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A cartridge based sensor array platform for multiple coagulation measurements from plasma

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This paper proposes a MEMS-based sensor array enabling multiple clot-time tests for plasma in one disposable microfluidic cartridge. The versatile LoC (Lab-on-Chip) platform technology is demonstrated here for real-time coagulation tests (activated Partial Thromboplastin Time (aPTT) and Prothrombin Time (PT)). The system has a reader unit and a disposable cartridge. The reader has no electrical connections to the cartridge. This enables simple and low-cost cartridge designs and avoids reliability problems associated with electrical connections. The cartridge consists of microfluidic channels and MEMS microcantilevers placed in each channel. The microcantilevers are made of electroplated nickel. They are actuated remotely using an external electro-coil and the read-out is also conducted remotely using a laser. The phase difference between the cantilever oscillation and the coil drive is monitored in real time. During coagulation, the viscosity of the blood plasma increases resulting in a change in the phase read-out. The proposed assay was tested on human and control plasma samples for PT and aPTT measurements. PT and aPTT measurements from control plasma samples are comparable with the manufacturer's datasheet and the commercial reference device. The measurement system has an overall 7.28% and 6.33% CV for PT and aPTT, respectively. For further implementation, the microfluidic channels of the cartridge were functionalized for PT and aPTT tests by drying specific reagents in each channel. Since simultaneous PT and aPTT measurements are needed in order to properly evaluate the coagulation system, one of the most prominent features of the proposed assay is enabling parallel measurement of different coagulation parameters. Additionally, the design of the cartridge and the read-out system as well as the obtained reproducible results with 10 μ l of the plasma samples suggest an opportunity for a possible point-of-care application.

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Introduction

Blood coagulation tests measure the time of onset of blood clotting, which involves an activating cascade of coagulation factors that ends with the formation of a cross-linked fibrin clot. Coagulation time tests are required for patients who are receiving anticoagulant therapy,¹ undergoing pre-operation evaluation,² suffering from hepatic or renal disorders,³ or being monitored for disease progression such as dengue hemorrhagic fever.⁴ Also patients with a risk of embolism, stroke or atrial

fibrillation require their coagulation time to be measured and adjusted periodically by suitable oral anticoagulants. Thus, fast, reliable and simple assays are needed to monitor the coagulation parameters.^{5,6} Prothrombin time (PT) and activated partial thromboplastin time (aPTT) are the most frequently performed tests to determine the functionality of the blood coagulation system.⁷ The measurement of PT and aPTT depends on the activation by the tissue factor (extrinsic system) or the surface (intrinsic system), respectively. In clinical use, aPTT is performed by manually mixing blood plasma samples with phospholipids and an activator (silica, celite, kaolin or ellagic acid). For the PT test, a reagent containing the tissue factor is added to a plasma sample to induce coagulation through the extrinsic pathway.

In general practice, patients need to visit a hospital or a central laboratory periodically for coagulation tests. Such a procedure puts a significant burden on the health care provider and increases the return time and the cost of the test. To alleviate these problems, some portable devices have been developed for PT measurements.^{8,9} Although these systems

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are practical and require low sample volumes, they are capable of conducting only one type of measurement on one sample. There is no commercial device which is suitable for both PT and aPTT measurements. These tests give specific information about intrinsic and extrinsic pathways which are not independent of each other.¹⁰ Hence obtaining both of the results enables a more comprehensive assessment. Furthermore, these devices require electrical connections between the disposable cartridge and the analyser unit which can create reliability problems in long-term use. On the other hand, the system developed in this study measures the clot time directly by monitoring the mechanical properties of blood with a non-contact actuation and detection method. Also it is the only platform that can perform multiple tests on the same sample.

