Colloids and Surfaces B: Biointerfaces 116 (2014) 446–451

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Real-time monitoring of surface-confined platelet activation on TiO_2

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ARTICLE INFO

Article history:
Received 8 November 2013
Received in revised form 10 January 2014
Accepted 19 January 2014
Available online 25 January 2014

Keywords:
Platelet activation
Aggregation
TiO_2
QCM-D

ABSTRACT

For the development of advanced hemocompatible biomaterial functions, there is an unmet demand for *in vitro* evaluation techniques addressing platelet-surface interactions. We show that the quartz crystal microbalance with dissipation (QCM-D) monitoring technique, here combined with light microscopy, provides a surface sensitive technique that allows for real-time monitoring of the activation and aggregation of the surface-confined platelets on TiO_2. The QCM-D signal monitored during adhesion and activation of platelets on TiO_2 coated surfaces was found to be different in platelet-poor and platelet-rich environment although light microscopy images taken for each of the two cases looked essentially the same. Interestingly, aggregation of activated platelets was only observed in a protein-rich environment. Our results show that a layer of plasma proteins between the TiO_2 surface and the platelets strongly influences the coupling between the platelets and the underlying substrate, explaining both the observed QCM-D signals and the ability of the platelets to aggregate.

1. Introduction

The understanding of platelet interactions with blood components and the vessel wall, as well as with biomaterials, is of fundamental interest for platelet function and also for the development of blood-contacting medical devices. In particular, the adhesion of platelets followed by aggregation or thrombus formation around a biomaterial is usually unwanted. Clinical trials and *in vivo* studies will always be required to assess hemocompatibilty and thrombogenicity of biomaterials, but in order to promote biomaterial development there is a demand for advancement of *in vitro* evaluation techniques, which can be used to screen biomaterial function.

Commonly, the method of choice for the study of the interaction between platelets and a biomaterial surface has been electron or fluorescence microscopy. These studies have mostly been performed as end-point experiments following upon fixation of platelets, and there are only few examples in the literature where activation or aggregation of surface-confined platelets have been monitored in real-time using, e.g., video microscopy [1].

The development of surface-sensitive analytical techniques will change this situation, and especially acoustic sensing techniques, such as the quartz crystal microbalance (QCM) technique, are emerging as tools to study cell attachment and spreading as well as the responses of surface-confined cells to external stimuli (leading to cytoskeletal rearrangements) [2,3]. In the first QCM platelet report, the adhesion of washed platelets onto sensors coated with bovine serum albumin or collagen was studied [4]. As in many of the early QCM studies of cells interacting with surfaces, the interpretation of the observed signals in this study was difficult. Over the years, the QCM technique has been further developed, and models to describe the QCM signals in liquid have been introduced [5–7]. Yet, the interpretation of cell experiments performed by QCM remains ambiguous.

With respect to platelets, the main aim for QCM studies this far has been to monitor platelet adhesion and to relate the QCM signal to the degree of platelet aggregation in the sample. Thus, platelets were stimulated prior to the QCM experiments, and injected to the sensor surface after aggregation. For example, Fatisson et al. studied platelet adhesion to protein–coated sensor surfaces (using human serum albumin and fibronectin) and correlated the signal to the degree of spreading on the sensor surface [8,9]. Sinn et al. made a direct comparison between QCM and optical aggregometry [10]. They showed the QCM signal to be different for aggregated and non-aggregated platelets on Au or fibrinogen-coated sensors. In the most recent study, Santos-Martinez et al. quantified the aggregation of platelets that had been pre-incubated with different types of nanoparticles using the QCM technique [11]. All of these studies clearly show that QCM can be used to distinguish the adhesion of aggregated platelets from that of non-aggregated platelets, but the interpretation of the observed signals remained elusive. One reason for these difficulties is that the observant has

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0927-7765
http://dx.doi.org/10.1016/j.colsurfb.2014.01.025

been literally blind, as the use of a closed module has not allowed imaging of cell attachment during the QCM experiment at the sensor surface. We [12] and others [13,14] have recently demonstrated that a combined QCM-D/microscopy set-up can be used to overcome this problem. Lord et al. used QCM-D combined with fluorescence microscopy to monitor cell adhesion to tantalum and oxidized polystyrene coated with various proteins [13] and furthermore to show remodeling of the extracellular matrix during cell adhesion on these surfaces [14]. In the most recent study by Tymchenko et al., it was demonstrated that changes in the monitored QCM-D signals correlated to morphological changes, induced by a cytomorphic agent, in cells observed simultaneously by light microscopy.

In the present study, we have made use of a combined QCM-D/light microscopy set-up (including a windowed QCM-D module) to monitor the activation of surface-confined platelets on TiO_2-coated QCM-D sensors. TiO_2 is one of the most frequently used materials for various implants due to its outstanding biocompatibility [15,16].

