A sample-to-result system for blood coagulation tests on a microfluidic disk analyzer

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In this report, we describe in detail a microfluidic analyzer, which is able to conduct blood coagulation tests using whole blood samples. Sample preparation steps, such as whole blood aliquoting and metering, plasma separation, decanting, and mixing with reagents were performed in sequence through microfluidic functions integrated on a disk. Both prothrombin time (PT) and activated partial thromboplastin time (aPTT) were carried out on the same platform and the test results can be reported in 5 min. Fifty clinical samples were tested for both PT and aPTT utilizing the microfluidic disk analyzer and the instrument used in hospitals. The test results showed good correlation and agreement between the two instruments. © 2014 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4893917>]

I. INTRODUCTION

Centrifugal microfluidics has demonstrated its ability as a fully automated system for point-of-care applications.^{1–7} It is able to actuate low-volume liquid without an external pump and can separate whole blood under centrifugation. Many microfluidic functions, such as aliquoting, metering,⁸ and mixing,^{9–11} were developed on the centrifugal platform for sample preparation. Therefore, an automated assay protocol can be performed by integrating a series of microfluidic functions. As a result, an increasing number of whole blood analyses have been developed on the centrifugal platform in recent years.^{12–16}

Prothrombin time (PT) and activated partial thromboplastin time (aPTT) tests are the most commonly requested blood coagulation tests in hospitals. An isolated prolongation of PT indicates deficiency or inhibition of the clotting factors in the extrinsic pathway. An isolated aPTT prolongation suggests deficiency or inhibition of the clotting factors in the intrinsic pathway. Prolongation of both PT and aPTT leads to the possibility of deficiency or inhibition of the clotting factors in the common pathway.¹⁷ PT tests have been used mostly to monitor patients on oral anticoagulants, while aPTT tests have been used to monitor the effectiveness of unfractionated heparin therapy. Regular monitoring of prothrombin time is also needed in patients with conditions such as chronic liver disease, vitamin K deficiency, factor VII deficiency, and patients on anticoagulant (warfarin) therapy.¹⁸ Patients taking anticoagulants include those with, or those at risk for development of, thromboembolic disease, including deep vein thrombosis, pulmonary embolism, myocardial infarction, and stroke. The use of warfarin in older patients requires special consideration because some have multiple medical co-morbidities and are taking multiple medications that may interact with warfarin. Heparin is indicated in the acute treatment and/or prevention of thromboembolic disease. However, heparin has a narrow therapeutic

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window between adequate anticoagulation and hemorrhagic side effects. Moreover, the anticoagulation response varies widely among patients. Therefore, the effect of heparin treatment needs to be frequently monitored with aPTT.

For the automation of the blood coagulation test, a repeatable and fast mixing mechanism is required since a complete reaction kinetics needs to be captured in order to calculate the blood coagulation time.^{6,19} However, the normal value of PT for a normal person is about 10 s. A fully automated system is required in order to achieve consistent test results and to minimize possible human errors during measurement. In the past, we developed a few approaches for PT measurement on a centrifugal platform based on different mixing mechanisms.^{4,6} In this study, we further extend our efforts to perform two blood coagulation tests, PT and aPTT, on one platform. Comparing to our previous work, a few improvements are made in this study. An aliquoting and metering fluidic design is introduced so that the whole blood sample can be distributed into multiple testing structures with one sample loading step. In addition, the metering fluidic design reduces the variation in sample volume resulted from pipetting and aliquoting. Furthermore, since aPTT tests require two reagent-adding steps, a novel sequential flow control scheme, which utilizes the motion induced by alternate spinning, is developed in this work. The advantage of this flow control scheme is that it does not rely on any valving function so that it can maintain its function after high-speed spinning, which is required for blood separation. This approach has never been published before. An automated blood coagulation test, which is able to yield consistent test results, can be achieved using this system.

II. EXPERIMENTAL

A. Sample collection

The Institutional Review Board of Taichung Veterans General Hospital approved this trial and the whole procedure of clinical trial was conducted in conformity to the declaration of Helsinki and the ICH guidelines. Patients who received PT and aPTT tests in the hospital for medical indications were invited to participate in the trial. All enrolled patients signed an informed consent form before collection of whole blood.