There have been recent studies which measured clot time with low sample volumes using MEMS and microfluidics technology. Magnetoelastic sensors,^{11,12} Quartz Crystal Microbalance (QCM)^{13–15} and Contour-Mode Film Bulk Acoustic Resonator (C-FBAR) based techniques¹⁶ have been utilized to measure coagulation times. However in these studies mixing of the blood samples with the reagents was conducted manually on the sensors. Hence, functionalized sensors with microfluidic platforms are not proposed. A commercial device¹⁷ based on static mode microcantilevers is in preparation for launch soon. These cantilevers require electrical connections to the cartridge due to the use of piezoresistive elements for sensing and actuation.¹⁸ Microfluidic devices have also been used for aPTT¹⁹ and PT tests.²⁰ To the best of our knowledge, the studies in the published literature are limited to single tests and are not multiplexed. Most of the clinical applications require both PT and aPTT tests. A MEMS-based microfluidic device enabling both tests on one sample is a promising tool for point-of-care use.

In our previous studies, we conducted blood plasma viscosity measurements through phase measurements with high sensitivity using the proposed scheme.^{21,22} During blood coagulation, a sudden change in the blood plasma viscosity

occurs due to fibrin generation.¹³ By monitoring the viscosity over a course of time, the sudden increase in viscosity can be used as an indication of coagulation time for the given sample.

In this study we introduce a novel method using microcantilevers and a sensing method for fast and convenient blood coagulation time measurements. To our knowledge, this is the first study using dynamic-mode cantilevers for coagulation time monitoring. The principle of the assay was introduced and the assay was tested on known plasma controls; the results were then compared with a reference datasheet and a reference commercial device. Additionally, for further implementation, the microfluidic channels of the cartridge were functionalized for PT and aPTT tests by drying specific reagents in each channel. PT and aPTT testing of control plasma samples revealed that coagulation times could be successfully measured for normal and abnormal plasma controls.

Materials and methods

Sensor and cartridge fabrication

Sensor and cartridge fabrication was performed using a procedure as reported in our earlier studies.^{21,23,24} Briefly the nickel cantilevers were fabricated by a simple one mask process utilizing sputter, photolithography, electrodeposition and wet etching steps. The fabricated nickel cantilevers were 200 μm long, 20 μm wide and 2 μm thick. These dimensions were chosen to keep the resonant frequency within the operating frequency of the lock-in amplifier that was used in the experiments.

After the microfabrication, the MEMS dies were placed in the microfluidic cartridge. The microfluidic cartridge consisted of two parts: the substrate and the lid. Both of the parts were made of polymethyl methacrylate (PMMA) and the specific geometries on each were patterned by precision machining. We bonded MEMS dies to the wells on the substrate. UV cured epoxy (Norland Optical Adhesive 81) was used for bonding. The microfluidic channels were patterned on the lid part. Each microchannel was 200 μm deep and 1 mm wide. These channels can be functionalized for different tests. The lid and substrate

Fig. 1 (A) Schematic view of the measurement setup. An electro-coil is used for actuating the nickel cantilevers. There is no electrical connection between the cartridge and the reader. (B) A photo of the measurement setup.

were bonded to each other by UV cured epoxy. This type of epoxy bonding also provides excellent sealing between the parallel channels. Each channel has a total volume of 10 μL . This volume can be further reduced with an optimized cartridge geometry.

Measurement setup

The schematic of the experimental setup and the principle of operation are shown in Fig. 1a. The PMMA cartridge contained the MEMS dies with a 2.5 mm $\times$ 2.5 mm size. The nickel cantilevers were actuated magnetically with a coil placed in close proximity to the backside of the PMMA flow cell.²¹ The coil was driven with a high power broadband current amplifier. Since no direct electrical contacts are required on the chip, magnetic actuation with an external coil has a significant advantage for in-liquid operation. The optical read-out can also be conducted remotely with laser-based techniques. The results presented in this study were obtained with a Laser Doppler Vibrometer (LDV). The vibration amplitudes were measured with the LDV. Optical measurement techniques based on a grating interferometer and knife-edge methods have also been successfully implemented by our group. Therefore, the LDV can be replaced with other techniques to provide measurement parallelism.^{21,25} The phase difference between the coil drive and the LDV was monitored with a lock-in amplifier (Zurich Instruments HF2LI) and recorded simultaneously using a LabVIEW interface. All experiments were conducted at 37 ± 0.1 °C. Temperature stability is an important aspect since it has a strong effect on viscosity.²⁶ A temperature controller unit was built around the cartridge as shown in Fig. 1b. The unit consisted of an AD590 temperature sensor, a controller circuit and heating wires entangled inside the aluminium block as shown in Fig. 1b. The system is capable of controlling the temperature with a 0.1 °C precision on the microfluidic channel.