Our aim was to monitor the activation of surface-confined platelets in real-time and to characterize the adhesion of platelets to TiO_2-coated surfaces. A comparison of results obtained for protein-rich and protein-poor conditions was important not only to show the influence of plasma proteins at the interface, but also to let us make an assumption about the nature of the linking of the platelets to the underlying substrate.

2. Material and methods

2.1. Chemicals

Unless otherwise specified, analytical grade of commercially available reagents were used. Water was deionized (resistivity >18.2 mΩ/cm) and purified (MilliQ plus, Millipore, France). Phosphate-buffered saline (PBS) was prepared by dissolution of tablets from Sigma in MilliQ water. Platelet additive solution (PAS, 2.94 g NaCitrat × $2H_2O$, 4.08 g NaAcetat × $3H_2O$, 6.75 g NaCl ad 1000 mL, pH 7.2) was purchased from MacoPharma Nordic AB, Helsingborg, Sweden. Oligo-ethylene glycol (OEG) disulfides with biotin groups (dS-OEG-biotin, structure: $-[S-C_2H_4-CO-NH-(CH_2-O-CH_2)_9-NH-CO-C_4H_8-Biotin]_2$, MW: 1539.9 Da) were purchased from Polypure, Norway. Streptavidin (Sigma) were aliquoted in water and stored at −20 °C. Thrombin receptor activation peptide 6 (TRAP-6, Sigma) was dissolved in water and stored as aliquots for maximum 4 weeks. Human serum albumin (HSA, Sigma) was dissolved in PBS prior to fluorescence microscopy experiments. CD62-PE and FITC conjugated CD61 antibodies were purchased from BD Biosciences.

2.2. Human platelet isolation

All platelets were derived from blood donors at the Regional Blood Bank at Sahlgrenska University Hospital. The blood donors were selected according to the standard procedure of the blood bank. Blood donors gave informed consent for the use of the blood components in the study by signing the health questionnaire.

2.2.1. Pooled buffy coat platelets

The buffy coat derived platelets were prepared from buffy coats donated by four regular whole blood donors. Whole blood units were collected in bottom-and-top bags containing 63 mL citrate-phosphate-dextrose (CPD) as anticoagulant (MacoPharma Nordic AB, Helsingborg, Sweden). The whole blood units were hard spin centrifuged (4880 × g for 11 min at 23 °C; RotoSilenta RS, Hettich Lab Technology, Sollentuna, Sweden) and the buffy coat was separated from the red blood cells and the plasma using Macopress Smart (MacoPharma Nordic AB, Helsingborg, Sweden). Next, the buffy coats were pooled together with platelet additive solution (PAS, SSP) and the pool was processed in TACSI (Terumo BCT Europe, Zaventem, Belgium) to separate the remaining red cells. The residual plasma content was approximately 20%.

Samples from the pooled platelets were taken aseptically from the bag using a separate sampling bag. The platelet count was measured with clinical standard methods.

The washed platelets were obtained by centrifugation (200 × g for 5 min) of the collected sample. The supernatant was discarded and PAS added. The procedure was repeated twice. Finally, the platelet pellet was dissolved in 3 mL of PAS and the platelet count was measured.

2.3. Platelet poor plasma

Whole blood units were collected from regular blood donors in bottom-and-top bags (MacoPharma Nordic AB, Helsingborg, Sweden) containing 63 mL citrate-phosphate-dextrose (CPD) as anticoagulant. The whole blood units were hard-spin-centrifuged (4880 × g for 11 min at 23 °C) and the buffy coat was separated from the red blood cells and the plasma using a blood expander platform (Macopress Smart, MacoPharma Nordic AB). Plasma was collected from AB RhD+ donors and stored in −20 °C until use.

2.4. Modification of QCM-D sensor surfaces

AT-cut quartz crystals with a fundamental frequency of 5 MHz coated with Au were purchased from Q-Sense AB.

A 65 nm thick layer of TiO_2 was sputtered on the top electrode of the Au crystals. Prior to the experiment the crystals were cleaned in a 10 mM sodium dodecyl sulfate aqueous solution (overnight), rinsed thoroughly with water, dried under N_2, and treated with UV/ozone for 3 times 15 min with rinsing and drying in between.

2.5. QCM-D

The principle of QCM-D relies on the sharp and stable oscillation resonance when an electric AC field is applied over the electrodes. The surface of the disk-shaped sensor performs a shear oscillation, i.e., a periodic motion parallel to the sensor surface. The frequency of this oscillation is reduced when mass is attached to the surface and increases when mass is detached. Recording of this frequency shift, Δf, thus gives information about the attached/detached mass to the sensor surface. The second measured quantity in QCM-D is the energy dissipation, ΔD, (or damping) of the oscillator. Formally ΔD is defined as the fraction of the total energy stored in the oscillator that is dissipated during one oscillation cycle. This means that the ΔD factor is important to characterize the viscoelastic properties of the mass coupled to the oscillator.

