Shortened 'Thrombin Time' Monitoring on QCM-D: A Better Substitute of 'Gold Standard'

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Abstract

Thrombin is the central enzyme in the coagulation cascade. Recently, modern technologies for thrombin generation measurement have shown a multidisciplinary interest in methods and assays. This is first report to recognize and optimize for human plasma application of 'thrombin time' (TT) assay for quartz crystal microbalance with dissipation (QCM-D) technique. QCM-D technique has been studied comparatively with mechanical coagulometer (which is regarded as 'gold standard' for coagulation assays). The optimized protocol for TT has been applied for plasma samples (n=54) on both platforms. Thrombin times (TTs) on QCM-D platform versus those of 'gold standard' yielded a promising correlation line with R² value of 0.86. For TT assay, QCM-D platform proved superior as compared to 'gold standard' platform due to following four edges. Firstly, QCM-D platform provides whole kinetic information, including monitoring of total coagulation on its measurement curve. 'Gold standard' cannot yield this information because it picks one point during coagulation. Secondly, TTs on QCM-D platform are 30% shorter as compared to those of 'gold standard'. Thirdly, TTs on QCM-D technique produced 16% lower %RSD demonstrating lower variability. Lastly, a historical lowest sample volume consumption of 2.50 µl has been applied on QCM-D platform. 'Gold standard' employs 40 times greater sample volume consumption for laboratory experiments of TT. Additionally, 40 times lower reagent volume consumption has been employed on QCM-D platform in comparison to its counterpart's. These advantageous features are substantial support for point of care (POC) settings for TT assay via QCM-D technique.

1 Introduction

Thrombin is the central enzyme in the coagulation chain reaction. Estimation of an individual's potential to produce thrombin could correlate with a hyper- or hypo-coagulable phenotype. Furthermore, modern technologies for thrombin generation measurement lead to a multidisciplinary interest in the assays and methods. Thrombin assays vary depending on the defect to be investigated. For instance, fluorogenic thrombin generation assays show acceptable intra-laboratory deviations but have a higher inter-laboratory deviation. Thrombin production is extremely variable among individuals. It requires individualized procedure for global haemostatic response, especially in the cases of bleeding disorders or on anticoagulant therapy.

Clinical standards assays, such as prothrombin time (PT) and activated partial thromboplastin time (aPTT) do not determine the complete coagulation. These assays use clot formation as their endpoint, which occurs only at the stage of formation of around 5% of all physiologically relevant thrombin\(^1\). Furthermore, these are insensitive to prothrombotic states. Coagulation factor assays can identify specific deficiencies, but they do not precisely correlate with the clinical phenotype. Further limitations of PT and aPTT tests\(^4\) include thrombin generation variations up to 40 times. These variations occur in cases of individual coagulation factors measurements at the extremes of the normal ranges in a synthetic plasma system. It is important to mention here that PT, aPTT and TT
are technically different assays and TT should not be mixed with PT and aPTT assays.

QCM has an outstanding potential for real application of pharmaceuticals and clinical relevant studies. It is attractive because of its cost-effectiveness on comparing to its counterpart sensor platforms. Furthermore, QCM-D offers monitoring of whole kinetics of the coagulation process via frequency and dissipation shifts. Recently, several reports have been documented on recognition of QCM-D platform for PT, aPTT, Prothrombinase induced Clotting Time (PICT) and related studies. Owing to fundamental importance, thrombin detection has caught multidisciplinary attraction in different domains recently. But, thrombin time (TT) assay, specifically for application of human plasma samples has not been recognized and studied on QCM-D platform yet. TT assay has been selected for present studies on QCM-D technique. This is first report to recognize (and optimize) TT for QCM-D platform and it is compared with that of ‘gold standard’ in parallel. Present study is also first in the terms of the lowest sample volume consumption of 2.50 µL applied ever for TT test. This report demonstrates the lowest thrombin reagent consumption of 2.50 µL for TT assay too. These factors are crucial support for POC settings for cost-effectiveness of TT assay. Additionally, TT based QCM-D measurement curve offers visualization and monitoring of the whole process of coagulation, including total coagulation point. ‘Gold standard’ cannot yield this information because it picks one point during coagulation. This factor is substantial in the perspectives of robustness and straightforwardness of the assay. TT based QCM-D platform yielded a promising correlation with that of ‘gold standard’. This is first report that demonstrates that TT based QCM-D assay offers a better alternative to that of ‘gold standard’. This is fundamentally due to two reasons. Firstly, QCM-D platform yielded shortened TTs as compared to those of ‘gold standard’. Shortened coagulation times are preferred in laboratory and clinic practice to avoid time consumption. Secondly, TTs on QCM-D platform yielded lower variability comparatively. This study is a proof of principle for TT assay on QCM-D platform, and it ultra-refines the POC settings for laboratory method.