PT and aPTT measurements from control plasma samples

PT and aPTT measurements were performed on a healthy donor's plasma samples and commercially available control plasma samples. Healthy volunteers signed an informed consent and the study was approved by the Koç University Ethical Committee.

For the PT and aPTT tests, Calcium Freeze Dried Rabbit Brain Thromboplastin (DIAGEN, UK, lot no: T74) and Micro-nised Silica Platelet Solution (DIAGEN, UK, lot no: SP63) reagents were used, respectively. Three different control plasma samples were used: normal (lot no: KP93, DIAGEN, UK), abnormal 1 (lot no: AB1-80, DIAGEN, UK,) and abnormal 2 (lot no: AB2-79, DIAGEN, UK,). The blood samples were taken from a healthy 28 year old male donor into a citrated vacutainer tube (Greiner Bio One Vacuette 454326 polyethylene terephthalate sodium citrate blood collection coagulation tube, 3.2% sodium citrate). Afterwards the plasma was separated by centrifugation at $5000g \times 10$ min. Before the measurements, all of the reagents and samples were pre-warmed at 37 °C. Briefly, for the aPTT tests, 10 μL of the test plasma (or control) was mixed with 10 μL

of the micronised silica/platelet substitute mixture and the tube was gently tilted at intervals for exactly five minutes. After the incubation, the sample was mixed with 10 μL of 25 mM CaCl_2 solution and the clock was started ($t = 0$). Immediately we placed 5 μL of this mixture on the MEMS chip through manual pipetting. Real-time phase and amplitude read-out was conducted. For the PT tests a similar strategy was followed. The reagent used in PT measurements (Calcium Freeze Dried Rabbit Brain Thromboplastin) contains Ca^{2+} . Briefly, 20 μL of Calcium Rabbit Brain Thromboplastin was mixed with 10 μL of plasma and a stopwatch was started ($t = 0$). After mixing the mixture was introduced to the system as in the aPTT test.

The PT and aPTT were calculated when the slope of the first-order derivative of the coagulation curve recorded from the change of the phase was maximum.

Sample preparation for scanning electron microscopy (SEM)

The samples were prepared as described for the test on the MEMS chip and then fixed for 4 h in 2.5% glutaraldehyde at room temperature. Dehydration with increasing ethanol concentration (30–100% v/v) was followed by drying. The samples were coated with 3 nm Au–Pd and imaged by a Zeiss Ultra Plus FE-SEM.

Results and discussion

Characterization of the system

When the cantilevers are operated in viscous liquid media, the viscosity influences the resonant frequency and the quality factor of the system. In our previous studies we measured the viscosity changes with a sensitivity of 0.01 cP by tracking the frequency at a constant phase difference²¹ or by tracking phase changes at a constant frequency.²² During blood coagulation a sudden change in blood plasma viscosity occurs. When the coagulation is initialized, thrombin acts on fibrinogen, resulting in fibrin generation; then the fibrins aggregate and form insoluble clots. After coagulation measurement, the MEMS chips were investigated by SEM and clot formation was clearly observed on the MEMS cantilevers (Fig. 2). Changes in the viscosity of plasma were caused by the formation of the fibrin clot,¹⁴ and we monitored this significant change in the viscosity by real-time tracking of the phase and amplitude.

