PiCT: 1st Recognition for Human Whole Blood on QCM-D Platform

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Abstract
Instruments for point-of-care (POC) coagulation monitoring for whole blood could reduce fundamental limitations of routine coagulation tests. ‘Prothrombinase induced Clotting Time’ (PiCT) is considered a universal assay for anticoagulants monitoring in laboratory and clinics but it has not been evaluated yet. It could reduce the inherited drawbacks of highly variability of results among different individuals in the activated partial thromboplastin time (aPTT) which is the most applied and clinical standard assay. In this report, PiCT for whole blood on quartz crystal microbalance with dissipation (QCM-D) platform for anticoagulant monitoring has been recognized for the first time. The present study demonstrates the lowest historical sample volume of human whole blood (as well as each reagent) consumption of 1.66 µL employed ever for coagulation. This is substantial support for launching spot test via QCM-D in laboratory and clinics and ultra-refining of POC settings. Different doses of danaparoid anticoagulant in blood samples (n=20) have been studied on QCM-D platform in parallel to ‘gold standard’. Both techniques demonstrated lower variability for anticoagulant with %RSD values between 3 and 8.5 depending on different anticoagulant doses. Data could be considered as precise and accurate for an anticoagulant recognition directly in blood using a clotting assay on QCM-D platform. Present study is crucial in the perspectives of robustness due to direct whole blood method on QCM-D platform and its cost-effectiveness due to lowest sample (as well as reagents) volume consumption.

Keywords: Whole blood, PiCT, Danaparoid, QCM-D, qCell T

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1 Introduction
Point-of-care (POC) coagulation monitoring devices for whole blood could reduce several limitations of routine coagulation tests. These devices are attaining popularity in clinical practice, especially in the cases of patients undergoing cardiac or liver surgery. They provide important information in a large variety of clinical scenarios such as massive hemorrhage, assessment of hypo- and hyper-coagulable states, therapeutic directions in pro- and anticoagulant therapies and diagnosis of a surgical bleeding. A surgical etiology of bleeding has to be considered only when viscoelastic assay results are normal. Furthermore, accurate monitoring of anticoagulant is a considerable burden on healthcare providers.

Quartz Crystal Microbalance (QCM) has outstanding potential by employing suitable sensor thin films for pharmaceuticals and clinical studies. It is remarkable due to its cost-effectiveness on comparing to its counterpart biosensor platforms. Quartz Crystal Microbalance with Dissipation (QCM-D) is unique emerging device for POC. It could monitor the whole coagulation process, starting from fibrin formation till the total coagulation point. The coagulation status in whole blood is advantageous over in plasma because whole blood coagulation allows the plasmatic coagulation system to interact with platelets and red cells. Therefore, it provides useful additional information on platelet function. Direct monitoring of anticoagulant in blood is preferable over in plasma to avoid the efforts for making plasma and pre-analytical contribution of different factors during plasma generation process.

PiCT is a universal coagulation assay and has potential to monitor all types of anticoagulants except vitamin K based e.g. Marcoumar or
Warfarin\textsuperscript{12}, aPTT is the most commonly used coagulation assay but its results suffer high variability among different individuals and it lacks precision and accuracy. Additionally, it is inapplicable to drugs such as low molecular weight heparin and direct factor Xa inhibitors. The PiCT does not correlate with aPTT\textsuperscript{13} but it could reduce the flaws of aPTT\textsuperscript{14}. Very few reports have been documented on PiCT. For example, Fenyesi et al studied the effects of lepirudin, argatroban, melagatran and heparin in normal and oral anticoagulant in plasma\textsuperscript{15}. Schöni et al studied correlation between PiCT-ROTEM and heparin levels\textsuperscript{16}. Clemens et al investigated dabigatran anticoagulant activity by employing PiCT assay\textsuperscript{17}. PiCT is unsuitable for monitoring of rivaroxaban\textsuperscript{14,15}.

Danaparoid anticoagulant is used in different clinical settings and its monitoring is substantial. For example it is used hip surgery, heparin-induced thrombocytopenia\textsuperscript{20} and in Kasabach-Merritt syndrome.