2 Materials and Methods

2.1 Chemicals and reagents

Thrombin reagent (STA trade mark of Diagnostica Stago Roche), was purchased from Roche Diagnostics, GmbH, Mannheim, Germany (website: https://www.roche.de). Dimethylformamid (DMF), 1-Vinyl-2-pyrrolidone (VP), N-methyl pyrrolidone, Di vinyl benzene (DVB), acetone and a,a’-azobisisobutyronitrile (AIBN) were purchased from Sigma-Aldrich. All chemicals were purchased at their highest purity availability.

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2.2 Instrumentation

Mechanical coagulometer Merlin MC 1 (Merlin Medical, Germany), QCM-D ( "qCell T" 3T Analytik, Tüttlingen, Germany), Centrifuge (Thermo Scientific, Germany), UVACUBE 100 (λₘₐₓ 350 nm, Hönlle UV Technology Germany) and Spin-coater (Semiconductor Production Systems, Germany)

2.3 Polymer generation

Polymer generation and relevant studies on surface morphology have been demonstrated in recently. Briefly, 30 µL VP, 70 µL DVB, 1.0 mg AIBN, 50 µL acetone and 50 µL DMF was taken in an Eppendorf tube and completely mixed by using sonication. After mixing, the mixture was incubated under UV lamp for 23 minutes. After incubation, the polymer was diluted with 700 µL acetone before spin coating it onto the front electrode of quartz crystal.

2.4 Quartz crystal thin films

Quartz crystals were treated with N-methyl pyrrolidone. Afterwards, they were further cleaned with acetone. After cleaning, 12 µL of polymer was spin coated at the speed of 5000 rpm for 90 seconds onto the front electrode of quartz crystals. QCM-D was employed to measure the layer height of (a 10 nm) coating on each quartz crystal. Afterwards, these quartz crystals were incubated under UV for 2.5 h. The purpose keeping under UV was to achieve rigid and sold coatings. These quartz crystals were stored in desiccator or subjected to coagulation measurements on QCM-D.

2.5 Human plasma samples

The human whole-blood-donations from healthy volunteers were collected from the university hospital of Tuebingen, Germany. These donations were collected in appropriate tubes possessing 1.0 ml of 0.106 mol l⁻¹ citrate. These samples were centrifuged at 2500 x g for 15 minutes at room temperature to obtain platelets poor plasma (PPP).

2.6 Thrombin-QCM-D measurements

Quartz crystals having coatings were confronted to TT test on QCM-D. For this purpose, quartz crystals were calibrated at 37 °C for getting a stable baseline. 5 µl of PPP in an Eppendorf was subjected to incubation step at 37 °C for three minutes. This was achieved in the incubation chamber of QCM-D. With the help of micropipette, 5 µl of thrombin reagent at 37 °C was mixed into the incubated PPP and 5 µl from resultant mixture was injected onto the centre of incubated quartz crystal. 5 µl from resultant mixture containing 2.50 µl of human PPP is the lowest sample volume used in the history of TT test to date. This also contains the lowest thrombin reagent volume consumption employed ever for TT. TT based QCM-D platform provides outstanding support for POC settings for future of
bioelectronics of QCM-D technique. This factor is substantial for implementation of QCM-D technique in clinics or laboratory settings. After TT-QCM-D measurements, quartz crystals were not reused. This was done because today's laboratory and clinic set ups don't rely on reusing to avoid contamination.

2.7 Thrombin-mechanical coagulometer ‘gold standard’ measurements

TT measurements were carried out on ‘gold standard’ platform in parallel to QCM-D platform. ‘Gold standard’ measurements were done by using 100 µl volumes of PPP and thrombin reagent with same incubation times.