B. Materials

Both PT and aPTT reagents were purchased from Dade Behring (Newark, DE, USA). The PT reagent, Dade[®] Innovin[®], is prepared from purified recombinant human tissue factor produced in *E. coli*, combined with synthetic phospholipids, calcium, buffers, and stabilizers. The aPTT reagents contain purified soy phosphates and rabbit brain phosphatides, buffers, and stabilizers.

Model fluids with various viscosities were prepared by mixing glycerol and water. Food coloring and fluorescent dye were added to facilitate the observation of fluid flow and quantification of the degree of mixing.

The detailed information about the microfluidic disk fabrication was provided in our previous report.⁴ The microfluidic disk has a diameter of 80 mm and a thickness of 2 mm. It is composed of two layers. The layer with the microfluidic pattern was fabricated through injection molding using conventional manufacturing equipment. It is sealed with 25- μ m thick tape (Advanced polyolefin diagnostic tape, 3M, USA).

C. Instrumentation

The microfluidic disk analyzer developed in house is capable of controlling the rotation of the disk and optical signal detection through a software interface written in LabVIEW (National Instruments, TX, USA). In addition, this analyzer is capable of maintaining temperature control in the testing environment at 37 °C. When operating the microfluidic disk analyzer, users are required to inject whole blood and reagents into the disk, then insert the disk into the analyzer. The analyzer then begins programmed spinning and the test results can be obtained in a few minutes.

D. Flow visualization

In order to characterize the flow field under high speed spinning, an image capturing system, which combines a high speed CMOS camera (SV-642, EPIX), a zoom lens system (Zoom 70XL, Optem), and a stroboscope (DT-311N, SHIMPO), was used. The digitized images captured from the high speed camera were analyzed using computer software (NI Vision) to acquire information required for liquid volume quantification and flow field analysis.

E. Blood coagulation analyses

Both the PT and aPTT measurements were determined by the time required to reach the targeted light transmission, which was defined as the average of the transmission changes in the measuring process. The PT measurement is usually converted to the international normalized ratio (INR) to justify the variation among different reagent and instrument combinations. On the other hand, the normal range of the aPTT measurement is usually provided by the reagent vendor according to the instrument used.^{20–25}

III. FLUIDIC DESIGN AND OPERATING PROCEDURES

The microfluidic design for blood coagulation tests is shown in Fig. 1. Six assay structures, three for PT tests (Fig. 1(a)) and three for aPTT tests (Fig. 1(b)), were integrated on a microfluidic disk. The fluidic design for PT measurement consists of a sample aliquoting unit, including 6 aliquoting chambers, and a whole blood processing unit, including a decanting chamber, a collecting chamber, a waste chamber, and a detection chamber. For aPTT measurement, a reagent decanting chamber is added to allow the addition of the secondary reagent (aPTT reagent II).

The operating protocol for conducting both the PT and aPTT tests simultaneously is illustrated in Fig. 2. In the loading stage (Fig. 2(a)), PT reagent ($11\ \mu\text{l}$), aPTT reagent I ($5.5\ \mu\text{l}$), and aPTT reagent II ($9.0\ \mu\text{l}$) were loaded into the disk separately. Venous blood samples from patients were collected in a test tube containing sodium citrate solution ($0.106\ \text{mol/l}$). The citrated whole blood sample ($180\ \mu\text{l}$) was then injected into the sample aliquoting chamber through the central hole of the disk. The disk was then placed on the rotational stage of the microfluidic analyzer. During the first spinning program, the rotational speed of the disk was increased from 0 to 5000 RPM with acceleration of 20000 RPM/s, and maintained at 5000 RPM for 90 s. The whole blood sample was divided into 6 aliquots by the aliquoting unit and each aliquot was delivered into a collecting chamber. Our design allows any excess amount of whole blood sample to flow into the waste chamber, leaving a metered volume of whole blood in both the decanting and collecting chambers (Fig. 2(b)). In the mean time, rapid

FIG. 1. The fluidic design for blood coagulation measurements: (a) PT test and (b) aPTT test.