Present study is important because PiCT assay involves two steps. In the first step, the whole blood is mixed with PiCT reagent and mixture is incubated for three minutes. In the second step, PiCT starter (i.e. CaCl\textsubscript{2}) is mixed into the incubated mixture. For PiCT based QCM-D platform, the platelets and red blood cells in whole blood samples may complicate the assay over in plasma. This factor is substantial because ‘gold standard’ coagulometer employs a steel ball which revolves throughout the PiCT measurement for effective mixing, while QCM-D studies are done in static conditions. PiCT application to whole blood for (any) anticoagulant monitoring on QCM-D platform has not been investigated. Therefore, anticoagulant danaparoid doses in whole blood have been selected for PiCT studies on QCM-D platform. This is first report to recognize PiCT for determination of anticoagulant (danaparoid) in whole blood on QCM-D platform and it is compared in parallel with ‘gold standard’ (i.e. mechanical coagulometer). This is continuous work on haemostasis\textsuperscript{31,32} and the lowest historical sample volume consumption of 1.66 µL of human whole blood for PiCT has been demonstrated for the first time. This could be a substantial support for POC settings for practical, precise and accurate monitoring of anticoagulant directly in whole blood. On considering PiCT-QCM-D platform for anticoagulant monitoring in whole blood in the perspectives of precision and accuracy, it paves a path as clinical routine. Present study is crucial in the perspectives of robustness due to direct whole blood method and its cost-effectiveness due to lowest sample (as well as reagents) volume consumption. Furthermore, it gives a proof of principle for whole blood based PiCT assay on QCM-D platform for anticoagulant monitoring. This is substantial for ultra-refining of POC settings for laboratory method too.

2 Materials and Methods

2.1 Reagents and chemicals

PiCT reagents (PiCT activator and PiCT starter (25 mM CaCl\textsubscript{2})) i.e. pefakot PiCT controls UFH 505-22 were purchased from DSM Nutritional Products Ltd, Switzerland. 1-Vinyl-2-pyrrolidone (VP), N-methyl pyrrolidone, Di vinyl benzene (DVB), acetone, dimethylformamid (DMF), a,a’-azobisisobutyronitrile (AIBN) and Danaparoid Sodium were purchased from Sigma-Aldrich. 50 mM TRIS buffer pH 7.4 was prepared from Sodium Chloride (VWR International BVBA) and Tris (2-hydroxy ethyl) amine hydrochloride (TRIS) (PAESEL+LORI GMBH & CO). Danaparoid dilutions were prepared in 50 mM TRIS buffer having pH 7.4.

2.2 Equipment and instruments

QCM-D (qCell T, 3T Analytik, Germany). QCM transducer having 10 MHz frequency is a piezoelectric AT-cut-quartz coated with two gold electrodes. It contains one electrode on each side with 8 mm diameter and 5 mm diameter respectively. UVACUBE 100 (λ\textsubscript{max} 350 nm, Hönle UV Technology Germany), Mechanical coagulometer Merlin MC 1 (Merlin Medical, ABW Medizin und Technik, Lerngo, Germany) and Spin-coater (Spin150-v3, Semiconductor Production Systems, Germany).

2.3 Polymer synthesis

30 µL VP, 70 µL DVB, 50 µL DMF, 50 µL acetone and 1.0 mg AIBN were mixed in the reaction vial. The resulting homogenous solution was kept under UV lamp for 23 minutes. The polymer was further diluted with 700 µL acetone prior to spin coating.

2.4 Sensor thin films

QCM electrodes were cleaned with N-methyl pyrrolidone and subsequently by acetone. After cleaning, 15 µl of polymer was spin coated at 6000 rpm for 100 seconds onto QCM-electrode. QCM-D (qCell T) was employed to check the layer height (a 10 nm) of thin film on each QCM. QCMs having sensor thin films were kept under UV for 2.5 hours for complete hardening of thin films. Afterwards, QCMs were subjected to PiCT measurements, or stored in desiccator prior to PiCT measurements on QCM-D.

2.5 Human blood samples

Whole blood samples from healthy donors were donation from the university hospital of Tuebingen, Germany. These fresh blood samples from healthy donors were taken in syringes having 1.0 ml of 0.106 mol 1\textsuperscript{-1} citrate.

2.6 PiCT QCM-D measurements

QCMs were mounted on QCM-D and were calibrated at 37 °C to achieve stable baseline. Blood samples were induced with 0.00, 0.25, 0.50, 0.75 and 1.00 IU/mL doses of danaparoid. 5 µl of PiCT
activator was incubated at 37 °C for one minute in an Eppendorf and followed by mixing of 5 µl of blood. This mixture was again incubated at 37 °C for three minutes in the incubation chamber of QCM-D. With the help of micropipette, 5 µl of PiCT starter (CaCl2) at 37 °C was mixed into the incubated mixture and 5 µl from resultant mixture was placed into the centre of incubated QCM. 5 µl from end mixture means 1.66 µl of human blood sample (i.e. is the lowest whole blood sample volume used in the history of haemostasis to date). 1.66 µl of each reagent is also the shortest reagent volume employed for PiCT. The lower whole blood sample volume consumption is important support for POC settings. After PiCT studies on QCM-D platform, QCMs were discarded into wastage. This factor is substantial because today's clinical set up employs disposals rather than reusable systems to avoid contamination.