Before the coagulation measurements we characterized the system in air, deionized (DI) water and blood plasma. Fig. 3 shows the frequency response and the phase of the system in different media. The resonant frequency of the cantilever is 39 206 Hz when it was operated in air. Also the quality factor (Q) is around 178. However when it was operated in DI water the frequency drops to 22 185 Hz and Q is around 5. This sharp decrease is due to hydrodynamic loading which has both inertial and viscous components.²⁷ The response in blood plasma before coagulation is similar to that in DI water. The resonant frequency is 20 267 and Q is around 3.6. This is due to the higher viscosity and density of

Fig. 2 SEM images of fibrin networks formed after coagulation on the MEMS chip (A). More detailed (B) and less detailed (C) images of the fiber structure. Dried fibrins pull the cantilever and stick it to the silicon surface.

blood plasma with respect to DI water. Detailed information can be obtained from our previous studies on blood plasma viscosity.^{21,22}

During blood coagulation, a sudden change in the blood plasma viscosity occurs due to fibrin generation.¹³ This kind of viscosity change creates an increase in the hydrodynamic loading of the vibrating structures in viscous liquids according to Sader's theory.²⁷ For example, increasing viscosity reduces the vibration amplitude of the vibrating mechanical structure driven by a constant amplitude. Also, the effect of viscosity change can be observed as a phase shift between the drive signal and the phase of the mechanical displacement. Depending on the drive frequency the phase shift could be either positive or negative. The effect of viscous loading on mechanical resonators has been further investigated in the literature.^{28–30} After coagulation, the quality factor and the resonant frequency changed. The significant difference in the phase curve after coagulation is shown in Fig. 3a. According to our method we excited the system at a certain frequency around the resonant peak. The phase difference at that certain frequency changed during coagulation. Hence simultaneous monitoring of the phase gave a unique signature which indicates the coagulation time.

Fig. 3b shows the long-term phase stability of the system in citrated blood plasma. The cantilever was driven at its resonant frequency. The phase was recorded for 30 minutes. The standard deviation of the phase during this measurement is 0.015° which can be defined as the noise floor of the system. As shown during coagulation measurements, this value is highly sufficient for sensing blood coagulation.

Fig. 3 (A) The frequency response and phase of the system in air, in DI water and in human blood plasma before and after coagulation. (B) Phase stability results for citrated (non-coagulating) blood plasma. $f = 20$ kHz, standard deviation = 0.015° .

Experimental procedure

In this study we conducted both the aPTT and PT tests with our system. In order to investigate the sensitivity and reproducibility of the system, we used commercially available control plasma samples. The coagulation time measurements from normal, abnormal 1 and abnormal 2 control plasma samples were conducted and the results were compared with the standard values provided by the manufacturer and a commercial reference device, CoaguChek XS (Roche, Germany). Afterwards different channels of the same cartridge were functionalized for different tests and both PT and aPTT tests were performed on the same cartridge.

Briefly, for the PT and aPTT tests, $10\ \mu\text{L}$ of test plasma (or control) was mixed either with $20\ \mu\text{L}$ of the Calcium Freeze Dried Rabbit Brain Thromboplastin reagents or with $10\ \mu\text{L}$ of the Micronised Silica/Platelet Substitute mixture, respectively. A stopwatch was started ($t = 0$) and $5\ \mu\text{L}$ of this mixture was placed immediately on the MEMS chip. Real-time phase and amplitude read-out was conducted.

The phase and amplitude of the system during a PT test are shown in Fig. 4a. The sharp change in the phase and

Fig. 4 (A) The change in the phase and amplitude during the PT test for an abnormal 1 control plasma sample. (B) Polynomial fit to the phase data and the first derivative of the fit function; the time where $df/dt \rightarrow 0$ indicates the onset of the coagulation is reported as the coagulation time. (C) PT tests for the normal, abnormal 1 and abnormal 2 control plasma samples ($n = 3$ for each measurement). (D) First derivatives of the phase curves.

amplitude during coagulation can be seen. The first 5 seconds is the time passed during the mixing of the reagent and plasma and introduction of the mixture to the system. The amplitude data are noisier than the phase read-out. This can be related with the relatively small vibration amplitudes. The RMS value of the displacement is in the order of 10 nm. This is a very small value compared to the cantilever dimensions (length: 200 μm , width: 20 μm , thickness: 2 μm). Also during coagulation, as a result of increased viscosity, the vibration was damped and the amplitude decreased to even lower values. The amplitude read-out is susceptible to environmental noise whereas the phase read-out approach is more immune to external noise sources since a reference signal (coil input) is used. The phase gives clearer information since the signal-to-noise ratio (SNR) is higher. It is more convenient to use the phase for tracking coagulation.

