Determination of surface-induced platelet activation by applying time-dependency dissipation factor versus frequency using quartz crystal microbalance with dissipation

Julien Fatisson1,2, Sania Mansouri1,2, Daniel Yacoub5, Yahye Merhi2,5 and Maryam Tabrizian1,2,3,4,*

1Department of Biomedical Engineering, 2Centre for Biorecognition and Biosensors, 3Faculty of Dentistry, and 4McGill Institute for Advanced Materials, McGill University, Montreal (QC), Canada
5Laboratory of Experimental Pathology, Montreal Heart Institute, Université de Montréal, Montreal (QC), Canada

Platelet adhesion and activation rates are frequently used to assess the thrombogenicity of biomaterials, which is a crucial step for the development of blood-contacting devices. Until now, electron and confocal microscopes have been used to investigate platelet activation but they failed to characterize this activation quantitatively and in real time. In order to overcome these limitations, quartz crystal microbalance with dissipation (QCM-D) was employed and an explicit time scale introduced in the dissipation versus frequency plots (Df–t) provided us with quantitative data at different stages of platelet activation. The QCM-D chips were coated with thrombogenic and non-thrombogenic model proteins to develop the methodology, further extended to investigate polymer thrombogenicity. Electron microscopy and immunofluorescence labelling were used to validate the QCM-D data and confirmed the relevance of Df–t plots to discriminate the activation rate among protein-modified surfaces. The responses showed the predominant role of surface hydrophobicity and roughness towards platelet activation and thereby towards polymer thrombogenicity. Modelling experimental data obtained with QCM-D with a Matlab code allowed us to define the rate at which mass change occurs (A/B), to obtain an A/B value for each polymer and correlate this value with polymer thrombogenicity.

Keywords: quartz crystal microbalance with dissipation; blood platelet activation monitoring; P-selectin expression; immunofluorescence microscopy; polymer-coated QCM-D biochip; thrombogenicity assessment

1. INTRODUCTION

Platelet interactions with other blood components as well as with the vascular wall represent a fundamental aspect of platelet function [1]. These interactions are governed by specific adhesion molecules on their cell membrane enabling platelets to bind to the endothelium, to the extracellular matrix and to leucocytes, thus facilitating their adhesion and activation at sites of vascular injury [1,2]. The platelet adhesion and activation are frequently characterized to assess the thrombogenicity of biomaterials, which is a crucial step for the development of blood-contacting devices, such as endovascular implants or catheters [3–6].

*Author for correspondence (maryam.tabrizian@mcgill.ca).


Although platelet activation is essential for normal haemostasis, it may also be associated with numerous vascular complications, such as inflammation, thrombosis, atherosclerosis and restenosis [1]. For instance, it is well known that following protein adsorption, the initial steps in primary haemostasis leading to blood clotting are platelet adhesion, activation and aggregation [1]. There have been many studies reporting on individual events in platelet activation, but these have focused on protein and gene expressions that lack information on the temporal aspects of the overall activation mechanism and its consequences for platelet morphology. Among the large variety of receptors or proteins expressed during platelet activation, P-selectin and αIIbβ3 glycoprotein are the most studied proteins [1,7,8]. Rinder et al. [9] studied P-selectin expression, β-thromboglobulin release and glycoprotein IIIb/IIIa expression in stored platelet concentrates and correlated...
cell adhesion and spreading to be a simple and effective way to quantitatively study with frequency changes over time. QCM-D has proven in real time by measuring the dissipation factor coupled bodies against specific receptors [8,20–23]. For instance, or confocal microscopy with fluorescently labelled anti–platelet adhesion could be obtained using fluorescent spreading using SEM [16–19]. Semi-quantitative data on materials by characterizing the cell morphology and cell have compared the surface thrombogenicity of biomaterials by characterizing the cell morphology and cell spreading using SEM [16–19]. Semi-quantitative data on platelet adhesion could be obtained using fluorescent or confocal microscopy with fluorescently labelled antibodies against specific receptors [8,20–23]. For instance, confocal microscopy has been employed to study platelet–neutrophil interactions [20] or platelet aggregation [21]. Although very relevant, the microscopic techniques could not provide a real-time measurement of the various stages of platelet adhesion and activation as a dynamic and multiparametric-dependent process.

In platelet-adhesion studies, scanning electron microscopy (SEM) observations are commonly employed for qualitative analysis, for instance, to assess morphological changes and cell spreading during the adhesion process. Reports are available on the spreading behaviours of platelets as a function of exposure time to fibrinogen using electron microscopy techniques [12–14], Modin et al. [13] and Fatisson et al. [15] used electron microscopy to complement microgravimetric analysis results obtained on cell spreading and adhesion, respectively. Some researchers have compared the surface thrombogenicity of biomaterials by characterizing the cell morphology and cell spreading using SEM [16–19].

