell Vol. 53 (1988), p. 505-518.

[4] Fogelson, A. L., & Neeves, K. B.: Annual review of fluid mechanics Vol.47 (2015), p. 377-403.

[5] International Warfarin Pharmacogenetics Consortium.: New England Journal of Medicine, Vol. 360 (2009), p. 753-764.

[6] Douketis, J. D., Johnson, J. A., & Turpie, A. G.: Archives of Internal Medicine Vol. 164 (2004), p. 1319-1326.

[7] Ezekowitz, M. D., Bridgers, S. L., James, K. E., Carliner, N. H., Colling, C. L., Gornick, C. C., ... & Rickles, F. R.: New England Journal of Medicine Vol. 327 (1992), p. 1406-1412.

[8] Fihn, S. D., Callahan, C. M., Martin, D. C., McDonell, M. B., Henikoff, J. G., & White, R. H.: Annals of internal medicine Vol. 124 (1996), p. 970-979.

[9] Boston Area Anticoagulation Trial for Atrial Fibrillation Investigators*.:New England Journal of Medicine Vol.323 (1990), p. 1505-1511.

[10] Oral Anticoagulation Monitoring Study Group.: American Journal of Clinical Pathology Vol. 115 (2001), p. 288-296.

[11] Broze Jr, MD, G. J.: Annual review of medicine Vol. 46 (1995), p. 103-112.

[12] Hirsh, J., Dalen, J. E., Anderson, D. R., Poller, L., Bussey, H., Ansell, J., & Deykin, D.: Chest Vol. 119 (2001), p. 8S-21S.

[13] Quehenberger, P., Kapiotis, S., Handler, S., Ruzicka, K., & Speiser, W.: Thrombosis research Vol. 96 (1999), p. 65-71.

[14] Fischer, F., Appert-Flory, A., Jambou, D., & Toulon, P.: Thrombosis research Vol. 117(2006), p. 721-729.

[15] Yang, C. L., Huang, S. J., Chou, C. W., Chiou, Y. C., Lin, K. P., Tsai, M. S., & Young, K. C.: Talanta Vol. 116(2013), p.704-711.

[16] Erskine, J. G., Walker, I. D., & Davidson, J. F.: Journal of clinical pathology Vol. 33 (1980), p. 445-448.

[17] Iwata, H., & Kaibara, M.: Blood coagulation & fibrinolysis Vol. 13 (2002), p.489-496.

[18] Toulon, P., Ozier, Y., Ankri, A., Fléron, M. H., Leroux, G., & Samama, C. M.: Thrombosis and haemostasis Vol.101 (2009), p. 394-401.

[19] Zucker, M. L.: Point of Care Vol. 6(2007), p. 223-226.

[20] Müller, L., Sinn, S., Drechsel, H., Ziegler, C., Wendel, H. P., Northoff, H., & Gehring, F. K.: Analytical chemistry Vol. 82 (2009), p. 658-663.

[21] Guhr, G., Brunig, R., Schmidt, H., Gehrisch, S., Siegert, G., & Weihnacht, in Frequency Control Symposium, 2007 Joint with the 21st European Frequency and Time Forum. IEEE International (pp. 577-580). IEEE.

[22] Hansson, K. M., Vikinge, T. P., Rånby, M., Tengvall, P., Lundström, I., Johansen, K., & Lindahl, T. L.: Biosensors and Bioelectronics Vol. 14 (1999), p. 671-682.

[23] Toner, M., & Irimia, D.: Annu. Rev. Biomed. Eng. Vol. 7 (2005), p. 77-103.

[24] Hou, H. W., Bhagat, A. A. S., Lee, W. C., Huang, S., Han, J., & Lim, C. T.: Micromachines Vol. 2(2011), p. 319-343.

[25] Li, H., Han, D., Pauletti, G. M., & Steckl, A. J.: Lab on a Chip Vol. 14(2014), p. 4035-4041.

[26] Shim, J. S., Browne, A. W., & Ahn, C. H. Biomedical microdevices Vol. 12 (2010), p. 949-957.

[27] Martinez, A. W., Phillips, S. T., Butte, M. J., & Whitesides, G. M.: Angewandte Chemie International Edition Vol. 46(2007), p. 1318-1320.

[28] Dimov, I. K., Basabe-Desmonts, L., Garcia-Cordero, J. L., Ross, B. M., Ricco, A. J., & Lee, L. P.: Lab on a Chip Vol. 11(2011), p. 845-850.

[29] Wootton D M, Ku D N.: Annual review of biomedical engineering Vol.1 (1999), p. 299-329.

[30] Sheth S B, DiCicco R A, Hursting M J, et al.: Thrombosis and haemostasis Vol. 86 (2001), p. 435-440.