FIG. 2. Assay operating procedures for the blood coagulation tests on the centrifugal platform. (a) Whole blood and reagents (PT reagent, aPTT reagent I, and aPTT reagent II) were loaded into the disk. (b) The disk was rotated to 5000 RPM, the whole blood was metered and the excess amount of whole blood was overflowed into the waste chamber. The PT reagent and the aPTT reagent I were delivered into the detection chamber. (c) In the sedimentation stage, the whole blood was separated into platelet-poor plasma at the upper layer and cellular blood at the bottom layer. (d) In the decanting and mixing stage, the disk was rotated alternately in both clockwise and counterclockwise directions. The platelet-poor plasma was decanted into the detection chamber and mixed with reagents. (e) The PT protocol was completed and the disk was rotated under the detection optics for signal measurements. For the aPTT test, the mixture of the plasma sample and aPTT reagent I continued to incubate for 3 min. (f) The disk started alternate spinning. The aPTT reagent II was decanted into the detection chamber and mixed with the mixture of plasma sample and aPTT reagent I. (g) The aPTT protocol was completed. The disk was rotated under the measuring optics for aPTT measurement.

sedimentation caused the whole blood to separate into cellular blood, which was stored in the collecting chamber, and platelet-poor plasma, which was stored in the decanting chamber (Fig. 2(c)). In addition, the primary reagent (PT reagent, and aPTT reagent I) flowed into the detection chamber during this spinning program. In the next stage, the disk went through a spinning motion by alternating clockwise and counterclockwise rotation at a frequency of 10 Hz with amplitude of 180° for 2 s. Then, $5.5\ \mu\text{l}$ of the platelet-poor plasma was decanted

from the decanting chamber into the detection chamber and mixed with the primary reagent (Fig. 2(d)). However, the secondary reagent (aPTT reagent II), which was stored in the inner radial position, remained in the reagent decanting chamber under this spinning program. For the PT test, after the quick mixing process, the disk was rotated to a position where the detection chamber was under the measuring optics to start signal measurement. On the other hand, for the aPTT test, the mixture of plasma sample and aPTT reagent I continued to incubate for 3 min (Fig. 2(e)). After that the disk went through alternate spinning at a frequency of 15 Hz with amplitude of 360° for 2 s. Next, $5.5\ \mu\text{L}$ of the aPTT reagent II was decanted from the reagent decanting chamber into the detection chamber and mixed with the plasma and aPTT reagent I (Fig. 2(f)). Then the disk was rotated under the measuring optics for aPTT measurement (Fig. 2(g)).

IV. RESULTS AND DISCUSSION

A. Aliquoting

Aliquoting is the first sample preparation step and it divides the whole blood into 6 aliquots. In this report, the aliquoting structure was placed in the center of the disk to prevent uneven liquid distribution resulting from the Coriolis force. Our experimental results showed that the coefficient of variation of the volume between each aliquot was about 5%, which was slightly higher than the CV for injecting the sample into each reservoir by pipetting ($\text{CV} \sim 3\%$). However, as the number of tests increases, the aliquoting structure could greatly reduce the labor required to conduct this assay.

B. Metering

Metering plays an important role in this design. Variations of the whole blood volume from either pipetting or aliquoting fluidic functions would affect the decanted volume. Since we only decanted a portion of the plasma, which is about 25% of the volume of whole blood, to mix with the reagent, the variation in the volume of whole blood resulted in a much greater coefficient of variation (4 times, in this case) in the decanted volume. Therefore, a metering function for the plasma is required in order to yield consistent test results. As shown in Fig. 3, comparing the two fluidic designs, the one with metering design (Fig. 3(a)) showed much less variation in the decanted volume than the one without metering design (Fig. 3(b)). With our metering design, a very consistent decanted volume could be achieved (CV less than 5%).

FIG. 3. Comparison of the decanted volume between the microfluidic designs (a) with metering structure and (b) without metering structure. The design with metering structure showed much less variation in the decanted volume ($\text{CV} < 5\%$).

C. Analysis of flow under alternate spinning

The main fluidic operations, decanting and mixing, in this design were carried out by alternate spinning, which is defined as periodically changing the direction of spinning (clockwise or counterclockwise). Under this motion, the fluid inside the chamber flowed to either side of the chamber alternately, as shown in Fig. 4(a). The elevated volume ratio, which is defined as the ratio of the liquid volume that flows above the original water level (V_e), to the total liquid volume (V), was used to represent the intensity of the flow under alternate spinning (Fig. 4(b)).