Herein, we propose microgravimetric analyses using quartz crystal microbalance with dissipation (QCM-D) to investigate platelet activation in situ simultaneously in real time by measuring the dissipation factor coupled with frequency changes over time. QCM-D has proven to be a simple and effective way to quantitatively study cell adhesion and spreading in situ [13,24,25]. In a previous study [15], we also examined platelet adhesion in a quasi-three-dimensional space via subtle cytoskeleton changes, with protein composition of the QCM-D chip biointerface. Conclusive results were obtained using polyelectrolyte multilayered shells as an amplifier of the dissipation signal in order to discriminate between various platelet morphologies upon adhesion onto surfaces presenting different thrombogenicity.

To assess platelet activation, QCM-D was used in conjunction with immunodlabelling to determine the energy dissipation factor and the expression of P-selectin, respectively. Reports are available where reproducible dissipation factor (D) versus frequency (f) plots were obtained as a result of cell interactions with surfaces having various physico-chemical properties [13,24–28]. Thrombogenic human fibronectin (HFN) and non-thrombogenic human serum albumin (HSA) have been employed as model protein biointerface to understand the biochemical processes involved in the QCM-D response during platelet activation. To demonstrate that this methodology can eventually be employed in a thrombogenicity study of materials, we further extended this work to platelet activation studies on spin-coated QCM-D chips with two frequently used polymers in the manufacturing of medical devices. Using QCM-D, the platelet activation on polymers having different chemical compositions and surface wettability was assessed in real time and in situ. The effect of these parameters on surface thrombogenicity was investigated and results were validated by SEM and immunofluorescence labelling of platelets with appropriate activation biomarkers.

2. MATERIAL AND METHODS

2.1. Chemicals

Unless otherwise specified, the analytical grade of commercially available reagents was used. HSA, bovine serum albumin (BSA) and phosphate-buffered saline (PBS) were purchased from Sigma–Aldrich. HFN was provided by Chemicon International. Paraformaldehyde (PFA) from ACROS was employed to fix blood platelets. Deionized water (Nanopure Diamond system, Barnstead International) was used in the preparation of all solutions.

2.2. Human platelet isolation

Fresh venous blood was collected from healthy human volunteers, free from medications known to interfere with platelet function, at least 10 days before the experiments, in accordance with the guidelines of the ethical committee of the Montreal Heart Institute. Platelets were then isolated from the whole blood, as previously described [29,30] and their concentration was adjusted to 250 × 10⁶ cells ml⁻¹. They were kept at room temperature for 30 min before the experiments. For the reproducibility of the results and in order to limit the activation of platelets only to the adhesion process in contact with protein surfaces, platelets were suspended in the presence of the Ca²⁺ chelator ethylenediaminetetraacetic acid (EDTA) and prostacyclin (a platelet-activation inhibitor).

2.3. Surface preparation

Quartz crystal sensors were modified and prepared inside the QCM-D chamber under static conditions (i.e. stop flow). The glass slides for microscopy observations were prepared in a 24-well plate also under static conditions in order to compare all the results between these two techniques. Protein adsorption was performed on surfaces pre-cleaned in a UV-ozone chamber (Biofore Nanosciences, Inc.) for 2 min, followed by the incubation of the samples in protein(s) solution for 30 min at room temperature as described in a previous study [15]. Following protein adsorption, quartz crystals were rinsed with PBS and EDTA–prostacyclin solution, respectively. Protein-modified or chip surfaces were exposed to the platelet suspension as described above for another 30 min in EDTA and prostacyclin at room temperature to allow proper adhesion while avoiding platelet activation (electronic supplementary material, figure S1). The same rinsing and platelet-adhesion procedures
were used for polymer-modified surfaces. Either protein- or polymer-modified surfaces were subsequently rinsed with platelet-free EDTA–prostacyclin solution in order to remove any adhered platelets and placed in contact with modified Tyrode’s buffer (NaCl 150 mM, KCl 2.5 mM, NaHCO₃ 12 mM, MgCl₂ 2 mM, CaCl₂ 2 mM, BSA 1 mg ml⁻¹ and dextrose 1 mg ml⁻¹) for 5, 10 or 15 min to trigger proper platelet activation.

2.4. Confocal immunofluorescence

The glass slides were prepared as described above. They were then fixed with 2 per cent (v/v) PFA, washed with PBS and blocked with 2 per cent BSA for 30 min at room temperature prior to incubation with anti-human CD62P-Alexa 647 (BioLegend, San Diego, CA, USA) and anti-human CD41-FITC (Dako) for 60 min. Slides were rinsed with PBS and analysed with an LSM 510 confocal microscope (Zeiss, Oberkochen, Germany). CD62P (P-selectin) was employed as a standard marker of platelet activation and CD41 (α₂β₃) as a control and common marker of platelet specificity.