3 Results and Discussion

QCM-D is unique for mass, viscosity or density sensitive for liquids based on information from frequency and dissipation shifts.

3.1 TT-QCM-D exemplary curves

The quartz crystals having sensor coatings were subjected to TT coagulation measurements by using the setup demonstrated in experimental section. In this regard, TT exemplary measurements of human real plasma coagulation by employing 1:1 ratio (v/v) of plasma: thrombin reagent and two negative controls have been compared in Figure 1.

Figure 1: QCM-D-exemplary measurements of TT for plasma of one healthy donor employing 1:1 ratio of plasma: thrombin reagent (v/v) and two negative controls (i.e. negative control with plasma and without plasma). TT coagulation points and total coagulation points have been indicated by red star and black indicators in both cases of frequency and dissipation curves respectively.

On comparing TT measurement curves with negative control curves, we can differentiate TT coagulation in the terms of shapes and magnitudes of frequency and dissipation shifts. TT coagulation point has been shown as "red star", is the start of falling (can be called as down lift) of frequency curve after attaining stable frequency. In contrast, an opposite behaviour can be seen in the dissipation curve; here it is the start of uplift of dissipation signal after the stability of the dissipation curve. Total coagulation has been indicated as "black star" indicator, is the finishing point of the coagulation. This point can be monitored in both cases of frequency and dissipation signals respectively.

Kinetic information of total coagulation monitoring on QCM-D measurement curve is substantial for POC settings and this factor
makes QCM-D unique technique\textsuperscript{21}. Monitoring of total coagulation is impossible with standard coagulometer TT measurement because it picks one threshold point during coagulation. Total coagulation point in both cases of frequency and dissipation curves is crucial for monitoring the mass effects due to the mass of clot that directly attaches to the sensor coating. This reflects the suitable adhesion of plasma clot on the surface of sensor coating for yielding distinctive frequency (\(\Delta f\) (Hz)) and dissipation (damping) (\(\Delta \Gamma\) (Hz)) shifts.

It is important to mention here that Bandwidth (\(\Delta \Gamma\) Hz) and dissipation (\(D\)) has same meaning, and they are related according to following equation.

\[
\Delta \Gamma (\text{Hz}) = 2D/f_n
\]

\(f_n\) is the resonance frequency of quartz crystal at overtone \(n\).

In the next step, titration data for different ratios of plasma one healthy donor and thrombin reagent for TT has been demonstrated in figure 2. The figure demonstrates a comparison of tQCM (where “t” is TT on QCM-D) with tCoag (where “t” is TT on ‘gold standard’) for a plasma sample. Error bars of \(\pm\)SD of three measurements demonstrated superior TT for the ratio of 1:1 on both instruments. On the other hand, other ratios yielded spread in error bars but still error bars are within analytical limits of deviations.

![Figure 2](image2.png)

**Figure 2:** Titration data for different ratios (v/v) of plasma and thrombin reagent for TT of plasma of one healthy donor. Error bars are \(\pm\)SD of three measurements

3.2 TT-QCM-D vs TT-‘gold standard’

Further application of TT test to more real samples of human plasma is crucial due to extremely complex nature of human real plasma samples\textsuperscript{22}. After fundamental confirmation of successful of TT coagulation on QCM-D, the platform was subjected to more human plasma samples and compared in parallel with ‘gold standard’ coagulometer. **Figure 3** compares TTs obtained from QCM-D and ‘gold standard’ coagulometer. For 54 samples, tQCM yielded 17.58\(\pm\)2.16 seconds, while tCoag yielded 25.04\(\pm\)3.61 seconds. TTs on QCM-D technique are 30% shorter as compared to TTs on gold standard.

![Figure 3](image3.png)

**Figure 3:** TT obtained from QCM-D (tQCM-D) and from ‘gold standard’ mechanical coagulometer (tCoag) for plasma samples (n=54). Data have been presented from mean of three TT measurements

A plot of TTs on gold standard vs TTs on QCM-D is essential to see the correlation of two techniques. **Figure 4** demonstrates a plot directly comparing tQCM (where “t” is TT) of plasma samples (n=54) with corresponding tCoag (where “t” is TT on gold standard). A promising correlation line with \(R^2\) value of 0.86 is passing through the centre of data.