After the data were collected a polynomial was fitted to the phase data, as shown in Fig. 4b. The first derivative of this

polynomial was used to obtain quantitative data. The time where the derivative is zero ($df/dt \rightarrow 0$) indicates the coagulation start time whereas the local minimum ($df/dt \rightarrow \text{min}$) shows the mid-point of the coagulation process (Fig. 4b). In Fig. 4c the fit functions obtained from the repetitive PT measurements from normal, abnormal 1 and abnormal 2 plasma are shown. Fig. 4d shows the first derivatives of these functions. The difference between the coagulation times of different samples can be seen. They can be clearly distinguished from each other. The same data acquisition and processing protocol was also applied to the aPTT measurements. After the measurements, the values were normalized with respect to the mean value of the normal plasma sample. The commercial devices report the coagulation times in terms of the International Normalized Ratio (INR).⁵ There will be variations between the test results (in seconds) of a normal individual. This is due to the variations in the batches of reagents and test conditions. The INR was devised to

standardize these results. It is related to the tested individual's PT time and the normal PT time.²⁰ The International Normalized Ratio (INR) for PT measurements and the normalized ratio for the aPTT measurements were thus obtained. The mean value, standard deviation and coefficient of variation for the PT and aPTT measurements are shown in Table 1.

The starting point of the coagulation obtained with our system matches the reference data better than the coagulation mid-point. Also another reference PT measurement was conducted with a reference commercial device, CoaguChek XS. Again the coagulation times are in good agreement with the commercial reference device measurements. Our proposed novel assay can significantly differentiate coagulation times between normal, abnormal 1, and abnormal 2 samples ($p < 0.01$).

A reliable diagnostic assay should yield the same measurements for the same target. Various sources of variability affect overall cartridge variability: electronic and fabrication chip-to-chip variability and biological assay variability. All three of these variability sources contribute to the overall measurement variability. To check the reproducibility of the assay, all of the measurements were repeated three times in a day and on three different days. The percent coefficient of variation (CV%) for the tested samples is 7.28 and 6.33 for PT and aPTT measurements, respectively. These reproducibility values are comparable with those of the commercial devices available in the market³¹ and recent studies in the literature.²⁰ The results of the aPTT and PT measurements in comparison with the reference values are shown in Fig. 5. There is no statistical difference between our results and those of the reference device and the control sample datasheet ($p > 0.05$).

Measurements with surface functionalized channels

All above measurements were performed on samples prepared outside the cartridge and placed on MEMS chips. For further implementation, the microfluidic channels of the cartridge were functionalized for PT and aPTT tests by drying specific

Fig. 5 The INR and normalized ratio values of the PT and aPTT tests. The reference values are provided from the manufacturer's datasheets. The error bars for the reference values show the standard deviations obtained from the range indicated in the datasheet. The error bars for the MEMS sensor show the standard deviations of the measurements.

reagents in each channel. Then we performed PT and aPTT measurements with the surface functionalized cartridges.

The surface functionalized channels of the cartridge are shown in Fig. 6a. For the aPTT test, Micronised Silica Platelet Solution (lot no: SP63, DIAGEN, UK) was used whereas for the PT test Freeze Dried Rabbit Brain Thromboplastin (lot no: DT139, DIAGEN, UK) was used. Briefly, 10 μ l of each solution was poured into the microchannels. 10 μ l of 25 mM CaCl₂ was also poured into the channels at the opposite side (Fig. 6a). Afterwards the cartridge was placed in a vacuum chamber for drying. The vacuum drying operation was conducted at around 100 mmHg vacuum for 20 min to dry the reagents.

