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Measurement of the evolution of rigid and viscoelastic mass contributions from fibrin network formation during plasma coagulation using quartz crystal microbalance

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ABSTRACT

The coagulation of blood plasma and the effect of fibrinogen concentration were studied with a quartz crystal microbalance (QCM), where frequency and half-width at half-maximum (bandwidth) values measured from the conductance spectrum near resonant frequency were used. Bandwidth change is an indicator of energy dissipation, allowing for an understanding of qualitative changes occurring during fibrin clot formation. Both frequency shift (Δf) and bandwidth shift ($\Delta \Gamma$) were dependent on the concentration of fibrinogen in plasma. We defined a sum of squares function α ($= \Delta f^2/1000 + \Delta \Gamma^2/1000$) that measures absolute changes in QCM resonant characteristics to semi-quantitatively include an overall contribution of adsorbed mass and elastic modulus components and a function β ($= 1 - \Delta \Gamma/\Delta f$) that indicates qualitatively the nature of response based on its deviation from ideal Newtonian behaviour. Increasing concentration of fibrinogen resulted in an increase in the value of α, showing that a larger amount of fibrinogen results in larger amount of coupled viscoelastic mass. Changes in β indicated that the nature of changes occurring was very similar to Newtonian and that coupling of rigid-mass dominates the overall response in the early stage of coagulation and in the later stage growing elastic mass compensates some of the response.

1. Introduction

Fibrinogen has been identified as a major risk factor in cardiovascular disorders [1–5]. It is a soluble dimeric glycoprotein found in plasma, with a molecular weight of 340 kDa. Fibrinogen present in plasma is a major component of the coagulation cascade and its conversion to fibrin is usually used as an "end-point" in most clot-based assays, referred to as the clotting time [6]. The process of clot formation involves the enzymatic action of thrombin generated as a part of the coagulation cascade, which cleaves soluble fibrinogen to insoluble fibrin and subsequent polymerization of fibrin to form a gel-like network [7]. The Clauss fibrinogen assay, prothrombin time (PT)-derived fibrinogen assay and total clottable protein assay are the most commonly used methods to determine fibrinogen concentrations [8]. Clot-based assays, such as PT, activated partial thromboplastin time (aPTT) and activated clotting time (ACT) together with fibrinogen assays are used routinely as a diagnostic tool for a global assessment of coagulation function [8] in cases of haemostatic disorders. All of these assays are based on the measurement of a clotting time without any information on changes in the other physical properties or the kinetics of the forming clot.

Significant changes in viscoelasticity occurring due to conversion of liquid plasma to a soft fibrin gel network have been shown to be an indicator of various pathologies. Changes in viscoelastic properties during the process of clot formation have been studied by a number of methods discussed in detail in a review by Evans et al. [9]. Mechanical resonators such as a Quartz Crystal Microbalance (QCM) and magnetoelastic sensors have garnered a significant attention towards the study of blood coagulation. Puckett et al. [10,11] and Roy et al. [12–14] used magnetoelastic sensors to study clot formation and showed that changes in amplitude of magnetic flux at a fixed frequency can be related to changes in viscosity of plasma during coagulation. QCM is a piezoelectrically actuated shear mode oscillator that is extremely sensitive to changes in physical properties at the surface. QCM operates as a microgravimeter following the Sauerbrey relation [15] for a thin layer of mass, resulting in a change in frequency proportional to the attached mass. The QCM response in a liquid medium results in a change in frequency and also causes a damping of the oscillations. In addition to damping caused by viscosity of a liquid, QCM responses

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are also influenced by viscoelastic properties of a film, and have been studied in detail [16–18]. Several studies [19–28] have shown frequency shift of QCM as a measure of change in plasma viscosity during coagulation using different activation methods (PT [26,29], aPTT [19–21,30], thrombin clotting time (TCT) [28] and recalcification [24,31]). The shift in frequency after activation was the only parameter used in showing the process of coagulation. Bandey et al. [32], used equivalent circuit modelling of QCM to distinguish between different stages of coagulation in a whole blood sample and settling behaviour of red blood cells based on deviations from Newtonian behaviour. In another study, Muller et al. [29] used diluted plasma and PT-based activation and studied changes in frequency and bandwidth shifts and showed a near Newtonian fluid behaviour during coagulation. Previously we have shown [33] the ability of QCM to measure changes in frequency and bandwidth as a result of coagulation for different fibrinogen concentrations (1.2–9.2 mg/mL), with only one concentration in the physiological range. It was shown that the frequency and bandwidth shifts can produce signature coagulation traces for plasma similar to a thromboelastograph (TEG®), however the qualitative and quantitative analysis of frequency and bandwidth was not performed. In this article, we study the formation of a fibrin clot on a QCM by monitoring resonant frequency and bandwidth to analyze qualitative and semi-quantitative changes in the physical property of a forming fibrin clot depending on a range of concentrations in the physiological range. We show that increasing the concentration of fibrinogen results in larger frequency and bandwidth shifts. Two functions are described that compare the relative differences in frequency and bandwidth shifts to provide an insight in the qualitative characteristics of the forming clot.

2. Materials and methods

2.1. Reagents

Water (ACS reagent), sodium chloride (NaCl) and toluene were purchased from Sigma–Aldrich (Dublin, Ireland). TriniCHECK Fibrinogen Low Control and TriniCHECK Level1 Control plasma used in this study was obtained from Tcoag Ireland Limited. The lyophilized plasma was reconstituted in 1 ml of water (Sigma W4502) and left to stabilize for at least 20 min at room temperature prior to use. The HemosIL® APTT-SP (liquid) kit (Instrumentation Laboratory, Milano, Italy) contains 25 mM Calcium chloride ($CaCl_2$) and an aPTT liquid reagent consisting of synthetic phospholipids and micronized silica. Lyophilized human fibrinogen (HYPHEN BioMed, Neuville sur Oise, France) used to spike the low fibrinogen control plasma.