2.7 PiCT mechanical coagulometer ‘Gold Standard’ Measurements

PiCT whole blood measurements were carried out on mechanical coagulometer in parallel to QCM-D’s at the same time by using 50 µl sample volumes, with same incubation times and mixing ratios.

2.8 Scanning electron microscopy (SEM)

QCM electrodes having sensor thin films were visualized via SEM. QCMs were critical point dried (CPD), sputtered with gold palladium and were visualized by using SEM.

3 Results and Discussions

QCM-D are unique ultra-sensitive devices towards mass, viscosity or density of wetting liquids and they provide sensor-responses in the form of frequency and dissipation shifts. A standing mechanical shear wave within the quartz transducer is produced on applying alternating voltage to the electrodes of QCM. This half wavelength is equal to the thickness of the quartz. That is why QCM is also termed as Thickness Shear Mode Transducer.

3.1 Surface morphology of thin film

SEM image of polymer thin film on QCM transducer reflected flat surface morphology. SEM image of sensor thin on QCM depicted in figure 1A, while QCM without thin film shown in figure 1B. Surface morphology of polymer thin film on the QCM demonstrated slight roughness as compared to QCM without thin film. Surface morphology is suited for present studies (Figure 1).

3.2 PiCT-QCM-D exemplary curves

The QCMs thin films were subjected to whole blood PiCT measurements according to the protocol explained in experimental section. PiCT exemplary measurements for human whole blood coagulation on QCM-D along with negative controls demonstrated in figure 2.

An easy differentiation of PiCT coagulation can be done in each case of frequency (Δf (Hz)) and dissipation (damping) (ΔΓ (Hz)) signals on comparing with negative controls.

Figure 1A: SEM image of sensor thin film on QCM

Figure 1B: SEM image of QCM without sensor thin film

Bandwidth (Δ Γ Hz) and dissipation (D) have same meanings and they are related according to following equation.

\[ Δ Γ (Hz) = 2D/fn \]

fn is the resonance frequency of quartz crystal microbalance at overtone n

PiCT coagulation and without coagulation measurement, signals are different in the perspectives of shapes and magnitudes of frequency and dissipation shifts. PiCT coagulation point has been indicated as “red star” indicator, is the start of falling (down lift) of frequency after reaching stability of frequency signal. An opposite behaviour can be observed in dissipation signal, i.e. start of uplift of dissipation after the stability. Total coagulation is the end of the coagulation process, has been indicated as “black star” indicator, in both cases of frequency and dissipation signals respectively.

PiCT-QCM-D exemplary curves of whole blood from one healthy donor induced with danaparoid doses of 0.00, 0.25, 0.50, 0.75 and 1.00 IU/mL depicted in Figure 3.

QCM-D technique is unique because of its kinetic information of total coagulation monitoring on QCM-D signal. Kinetic information is substantial support to POC settings and is not possible in standard coagulometer measurement because it picks one threshold point during coagulation. Total coagulation point on frequency and
dissipation signals is crucial for assessing the mass effect from the clot that directly attaches to the sensor thin film on completion of coagulation process. Additionally, danaparoid doses affect the frequency and dissipation shifts as well as kinetics which are reflected in the form of PICT and total coagulation points. These effects are further controlled by the visco-elastic and hydrophobic properties of sensor thin film employed. An increase of the danaparoid dose within same blood sample of healthy donor leads to longer kinetics of PICT and total coagulation points. This causes a lower the frequency and dissipation shifts of 15-25%.

Figure 2: QCM-D-exemplary measurements of PICT for whole blood (from a healthy donor) coagulation along with two different negative controls i.e. blood without coagulation and PICT-coagulation activator without blood. PICT coagulation points and total coagulation points are indicted by red star and black indicators in both cases of frequency and dissipation curves respectively.

3.3 PICT-QCM-D vs PICT-‘gold standard’

Study of more real samples of human blood samples is essential due to highly complex nature of human real blood samples\(^5\). A plot directly comparing tQCM (where "t" is PICT) of blood samples (n=20 for each case) having different doses of danaparoid with tCoag (where "t" is PICT on gold standard) demonstrated in figure 4. A promising correlation line is passing through the origin remaining within analytical limits of deviations. Every dose of danaparoid yielded precise range and can be differentiated from other counterpart doses. Lower doses of danaparoid produced outstanding \(R^2\) values of 0.99, while higher doses of 0.75 and 1.00 yielded \(R^2\) 0.94 and 0.97 respectively.