Table 1 PT results in terms of INR and aPTT results in terms of normalized ratio. The results are compared with the reference datasheet of the control plasma samples and the reference commercial device

PT (INR)									
	MEMS results (INR)						Datasheet (INR)		Reference device (INR)
	Within day			Between day					
	Mean	σ	CV (%)	Mean	σ	CV (%)	Mean	σ	
Normal	0.95	0.07	7.73	1.00	0.08	7.79	1.03	0.07	1.30
Abnormal 1	1.47	0.16	10.54	1.60	0.11	6.85	1.74	0.35	1.60
Abnormal 2	2.74	0.26	9.63	2.75	0.20	7.20	2.83	0.40	3.30

aPTT (normalized ratio)									
	MEMS results (ratio)						Datasheet (ratio)		
	Within day			Between day					
	Mean	σ	CV (%)	Mean	σ	CV (%)	Mean	σ	
Normal	1.04	0.05	5.24	1.01	0.04	3.94	1.01	0.08	
Abnormal 1	1.19	0.04	3.10	1.19	0.11	9.11	1.36	0.20	
Abnormal 2	1.89	0.20	10.65	1.78	0.10	5.87	1.93	0.28

Fig. 6 (A) Scheme of the functionalization procedure. Channels are functionalized for aPTT, PT and reference tests. (B) aPTT and PT tests and the reference measurement conducted in different channels of the same cartridge.

For each test 10 μ l of the blood plasma sample at 37 $^\circ$ C was poured into the reagent coated channel. For the aPTT test 5 min of incubation time was chosen whereas for the PT test it was 1 min. After the incubation, the sample was pushed to the opposite channel that was coated with CaCl_2 . When the sample touches the channel walls, the sample is pulled back to the cantilever site and the clock is started ($t = 0$). Real-time phase and amplitude read-out was conducted. Although the tests were conducted one at a time within the scope of this paper, tests can be performed simultaneously using multiple photodetectors for the read-out. Since the electromagnetic actuation is at a constant frequency, using a detector array will enable simultaneous monitoring of the phases of different cantilevers in parallel channels.

The results of the PT and aPTT tests performed on the same cartridge are shown in Fig. 6b. As expected, when the coagulation started a sharp change in the phase was observed. The reference curve was obtained by addition of Ca^{2+} to the citrated blood plasma. According to the first derivative of the phase data the coagulation time is 645 s for the reference channel. This is an expected value since no external reagent was added to the system. According to the first derivative of the phase data the coagulation time for the aPTT test was 67 s whereas for the PT test it was 22 s. According to the reference values in the reagent datasheets these values are supposed to be in the ranges 35–40 s and 14–17 s for aPTT and PT tests,

respectively. This slight difference is probably due to reagent mixing inefficiency in the microchannels and further improvements are needed to obtain more accurate PT and aPTT values.

Conclusions

In this paper we demonstrated, a LoC (Lab-on-Chip) sensor array platform enabling multiple coagulation tests. The system has independent reader and cartridge units. The cartridge consists of microfluidic channels and MEMS microcantilevers placed inside them. The magnetic microcantilevers are made of electroplated nickel. In different channels of the cartridge aPTT and PT tests were conducted with blood plasma taken from a healthy donor. The sample volume used for each test was 10 μ l which could be decreased with further design improvements. In order to improve the system to the commercial medical device level, standardized clinical tests are required. The effect of surface properties will be investigated and the mixing conditions will be optimized to obtain high repeatability. Nevertheless, the obtained results gave satisfactory information about the proof of concept. To the best of our knowledge this is the first demonstration of the use of a dynamic microcantilever based system for coagulation time measurement. The independence of the cartridge and the reader unit increases the potential of this approach as a point-of-care system.

In this study we used a bench-top prototype reader unit based on an LDV. In the future, the LDV will be replaced by an optical read-out based on the optical lever method. The reflected beams will be measured using a photodetector array. This will enable manufacturing of a handheld device capable of performing multiple tests. The ultimate goal of this project is to develop a system that is capable of making measurements from whole blood. Towards this goal, in this study we demonstrated our system using blood plasma. In the future, the tests will be conducted also on whole blood samples.

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