2.5. Scanning electron microscopy

The blood platelet morphology was investigated with a Hitachi S-4700 field emission gun scanning electron microscope (FEG-SEM). A protocol identical to the confocal microscopy samples was used for SEM sample preparation prior to fixation. After fixation with PFA, surfaces were dehydrated using an ethanol–water solution (increasing the ethanol proportion from 30 to 100%) and then fixed with 2 per cent (v/v) PFA, washed with PBS and blocked with 2 per cent BSA for 30 min. Polymer-coated samples were then fixed with 2 per cent (v/v) PFA, washed with PBS and blocked with 2 per cent BSA for 30 min. Polymer-coated samples were then fixed with 2 per cent (v/v) PFA, washed with PBS and blocked with 2 per cent BSA for 30 min.

2.6. Quartz crystal microbalance analyses

QCM-D measurements were performed with a Q-Sense E4 unit (Q-Sense AB, Göteborg, Sweden) by simultaneously monitoring the changes in frequency (Δf) and energy Δ-factor (ΔD) after each deposition step. The QCM-D chip was excited to oscillate in the thickness-shear mode at its fundamental resonance frequency (f = 5 MHz) and odd overtones (ν = 3, 5, 7, . . .) by applying a radio-frequency voltage across the electrodes. Energy dissipation was recorded periodically by computer-controlled disconnecting of the oscillating crystal from the circuit. This allows the measurement of the decay time τ₀ of the exponentially dissipated sinusoidal voltage signal over the crystal caused by the switching of the voltage applied to the piezoelectric oscillator. QSOFT, the Q-Sense software, was then used to acquire the dissipation factor, D, via

\[
D = \frac{1}{\pi f \tau_0} = \frac{2}{\omega \tau_0},
\]

where \(f\) is the resonance frequency and \(\tau_0\) is the relaxation time constant. These data provide information on the adsorption process as well as on certain viscoelastic properties of the adsorbed film. Prior to protein layer deposition, the silica-covered quartz (QSX-303) was cleaned in a UV-ozone chamber for 10 min. The crystal was then mounted in the QCM-D measurement radial flow chamber, stabilized at 20.00 ± 0.02°C by means of a circulating water bath and O-ring seals with only one face in contact with the working solution. Frequency and dissipation shifts were continuously recorded during the adsorption and rinsing steps. Signal amplitude decreases with overtone number, as well as signal-to-noise ratio. However, the first overtone (fundamental resonance frequency) can be too sensitive and reflect several artefacts. In order to avoid these artefacts while keeping a good signal amplitude and signal-to-noise ratio, the third overtone was chosen to report the data in each Δf plot.

2.7. Polymer spin-coating

High-density polyethylene (HDPE) or polycarbonate (PC) was dissolved at 0.25 per cent in toluene (Aldrich) for HDPE or tetrahydrofuran (Aldrich) for PC, and spin-coated (Laurell Technologies Corporation, North Wales, PA, USA) either on silica-covered QCM-D chips (Q-sense, Göteborg, Sweden) for QCM-D studies or on glass coverslips for microscopy analyses. Polymer solution (polyethylene) was heated to approximately 120°C before casting at 3000 r.p.m. for 3 min. Polymer-coating characterization on silica was assessed by contact angle and QCM-D measurements. Table 1 displays the mean contact angles and the thicknesses measured with the Sauerbrey equation, using 0.95 g ml⁻¹ and 1.3 g ml⁻¹ for the densities of HDPE and PC, respectively. As expected, HDPE was found to be more hydrophobic than PC and SiO₂. SiO₂ was therefore used as the hydrophilic control surface. However, the differences in the two film thicknesses have not been taken into account in the microgravimetric measurements, as the polymer coatings are considered as rigid like the bare (or uncoated) silica crystals and should neither affect the crystal performance nor the dissipation energy factor.

<table>
<thead>
<tr>
<th>surface</th>
<th>HDPE</th>
<th>PC</th>
<th>SiO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>contact (°)</td>
<td>120.05 ± 0.44</td>
<td>65.24 ± 3.63</td>
<td>26.10 ± 1.40</td>
</tr>
<tr>
<td>thickness (nm)</td>
<td>1509 ± 15.4</td>
<td>1069 ± 10.2</td>
<td>n.a.</td>
</tr>
<tr>
<td>roughness (r.m.s) (nm)</td>
<td>121 ± 16</td>
<td>38.3 ± 6</td>
<td>0.95 ± 0.07</td>
</tr>
</tbody>
</table>

3. RESULTS AND DISCUSSION

In a previous study [15], using polyelectrolyte multilayered shells as a dissipation signal amplifier, we demonstrated real-time monitoring of subtle cytoskeleton changes in platelets induced by primary platelet adhesion on different protein surfaces by QCM-D. Platelets can be activated upon adhesion to a surface [1]. Our experiments were conducted in the presence of a platelet activation blocker (i.e. prostacyclin) during the platelet adhesion process in order to minimize the platelet activation. In a...
platelet activation on HFN-modified surface as a function of time. The figure shows the significance of time scale to differentiate the protein-induced platelet activation.