A historical lowest sample volume of 2.50 µl consumption for TT assay has been applied in comparison to ‘gold standard’ that uses 40 times greater sample volume (i.e. 100 µL) for laboratory experiments of TT. Additionally, 40 times lower reagent volume usage in comparison to that of mechanical coagulometer has been used on QCM-D technique. This is substantial support for POC settings in the perspectives of coagulation time shortening and the lowest sample (and reagent volume consumption). This factor is substantial because POC settings focus on shortened coagulation times and lowest sample and reagent volume consumption for laboratory and clinical practice.

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Figure 4: tQCM-D versus tCoag for plasma samples (n=54). Each data point is mean of three TT measurements

3.3 Bland-Altman plot

For present studies on TT, a Bland-Altman plot is necessary in order to compare the agreement of two techniques for a visual overview rather than correlation. For this purpose, a Bland-Altman plot\(^2\) for tQCM-D and tCoag has been plotted in Figure 5.

Figure 5: A Bland Altman plot for TT measured on QCM-D and 'gold standard' for plasma (n=54) samples

Both techniques demonstrated a linear line with \(R^2\) value of 0.67. The plot demonstrates a promising correlation of QCM-D technique with 'gold standard' technique for TT assay. The curve demonstrates 30% shorter values for tQCM-D as compared to tCoag remaining within analytical limits of deviations. In summary, a Bland-Altman

Figure 6: Comparison of %RSD data of plasma samples (n=54) for tQCM-D and tCoag

Quartz crystals are gaining popularity due to their viscoelastic and mass sensitive properties. By applying suitable sensor coatings, QCM-D technique could yield improved haemostasis kinetics in the perspectives of precision, accuracy and lower variability. QCM-D technique could reduce the flaws of conventional techniques, which are clinical and laboratory standards. Present study for TT assay is a promising example that reduces the limitations of 'gold standard'. On considering TT test on QCM-D platform in the perspectives of coagulation time shortening, precision, accuracy and lower variability, it paves a path towards routine laboratory method. Frequency and dissipation shifts on QCM-D provide additional advantage of total coagulation, which is impossible on mechanical coagulometer. Overall, QCM-D technique for TT proved a better and promising alternative to the gold standard.

4 Conclusion

TT has been optimized for human plasma applications on QCM-D platform, and it is compared in parallel with ‘gold standard’
mechanical coagulometer. Owing to lower deviations of TT and total coagulation points with 1:1 ratio (v/v) of plasma: thrombin reagent, it has been selected and applied for TT for plasma samples (n=54). Kinetic information of total coagulation monitoring on QCM-D measurement curve is substantial feedback to POC settings. ‘Gold standard’ cannot yield this information because it picks one point during coagulation. For 54 plasma samples, TT on QCM-D (tQCM) yielded 17.58±2.16 seconds, while TT on QCM-D (tCoag) yielded 25.04±3.61 seconds. Here, TTs on QCM-D are 30% shorter as compared to TTs on gold standard. A plot directly comparing tQCM of 54 plasma samples with corresponding tCoag yielded a promising correlation line with $R^2$ value of 0.86 passing through the centre of data points. TT on QCM-D platform for 54 plasma samples yielded %RSD of 12, while TT on ‘gold standard’ yielded %RSD of 14.5 respectively. The relative standard data on QCM-D technique is superior. Historical lowest sample volume consumption of 2.50 µl has been applied in comparison to ‘gold standard’ that uses 40 times greater sample volume (i.e. 100 µL) for laboratory experiments of TT. Additionally, 40 times lower reagent volume consumption in comparison to ‘gold standard’ has been employed on QCM-D platform. The lowest sample and reagent consumption, shortened coagulation times and lower variability of data on QCM-D are substantial support for POC settings. Furthermore, data presented above is promising for worldwide application of QCM-D technique for TT in laboratory and clinical practice.

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6 Competing interests

MH declares no competing interests.

7 Author's contributions

MH conceived idea, planned, performed research and wrote the manuscript.

8 References

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