Figure 3: QCM-D-exemplary measurements of PICT for whole blood (of one healthy donor) coagulation for blood having danaparoid 0.00, 0.25, 0.50, 0.75 and 1.00 IU/mL. PICT-coagulation and total coagulation points are indicted by red and black stars indicators in both cases of frequency and dissipation curves respectively.

The data in every case of blood probe was within analytical deviation limits and was on the ideal correlation line. This is outstanding support for POC settings for QCM-D technique because the lowest whole blood sample volume (as well as each reagent) consumption of 1.66 µL for QCM-D has been employed in comparison with ‘gold standard’. ‘Gold standard’ utilizes 30 times higher whole blood volume (as well as each reagent volume) of 50 µL for laboratory experiments for PICT. Blood samples (n=20) having 0.00, 0.25, 0.50, 0.75 and 1.00 IU/ml doses of danaparoid yielded precise PICT ranges of 25 ±3, 56±5, 102 ±11, 157 ±8 and 215 ±9 seconds respectively. Data could be considered as accurate and precise for an anticoagulant monitoring directly in blood using a clotting assay.

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3.4 Bland-Altman plot

A Bland-Altman plot[28] for t-QCM-D and tCoag demonstrated in figure 5 for further comparison into the techniques.

For whole blood PICT, Bland-Altman plot was crucial for a visual overview and comparing the agreement of two techniques rather than correlation. The Bland-Altman plot for tQCM-D vs tCoag depicted a linear line which was within ±2SD agreement at 0.00 and 0.25 IU/mL doses of danaparoid. It yielded a spread within analytical deviation limits for higher doses of 0.50 - 1.00 IU/mL danaparoid. On merging the discussion above, PiCT-QCM-D was promising in the terms of precision and accuracy for challenging anticoagulant monitoring directly in whole blood for laboratory practice.

3.5 Relative SD

The interesting part of the present study is the %RSD data of PiCT-QCM-D in comparison to that of PiCT-Coag. It is depicted in figure 6. PiCT on QCM-D platform for blood induced with danaparoid doses of 0.00, 0.25, 0.50, 0.75 and 1.00 IU/mL yielded %RSD of 12.08, 9.03, 10.88, 5.01 and 4.06 respectively. PiCT on ‘gold standard’ for blood having danaparoid 0.00, 0.25, 0.50, 0.75 and 1.00 IU/mL yielded %RSD of 12.33, 8.92, 11.47, 4.79 and 4.05 respectively. Both techniques yielded %RSD values between 4 and 12 with slight fluctuations on both sides. The %RSD data for both techniques demonstrated lower variability for anticoagulant monitoring directly in whole blood.

QCMs are outstanding sophisticated devices due to their viscoelastic[29,30] and mass sensitive properties. Viscoelastic and mass sensing of the haemostasis at non-molecular levels are fundamental properties for fibrin polymerization in coagulation process, platelets fibrinogen interactions and fibrinolysis. Furthermore, characteristics of sensor thin film employed could contribute in precise and accurate monitoring of anticoagulant and haemostasis kinetics. PiCT test in the perspectives of precision and accuracy is promising and it paves a path towards a clinical laboratory routine test. The present study is promising for PiCT test application on QCM-D technique.
4 Conclusions

Blood samples (n=20) having 0.00, 0.25, 0.50, 0.75 and 1.00 IU/ml doses of danaparoid yielded precise PiCT ranges of 25±3, 56±5, 102±11, 157±8 and 215±9 seconds respectively. PiCT on QCM-D platform for blood having danaparoid 0.00, 0.25, 0.50, 0.75 and 1.00 IU/mL yielded %RSD of 12.08, 9.03, 10.88, 5.01 and 4.06 respectively. PiCT on ‘gold standard’ for blood having danaparoid 0.00, 0.25, 0.50, 0.75 and 1.00 IU/mL yielded %RSD of 12.33, 8.92, 11.47, 4.79 and 4.05 respectively. Both techniques yielded % RSD values between 4 and 12 with slight fluctuations on both sides. Data could be considered as precise and accurate for an anticoagulant recognition directly in blood using a clotting assay. Data of PiCT on QCM-D platform for whole blood presented above for haemostasis studies is promising for POC settings in clinical and laboratory applications. PiCT application on QCM-D platform could be potential candidate for routine laboratory method worldwide.

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6 Competing interest

Author has no competing interests.

7 Author’s contributions

The study was conceived, planned, performed and written in the form of manuscript by MH.

8 References