In order to understand the flow mechanism, we conducted a dimensional analysis of the parameters such as operating conditions (frequency and amplitude of spinning), chamber geometry (chamber depth), and fluid properties (viscosity). The dimensional analysis showed that the intensity of the flow could be characterized by the product of the modified Reynolds and Bond numbers, which are defined as

$$\text{Re}^* = \frac{\rho(\bar{R}Af)H}{\mu}, \quad (1)$$

$$\text{Bo}^* = \frac{\rho(\bar{R}Af^2)H^2}{\sigma}, \quad (2)$$

$$\text{Re}^*\text{Bo}^* = \frac{\rho^2\bar{R}^2A^2f^3H^3}{\sigma\mu}, \quad (3)$$

where ρ is the density, $\bar{R}$ is the average radius of the fluid, H is the depth of the chamber, σ is the surface tension of the fluid, and μ is the viscosity of the fluid. A and f represent the amplitude and frequency of the alternate spinning, respectively. The product of these two modified dimensionless numbers represents the interaction between the driving force induced by centrifugal force and inertial force to the resistance from surface and viscous forces.

A series of experiments were conducted to verify the relationship between the elevated volume ratio and the product of the modified dimensionless numbers. As shown in Fig. 5, it is clear that the elevated volume ratio, which represents the intensity of the induced flow, was positively related to the product of the modified dimensionless numbers. The flow under alternate spinning was minimal when the product was less than 100, but it increased rapidly when the product exceeded 100. Therefore, we could estimate the flow intensity according to the product of the dimensionless numbers.

FIG. 4. (a) The illustration of alternate spinning. (b) The elevated volume ratio is defined as the ratio of the fluid volume elevated above the original fluid level (V_e) to the total fluid volume (V).

FIG. 5. The relationship between the elevated volume ratio and the product of the modified Reynolds and Bond numbers by alternate spinning for fluids with viscosities ranging from 1 to 5 cP, operated under various frequencies (5–15 Hz) and amplitudes (100°–400°) in a shield-shaped chamber with depths ranging from 100 to 800 μm .

D. Decanting

The decanted volume ratio, which is defined as the ratio of the decanted volume to the total liquid volume, was also analyzed using the product of the modified Reynolds and Bond numbers under the same conditions mentioned in Sec. IV C. As shown in Fig. 6, a positive trend similar to that of the elevated volume ratio was observed. However, the data points in Fig. 6 are more scattered than those in Fig. 5. Comparing these two figures, it is clear that the decanted volume ratio is more prominent than the elevated volume ratio in the cases of low chamber depths (100 μm and 200 μm). This could be due to the differences in flow resistance. During alternate spinning, the liquid that flows above the original water level has to overcome the viscous drag force from the solid walls, and resistance from the liquid which has already flowed above the original water level. On the other hand, in the decanting process, the liquid

FIG. 6. The relationship between the decanted volume ratio and the product of the modified Reynolds and Bond numbers under various liquid viscosities (1–5 cP) and chamber depths (100–800 μm).

only has to overcome the viscous drag force since the initial volume of liquid that flowed over the original water level was delivered into the detection chamber and would not form a barrier for the later liquid flow. Therefore, the decanted volume ratio is higher than the elevated volume ratio under the same operating condition. This barrier effect of the initial volume of liquid on the later liquid flow is more prominent in cases of high flow resistance (as shown in Fig. 6 for chamber depths of $100\ \mu\text{m}$ and $200\ \mu\text{m}$) than low flow resistance (chamber depth = $800\ \mu\text{m}$).

E. Mixing

Fluorescent signal distribution was used to quantify the mixing efficiency. The images of the detection chamber were captured by a fluorescence microscope (IX71, Olympus) with a cooled CCD camera (DP71, Olympus). Each captured image was stored in a 16-bit grayscale bitmap. The degree of mixing was quantified by the standard deviation (σ) of fluorescent signal distribution (ranging from 0 to 1) in the detection chamber. The value of σ was 0.5 for completely segregated streams and 0 for completely mixed streams.