previous study [15], we clearly showed that cytoskeletal changes (probably resulting from platelet activation) were not substantially noticeable, suggesting that no or very limited platelet activation occurs upon adhesion of platelets on surfaces having the same protein. Figure S1 of the electronic supplementary material shows that platelet adhesion reached a steady state after 30 min. Moreover, the values obtained for platelet adhesion were significantly lower than for platelet activation. For example, $D$ and $f$ reached values of the order of $10 \times 10^{-6}$ and −50 Hz for platelet adhesion, while they were of the order of $300 \times 10^{-6}$ and −300 Hz for platelet activation on protein surfaces. From these findings, one can investigate mainly the platelet activation induced by appropriate medium after the initial stage of adhesion in the QCM-D flow chamber. As explained by Fredriksson et al. [27,28], the spatial distribution of cells on the quartz sensor surface might affect the reproducibility between measurements. As the lateral sensitivity of $Df$ and $\Delta D$ is the same [31], plotting the dissipation against the frequency is qualitatively independent of the cell spatial distribution and would attenuate the effect of irreproducibility between each measurement. It also suggests that $Df$ plots can possibly be independent of cell numbers as demonstrated in the electronic supplementary material, figure S1, where similar $Df$ plots could be obtained for platelet adhesion on protein-coated surfaces. This assertion was the basis of our approach for using the $Df$ plots to compare the platelet-adhesion behaviour on different surfaces.

3.1. Monitoring platelet activation rate using a supporting protein layer

Including the time scale as an independent or an explicit parameter in QCM $Df$ plot curves for the platelet activation on different protein layers provided us with the curves displaying the activation of platelets adhered onto surfaces containing various levels of HFN and HSA ($Df$–$t$ plot in figure 1a). Third overtone signals were chosen as the most stable instead of the fundamental ones. As signal penetration depth decreases with overtone number, the third overtone signals also provide higher frequency and dissipation shifts compared with higher overtone number. The increase in dissipation along with a decrease in frequency indicated an enhancement of viscoelastic mass at the sensor interface. The increase in viscoelastic behaviour of the surface can only be correlated with the formation of pseudopodia as a result of platelet activation. This is because the platelet activation induces cell spreading via the rapid formation of pseudopodia [1]. In addition, the penetration depth of the QCM-D signal is around 250 nm. Therefore, only cell fragments in the region close to the sensor surface can be detected [28]; the whole cell would be too large to be detected by the QCM-D crystal. The formation of pseudopodia would fall within the detection range and could therefore be the main cause of the increase in mass of the QCM-D chip leading to the frequency and dissipation shifts with time. Modin et al. [13,24,25] also showed that QCM-D and consequently $Df$ plots can be used to determine the spreading behaviour of cells through the increase in dissipation versus the decrease in frequency upon their contact with different surfaces even with the signal penetration depth limitation. Varying the experimental conditions such as cell type, buffer, temperature, etc., authors could obtain a typical $Df$ plot for each of the given conditions [27] with a general trend of increasing slopes for $D$ and decreasing slopes for $f$. This feature was then attributed to cell spreading, as cell spreading leads to a progressive increase of viscoelastic mass (owing to high hydration state of cells) on the sensor surface. Moreover, the curve corresponding to albumin layer increased more slowly with time when compared with that of fibronectin. This trend can also be observed when fibronectin is gradually added as a supporting protein. In accordance with further microscopic observations, these results indicated that specific interactions between the thrombogenic fibronectin and platelets are responsible for a higher rate of activation [1,7]. Figure 1b shows a typical curve for a surface modified only with HFN. This example was extracted from figure 1a for clarification of frequency and dissipation signals as a function of

Fatisson et al. Surface-induced platelet activation 991

Figure 1. (a) Plot of the third overtone dissipation, as a function of corresponding frequency and time, measured for platelet activation on protein-modified quartz sensors, against the corresponding frequency shift, normalized to the overtone number. The filled circle curve corresponds to fibronectin (HFN) and the open square curve represents the response for albumin (HSA) alone, whereas the filled triangle curve resulted from a surface concentration ratio of 7 HSA for 1 HFN, and the open triangle curve corresponds to fibronectin (HFN) and the open square curve represents the response for albumin (HSA) alone. The dashed line shows a typical curve for a surface modified only with HFN. This example was extracted from figure 1a for clarification of frequency and dissipation signals as a function of the order of 300 Hz for platelet activation. For example, $D$ and $f$ reached values of the order of $10 \times 10^{-6}$ and −50 Hz for platelet adhesion, while they were of the order of $300 \times 10^{-6}$ and −300 Hz for platelet activation on protein surfaces. From these findings, one can investigate mainly the platelet activation induced by appropriate medium after the initial stage of adhesion in the QCM-D flow chamber. As explained by Fredriksson et al. [27,28], the spatial distribution of cells on the quartz sensor surface might affect the reproducibility between measurements. As the lateral sensitivity of $Df$ and $\Delta D$ is the same [31], plotting the dissipation against the frequency is qualitatively independent of the cell spatial distribution and would attenuate the effect of irreproducibility between each measurement. It also suggests that $Df$ plots can possibly be independent of cell numbers as demonstrated in the electronic supplementary material, figure S1, where similar $Df$ plots could be obtained for platelet adhesion on protein-coated surfaces. This assertion was the basis of our approach for using the $Df$ plots to compare the platelet-adhesion behaviour on different surfaces.