2.2. QCM sensor preparation and experimental arrangement

The QCM sensor preparation and experimental arrangement has been described in detail in [33]. Briefly, the polished 5 MHz QCM sensor crystals with gold electrodes (Maxtek Inc., Torrance, CA were cleaned first in a piranha solution (H_2SO_4:H_2O_2 in 3:1, v/v ratio), followed by a thorough rinse with deionised water and dried in a stream of nitrogen. A hydrophobic polystyrene (PS) layer was then spin-coated (2%, w/w PS in toluene; 2400 rpm for 2 min) The PS-coated sensors were cured in an oven overnight at 60 °C and the resonant frequency of the QCM was monitored before and after the PS coating to evaluate the thickness of the polymer film. The coated sensors were stored in a desiccator until use and were used within 24 h of coating. After coagulation experiments, the fibrin clot was washed using water followed by ultrasonic cleaning in 2% (w/v) sodium dodecyl sulphate (SDS), water and 70% (v/v) ethanol, respectively. The remaining PS surface coating was dissolved in toluene. The regeneration of surface was ensured by recording frequency and bandwidth after all the cleaning steps. The experimental arrangement consisted of a QCM holder (SRS QCM200), an inlet–outlet housing and a Network/Spectrum/Impedance Analyzer (Agilent, 4395A-010). The QCM sensor was placed in the holder, with one surface in contact to the electrodes and the other surface in contact with the sample enclosed in a volume (220 μL) contained by the housing and the sensor, such that only one electrode is in contact with the liquid. The resonant frequency and 'bandwidth' (half width at half maximum) of the sensor were determined using a Lorentzian curve fit of the conductance spectrum in a frequency span of 2 kHz on either side of the resonant frequency. The conductance spectrum data from the impedance analyser, followed by curve fitting and continuous evaluation of both the resonant frequency (f) and bandwidth (Γ) as a function of time were done using a custom LabVIEW® interface. A Binder ED-53 oven (Bohemia, NY, USA) maintained at 37 °C was used to conduct all QCM measurements at a physiological temperature.

3. Results and discussion

The conductance spectrum for QCM has a peak at the series resonant frequency. The bandwidth for 5 MHz QCM crystals, in air, was in the range 15–20 Hz. Coating of PS resulted in a rigid film, with a decrease in resonance frequency of 1550 ± 50 Hz and small change in bandwidth. Thus, the Sauerbrey relation [15] could be used to calculate the thickness of the film (258 ± 8 nm). Addition of liquid on QCM results in a change of both the resonant frequency and bandwidth. In cases where there is no mass adsorption from components in liquid, storage modulus (G') and loss modulus (G''), indicative of elasticity and viscosity, respectively, is given by [17]:

$$G' = k \cdot [\Delta f^2 - \Delta \Gamma^2] \quad (1)$$

$$G'' = 2.k \cdot [\Delta f \cdot \Delta \Gamma] \quad (2)$$

where k is a proportionality coefficient, Δf is shift in resonant frequency and $\Delta \Gamma$ is change in bandwidth. The classic Kanazawa equation [34] is for the case where $\Delta f = \Delta \Gamma$, typical for a purely viscous load ($G' = 0$). However, differences in Δf and $\Delta \Gamma$ can occur due to several reasons such as surface roughness ($\Delta f > \Delta \Gamma$), slippage ($\Delta f \leq \Delta \Gamma$) or mass adsorption ($\Delta f > \Delta \Gamma$) from a liquid [35].

The change in conductance spectrum upon addition of undiluted plasma is shown in Fig. 1(A). Addition of plasma on QCM decreases both resonance frequency and bandwidth. In contrast to whole blood, which is non-Newtonian, platelet poor plasma is almost a viscous Newtonian liquid [36] and hence $\Delta f = \Delta \Gamma$ is expected [16,17]. Fig. 1(B) shows changes in f and Γ for DI water and undiluted platelet poor plasma. However, the frequency shifts observed ($\approx$978 Hz) were larger than bandwidth shifts ($\approx$820 Hz). A similar difference in Δf and $\Delta \Gamma$ is obtained when plasma is added in a 1:1 dilution, where the magnitude of changes in frequency and gamma are smaller [33]. This difference ($\Delta f > \Delta \Gamma$) can occur either due to surface roughness, which tends to increase the f response by mass trapped in roughness contours, or additional mass from components in the liquid coupling to the surface. However, it was observed that DI water resulted in $\Delta f \approx \Delta \Gamma$, confirming that the difference is mainly due to adsorbed mass of plasma proteins and not surface roughness. Interactions between proteins and a hydrophobic PS surface [27] leads to rigid mass accumulation and, thus, to the extra frequency shift (following the Sauerbrey equation [15]). In principle, such a situation can be modelled as a QCM load [16] consisting of a finite rigid-mass layer beneath a semi-infinite viscous layer. This can allow for the estimation of adsorbed protein mass ($m_i \approx 2$ μg/cm^2) and loss modulus ($G'' = 1.1$ mPa s) which are proportional to ($\Delta f - \Delta \Gamma$) and to $\Delta \Gamma^2$, respectively.