The relationship between the mixing efficiency (quantified by the standard deviation) and the product of the dimensionless numbers is shown in Fig. 7. When the product of the dimensionless numbers is low ($\text{Re} \cdot \text{Bo}^* < 0.1$), it is difficult to achieve mixing since only a minimal flow is induced. As the product of the dimensionless numbers increases ($\text{Re} \cdot \text{Bo}^* > 0.1$), the effect of mixing increases with the increment of the product of the dimensionless numbers. Accordingly, very good mixing results ($\sigma < 0.05$) were observed as the product of the dimensionless numbers exceeded 10.

F. Clinical tests

Fifty whole blood samples were collected from Taichung Veterans General Hospital. Each sample was used to conduct both PT and aPTT tests using the microfluidic disk analyzer (MDA) and Sysmex CA1500, which is the instrument used in the hospital. Each sample was tested three times and the average value was calculated. Since the blood coagulation time measurement varied depending on reagents and instruments used, the World Health Organization (WHO) recommended that PT results for patients on oral anti-coagulants should be reported as INR values. However, aPTT results are not standardized across different reagent/instrument systems, which may result in significant variability in aPTT results.²⁶

FIG. 7. The relationship between the mixing efficiency (σ) and the product of the modified Reynolds and Bond numbers.

FIG. 8. The comparison of the correlation of blood coagulation measurements by MDA and Sysmex CA1500. (a) The PT tests and (b) the aPTT tests. The correlation coefficients of the PT and aPTT tests were greater than 0.96, which means the two systems are highly correlated.

A normal range for aPTT was provided for each reagent and instrument combination. In this study, we did not convert the aPTT measured from MDA to the one measured by CA1500. The raw data was used in the analysis. The correlation of the test results for both instruments for both PT and aPTT is shown in Figs. 8(a) and 8(b). For the PT tests, the correlation coefficient from the Pearson's correlation test was 0.9876 ($y = 0.6511x + 0.3934$, $p < 0.001$). The correlation coefficient of the aPTT tests was 0.9776 ($y = 1.4889x - 25.042$, $p < 0.001$). These results indicate that the test results of these two instruments are highly correlated for both PT and aPTT.

The Bland-Altman plot was designed to compare the differences across the total range of clinical measurements and was used as a tool to analyze the agreement between the two test methods.²⁷ The y-axis represents the differences in clinical measurements between the two instruments, while the x-axis represents the average in clinical measurements between them. Fig. 9(a) shows that in the PT tests, the mean difference between the two instruments was about 0.09 INR and most of the test results were within a 95% confidence interval (between 0.42 and -0.24 INR). For the aPTT tests, the mean difference between the two instruments was about 10.20 and most of the test results were within a 95% confidence interval (between 18.30 and 2.10).

Therefore, significant agreement between the MDA and Sysmex CA1500 for both PT and aPTT tests was observed.

FIG. 9. The Bland-Altman plot for blood coagulation measurements by MDA and Sysmex CA1500. (a) The PT tests and (b) the aPTT tests. Most data points were within a 95% confidence interval. Good agreement between the two systems was observed.

V. CONCLUSIONS

A sample-to-result system for blood coagulation tests was successfully developed on a microfluidic disk analyzer. The user only needs to load the reagents and the whole blood sample (only once) into the disk, the programmed spinning control will induced the spinning motion to conduct sample preparation steps, such as aliquoting, metering, separation, decanting, and mixing, to yield clinical test results. Possible human error can be minimized and consistent test results can be achieved by using this analyzer. The decanting and mixing mechanisms induced by the alternate spinning were analyzed by dimensional analysis. We found that the intensity of the flow can be characterized by the product of the modified Reynolds and Bond numbers, which can used as a useful tool for fluidic design under alternate spinning motion. Finally, the blood coagulation tests on MDA were verified using clinical samples and test results were compared with the instrument used in medical centers. Significant correlation and agreement between the two instruments were observed. Since MDA showed similar performance to the instrument used in medical centers and the microfluidic disks can be fabricated at low cost by injection molding, it demonstrates great potential for commercialization in the near future.

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