3.1. Monitoring platelet activation rate using a supporting protein layer

Including the time scale as an independent or an explicit parameter in QCM $Df$ plot curves for the platelet activation on different protein layers provided us with the curves displaying the activation of platelets adhered onto surfaces containing various levels of HFN and HSA ($Df$–$t$ plot in figure 1a). Third overtone signals were chosen as the most stable instead of the fundamental ones. As signal penetration depth decreases with overtone number, the third overtone signals also provide higher frequency and dissipation shifts compared with higher overtone number. The increase in dissipation along with a decrease in frequency indicated an enhancement of viscoelastic mass at the sensor interface. The increase in viscoelastic behaviour of the surface can only be correlated with the formation of pseudopodia as a result of platelet activation. This is because the platelet activation induces cell spreading via the rapid formation of pseudopodia [1]. In addition, the penetration depth of the QCM-D signal is around 250 nm. Therefore, only cell fragments in the region close to the sensor surface can be detected [28]; the whole cell would be too large to be detected by the QCM-D crystal. The formation of pseudopodia would fall within the detection range and could therefore be the main cause of the increase in mass of the QCM-D chip leading to the frequency and dissipation shifts with time. Modin et al. [13,24,25] also showed that QCM-D and consequently $Df$ plots can be used to determine the spreading behaviour of cells through the increase in dissipation versus the decrease in frequency upon their contact with different surfaces even with the signal penetration depth limitation. Varying the experimental conditions such as cell type, buffer, temperature, etc., authors could obtain a typical $Df$ plot for each of the given conditions [27] with a general trend of increasing slopes for $D$ and decreasing slopes for $f$. This feature was then attributed to cell spreading, as cell spreading leads to a progressive increase of viscoelastic mass (owing to high hydration state of cells) on the sensor surface. Moreover, the curve corresponding to albumin layer increased more slowly with time when compared with that of fibronectin. This trend can also be observed when fibronectin is gradually added as a supporting protein. In accordance with further microscopic observations, these results indicated that specific interactions between the thrombogenic fibronectin and platelets are responsible for a higher rate of activation [1,7]. Figure 1b shows a typical curve for a surface modified only with HFN. This example was extracted from figure 1a for clarification of frequency and dissipation signals as a function of
time. However, this simple plot could not provide any relevant insight regarding the specific signature as a $D_f-t$ plot as does figure 1a.

As every cell under the given conditions will provide a distinguishable QCM-D dissipation and frequency signal [26,27], it was necessary to assess the cell morphology and P-selectin expression by electron microscopy and immunofluorescence labelling, respectively. The results were then correlated with QCM-D analyses to extract more qualitative data on platelet activation. Figure 2a shows SEM microphotographs of platelets on different protein layers after 10 min of platelet exposure to the medium. Cell spreading and platelet activation were higher on the thrombogenic HFN biointerface in
comparing with the surfaces treated with the non-thrombogenic HSA. By staining platelets for CD62P, a standard biomarker of α-granule secretion and platelet activation and their subsequent adhesion on the different surfaces, the confocal immunofluorescence microscopy showed that the expression of CD62P was most pronounced on the HFN-coated coverslips when compared to those coated with HSA (figure 2b). This result indicated that when specific interactions (herein owing to the presence of HFN) were promoted, the platelet activation was further triggered. In addition, as the contact time between platelets and nutrients (activation-inducing medium) increased, the cells were more spread as indicated by the relative increase of fluorescence intensity of anti-CD62P with time (figure 3). Upon activation, platelet pseudopodia are formed and caused an important shift in frequency and dissipation, as noted in figure 1.

Since P-selectin labelling is the method of choice to assess platelet activation, the increase in fluorescence intensity of anti-CD62P is used to correlate the observations made on figure 1, where the activation rate has been considered to be faster when specific interactions with the surface are involved due to the presence of HFN. Figure 3 shows two different fluorescent probes labelled with two different receptors: CD41 and CD62P (P-selectin). CD41, which was employed as a control platelet marker, was continuously expressed on platelets with positive staining for all samples. In contrast, immunofluorescence labelling of samples for P-selectin indicated that HFN induced a time-dependent P-selectin expression and cell spreading when platelets were allowed to adhere for a longer period of time on HFN (5–15 min.). Since the intracellular protein P-selectin is expressed only at the surface of membrane after α-granule secretion and not in platelets, its expression is therefore an indication of platelet activation [1]. These results simply imply that P-selectin was expressed as a function of platelet adhesion time on HFN owing to the fact that platelets contain fibronectin receptors capable of activating cells as they come in contact with components of the extracellular matrix. Fibronectin constitutes an important thrombogenic factor through which platelets can adhere to the subendothelium, predominantly by the platelet receptors αIIbβ3, αvβ3 and αvβ1 [32]. This has been observed in a setting of vascular trauma, for instance, in physiological (haemostasis) or pathological (atherosclerosis) upset. Wang et al. [11] showed that thrombin-activated platelets expressed a higher rate of P-selectin. Increased platelet adhesion on fibronectin over time under shear stress has also been reported [33]. In addition, the increase of P-selectin observed with HFN is in accordance with the inherent thrombogenic properties of HFN and proved the relevance of using this protein as a model for developing the method described here. Moreover, at t = 5 min, CD41 expression confirmed the presence of platelets where little CD62P expression was observed. At t = 10 and 15 min, CD41 expression seemed to mirror CD62P expression, which confirmed high platelet activation rate. This feature stresses the lack of accuracy in obtaining quantitative data by confocal microscopy and supports the adequacy of QCM-D for providing more accurate measurements with higher sensitivity within only a few minutes as presented in figure 1.

These microscopy observations along with P-selectin expression showing the dependency of activation on the nature of the surface and the exposure time, allowed us to correlate these results with the temporal microgravimetric data and to determine the kinetics of platelet activation on protein layer presented by \( Df-t \) plots.

In contrast to figure 1 where the \( Df-t \) plots were discernible from each other, the \( Df \) curves appear to merge, demonstrating the significance of an explicit time scale in the platelet activation rate as a function of the nature of the surface (figure 4a). The curves overlapping confirmed that the platelet activation process follows an identical pathway under equal conditions of stimulation (same buffer, same nutrients and same surface chemistry). The exponential growth of the \( Df \) plots could be related to the transport process of the nutrients present in the buffer, from the solution through the cell membrane. Figure 4b summarizes the results of linear regression of the dissipation curves as a function of the square root of the absolute value of frequency curves for each protein-modified surface presented in figure 4a. The values obtained for linear regression were almost identical, which led us to describe the \( Df \) plots in that figure using the following equation:

\[
D = 19.62(\pm 1.12)\sqrt{f}.
\]  (3.1)

This correlation can be subsequently used as a mathematical tool to elucidate the pathway of platelet activation by determining the contribution of each event in this equation.

### 3.2. Application of the model surface-modified protein to material thrombogenicity assessment

The above-mentioned findings were used to extend the methodology for the assessment of the thrombogenicity of PC and HDPE surfaces. Although in real applications proteins will immediately absorb on the polymeric devices in contact with body fluids, our aim was to evaluate the effect of intrinsic polymer properties such as surface chemical composition, roughness and wettability on the surface thrombogenicity. Literature reports on the effect of surface properties on the surface thrombogenicity are numerous [12,19,34], where the impacts of either the material surface roughness and/or its hydrophobicity on platelet adhesion/activation and therefore on material thrombogenicity have been evaluated [35–37]. Using our QCM-D-based methodology, we could obtain results which were in agreement with these previous findings. By the inclusion of the time scale and kinetics in QCM-D plots \((Df-t)\), we were able to compare the thrombogenic properties of the polymer-modified surfaces having similar polymer film thickness (table 1). The main objective of the present study is the evaluation of platelet activation after adhesion to different surfaces. However, further characterization of polymer-coating thickness was carried out by SEM (electronic supplementary material, figure S2), thus confirming the QCM-D measurements. Although the differences between \( Df-t \) plots were less pronounced when compared with the protein-modified surfaces
owing to more rigid polymer biointerfaces, the QCM-D response to platelet activation was distinguishable for the two surface-modified-polymer QCM-D crystals and for the bare SiO2 chip (figure 5a). The contact angle measurements (table 1) indicated a higher surface hydrophobicity for PC than SiO2. As the cell density on the surfaces was very similar (figure 4b, first row), the different features of $D_f(t)$ curves for the surfaces composed of SiO2 and those composed of PC pointed out the influence of material hydrophobicity. Aguilar et al. [16] observed that platelet activation decreased when the hydrophobicity of the polymer increased. Interestingly, on SiO2, the $D_f(t)$ curve appeared exponential, suggesting that the platelet activation rate was faster on silica at the beginning. As for the PC surface, the linear behaviour of the $D_f(t)$ curve suggested a slower activation rate in the first seconds. This observation is confirmed by greater P-selectin expression for SiO2 than for PC or HDPE-coated surfaces (figure 5b).

The dissipation factors observed in the case of protein-modified surfaces (figure 1) were higher than the polymer-modified surfaces (figure 5a) owing to the higher hydrophilicity of proteins versus polymers. This might also suggest that the activation process is taking place faster in the case of protein-modified surfaces.

In addition to surface composition and wettability, the platelet adhesion and activation were affected by surface roughness. An increase in surface roughness promoted the formation and anchorage of platelet pseudopodia, resulting in higher platelet adhesion, spreading and activation. This result is in agreement with the observations of Linneweber et al. [36] where they showed that rougher surfaces induced higher platelet adhesion rate. As presented in figure 5, the platelet activation rate on the smooth PC surface was less advanced than platelets adhered onto the SiO2 rough surface. As such, dissipation increased less rapidly with frequency and time when platelets adhered on PC than on SiO2. Conversely, the $D_f(t)$ plot for HDPE falls between the silica and the PC surface. This suggests that the effect of surface roughness is attenuated by surface hydrophobicity. We found that the surface roughness of HDPE was much higher (as measured by atomic force microscopy (AFM)). If the roughness effect could be investigated independently from the effect of hydrophobicity, the curves would have shown higher activation for rougher surfaces. Nevertheless, surfaces coated with HDPE resulted in a lower activation rate and a minimal increase of $D$ factor with frequency and time owing to its greater hydrophobicity. One could then reasonably conclude that HDPE induced less platelet activation and has less of a thrombogenic effect than SiO2 because of the higher surface hydrophobicity, which would explain the similarity in the linear features of the $D_f(t)$ plots between HDPE and PC.

P-selectin expression after 5 min of contact between platelets and nutrients also does not seem very different between PC- and HDPE-coated surfaces (figure 5b). These observations suggest again that QCM-D is a more sensitive technique to detect different platelet activation rates in the early stages (first minutes/seconds) and assess the material’s thrombogenicity.

### 3.3. Modelling quartz crystal microbalance with dissipation data in Matlab for quantification of the platelet activation rate

The curves presented in figures 3a and 4a were modelled using a Matlab code with a simple equation describing the dissipation as an exponential function of frequency and time:

$$D = \frac{\exp(\alpha f^A)}{\exp(\beta f^B)}$$  

(3.2)

where arbitrary constants $\alpha = 1 \text{ Hz}^{-1}$ and $\beta = 1 \text{ s}^{-1}$. In this equation $A$ and $B$ are two constants, where $A$ is related to frequency, therefore to the mass changes, and $B$ to time or to the rate at which mass changes occur. Table 2 summarizes $A$ and $B$ for each curve presented in figures 3a and 4a, and $A$ to $B$ ratio. Negative and positives values are obtained for $B$ and $A$. 

Figure 4. (a) Plot of the third overtone dissipation factor ($D_f^3$) measured for platelet activation on protein-modified quartz sensors, against the corresponding frequency shift, normalized to the overtone number. The filled circle curve corresponds to HFN and the open square curve represents the response for HSA alone, whereas the filled triangle curve resulted from a surface concentration ratio of 7 HSA : 1 HFN, and the open triangle curve was obtained for a 50 : 1 HSA/HFN ratio. The figure shows that the platelet activation curves overlap as a result of identical activation pathway. (b) Slopes of dissipation curves as a function of the square root of the absolute value of frequency as depicted in figure 1a and used as an indication for platelet activation on different protein-modified surfaces.
The filled square curve corresponds to the bare SiO2 surface, the filled triangle curve to the PC-coated sensor and the open diamond curve was obtained with the HDPE-coated crystal. (b) Same scale confocal microphotographs of platelets on non-coated (SiO2) or PC- or HDPE-coated glass slides with CD62P and CD41 immunofluorescence after 5 min of contact with nutrients. The figure shows the significance of the QCM-D method to differentiate the platelet activation induced by the polymeric surface properties in the early stages when compared with fluorescence microscopy.

Table 2. A and B are constants obtained from the dissipation curves in figures 3a and 4a as an exponential function of frequency and time as described by equation (3.2). The ‘corrected-roughness’ A/B values are the results of A/B values multiplied by the r.m.s. values listed in table 1. This correction has not been applied for the protein-coated surfaces as they showed similar roughness to the one measured for SiO2.

<table>
<thead>
<tr>
<th>surface</th>
<th>A</th>
<th>B</th>
<th>A/B</th>
<th>corrected roughness A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA</td>
<td>0.0580</td>
<td>−0.0159</td>
<td>−3.6478</td>
<td>n.a.</td>
</tr>
<tr>
<td>50 : 1</td>
<td>0.0505</td>
<td>−0.0226</td>
<td>−2.2345</td>
<td>n.a.</td>
</tr>
<tr>
<td>7 : 1</td>
<td>0.0393</td>
<td>−0.0232</td>
<td>−1.6940</td>
<td>n.a.</td>
</tr>
<tr>
<td>HFN</td>
<td>0.0222</td>
<td>−0.0186</td>
<td>−1.1936</td>
<td>n.a.</td>
</tr>
<tr>
<td>SiO2</td>
<td>0.1891</td>
<td>−0.0458</td>
<td>−4.1288</td>
<td>−3.9141</td>
</tr>
<tr>
<td>PC</td>
<td>0.1134</td>
<td>−0.0389</td>
<td>−2.9252</td>
<td>−111.179</td>
</tr>
<tr>
<td>HDPE</td>
<td>0.1293</td>
<td>−0.0395</td>
<td>−3.2734</td>
<td>−396.138</td>
</tr>
</tbody>
</table>

respectively, as the dissipation is directly proportional to both frequency and time. A higher value of A/B would then indicate that a greater mass change occurs within a shorter time. As such, one can foresee that the increased concentration of the thrombogenic HFN protein on the surface will lead to the faster spreading of the platelets, resulting in a higher mass detection on the surface. Therefore, increasing A/B values with the fibronectin concentration in the protein layer could be causally related to higher platelet activation rate and vice versa. The lowest A/B observed for 100 per cent albumin-coated surface could only be associated with the blocking properties of BSA. Albumin simply masks the charge on the bare SiO2 surface [38], which decreases the repulsion forces between the negatively charged SiO2 and the negatively charged cell membrane.

Since protein-modified surfaces had very similar roughness and hydrophobicity, their corresponding A/B ratios could directly reflect the surface thrombogenicity and did not require including the effect of physical parameters in the model. However, for polymer-modified surfaces, the compensatory effects of the surface roughness and surface hydrophobicity on platelet activation rate should be taken into account to determine the A/B ratios. By taking into account the roughness as determined by AFM to correct the A/B values, important differences were noticed among surfaces. Comparison of corrected A/B ratios showed that the surface roughness has a significant effect on platelet spreading and activation rate on polymer-modified surfaces. To include this effect in the A:B ratio, their A/B values obtained through modelling have been multiplied by the r.m.s. values reported in table 1 as measured by AFM. This led to a very interesting relationship between the surface roughness versus surface wettability and A/B values, and showed that the platelet activation rate is lower on SiO2 surfaces than on any of the HFN-modified biointerfaces but higher than the PC-modified and HDPE-modified surfaces. One can also notice that SiO2 A/B ratio is close to HSA A/B ratio. This can be explained by the fact that these surfaces have similar roughness and hydrophobicity and both are not thrombogenic. Likewise, the lowest A to B ratio obtained for HDPE then demonstrated the dominant effect of surface hydrophobicity versus surface roughness on platelet activation rates and confirming the data on platelet activation observed experimentally through the physical and biochemical analyses.

4. CONCLUSION

We introduced a temporal approach to platelet activation as a dynamic and multiparametric process instead of looking solely at a given event which is frequently reported in the literature. Protein-modified QCM-D quartz crystals were used as a means for
real-time and in situ monitoring of the blood platelet spreading and activation. This methodology involved plotting the variation of dissipation factor along with frequency as a function of time (DF-t) as an explicit parameter during these events. The DF-t plots obtained for fibronectin-modified quartz sensors corresponded to the highest activation rate among tested surfaces. This suggests that even though the pathway for platelet activation is the same, the activation rate can be altered when ‘specific interactions’ with the surface are involved.

The method was shown to be applicable to polymer-modified surfaces in order to assess polymer thrombogenicity. The shift in dissipation factor and frequency as a function of time on polymer-modified surfaces, owing to the effect of surface roughness and hydrophobicity, was related to the differences observed in platelet spreading and activation rate and therefore to the polymer thrombogenicity. Modelling on the methodology provided us with quantitative data on the mass changes to the rate of mass changes ratio on QCM-D sensors owing to platelet activation. The developed approach would offer a simple, versatile and sensitive tool for the study of biomaterial thrombogenicity in vitro as well as to diagnose platelet-related blood disorders in real time with a relatively fast response.

The authors thank Canadian Institutes for Health Research-Regenerative Medicine/Nano medicine and National Science Research Council (NSERC)-Discovery Grant and The Fonds que´be´cois de la recherche sur la nature et les technologies (FQRNT)-Center for Biorecognition and Biosensors for their technical expertise in AFM obtained by QCM-D, and Line Mongeon and Louis Villeneuve to QCM-D E4, Jamal Daoud for his help in modelling the data financial support. We thank Dr N. Tufenkji for giving us access (FQRNT) to QCM-D sensors owing to platelet activation. The developed approach suggests that even though the pathway for platelet activation is the same, the activation rate can be altered as well as to diagnose platelet-related blood disorders in real time with a relatively fast response.

The authors thank Canadian Institutes for Health Research-Regenerative Medicine/Nano medicine and National Science Research Council (NSERC)-Discovery Grant and The Fonds que´be´cois de la recherche sur la nature et les technologies (FQRNT)-Center for Biorecognition and Biosensors for their technical expertise in AFM obtained by QCM-D, and Line Mongeon and Louis Villeneuve for their technical expertise in AFM/SEM and in confocal microscopy, respectively.

REFERENCES