QCM-D experiments were performed using a Q-Sense E1 unit (BiolinScientific/Q-Sense, Göteborg, Sweden) equipped with a standard window module that allows for simultaneous visualization of platelets on the sensor surface. A polarized light microscopy (Leica DM4000M) equipped with a 10× objective was used for imaging.

All measurements were carried out at 37 °C. Platelets were injected at a concentration of 200,000 platelets/μL. QCM-D graphs display the frequency and dissipation shift recorded for the fundamental frequency of the oscillating quartz sensor. For platelet activation, TRAP-6 was injected at a final concentration of 64 μM.

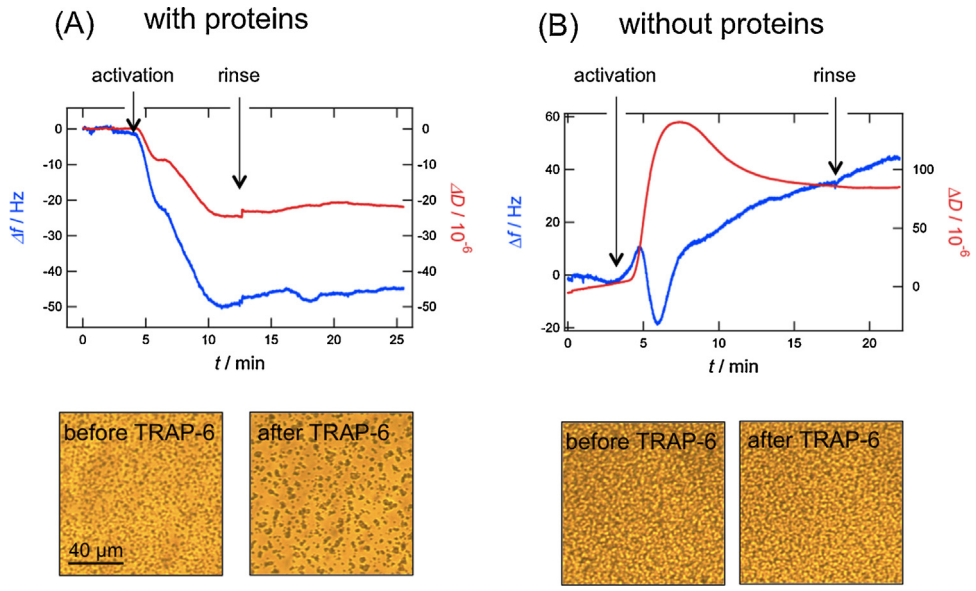

Fig. 1. QCM-D frequency and dissipation versus time curves (upper panels) and light microscopy (lower panels) images of platelets activation and aggregation on TiO_2 coated surfaces in (A) presence of plasma proteins and (B) without plasma proteins. Light microscopy images were taken during the QCM-D experiment, before and 10 min after injection of TRAP-6.

QCM-D signals were fitted to the viscoelastic model [6] implemented in QTools (Q-sense AB, Sweden) to estimate the thickness of the adsorbed viscoelastic layer (here plasma proteins).

2.6. Fluorescence microscopy

Fluorescence microscopy images were taken to verify platelet activation using an Axioplan 2E MOT microscope (Carl Zeiss AG, Germany) equipped with a 20× objective. QCM-D sensors sputter-coated with TiO_2 were cleaned following the protocol described above and placed in petri dishes. Sensors were incubated in either platelet poor plasma to allow for saturation with plasma proteins or in storage solution for 45 min at 37 °C. In the next step, platelets were added to the sensor surfaces at concentration of 200,000 platelets/μL either in plasma or storage solution and allowed to attach for 120 min at 37 °C. After washing with PPP and PAS, respectively, platelets were activated by addition of 64 μM TRAP-6. After 30 min, platelets were washed with PBS and incubated for 2 h with 1% paraformaldehyde at room temperature. Afterwards, the sensors were washed with PBS and incubated for 60 min with 1% human serum albumin at 37 °C for blocking of unspecific binding to the substrate. After a further washing step, the sensors were incubated with CD61 or CD62P antibodies, respectively. A FITC conjugated CD61- antibody was used for the detection of platelet glycoprotein IIIa. Platelet activation was detected with a PE-conjugated CD62P-PE antibody binding to P-selectin expressed on activated platelets.

3. Results and discussion

3.1. Activation and aggregation of surface-confined platelets

The aim of the present study is to show the potential of QCM-D for real-time monitoring of the activation of surface-confined platelets. We used TRAP-6 (thrombin receptor activation peptide 6), a synthetic peptide that can reproduce thrombin-induced cellular effects, for the activation of platelets in plasma (PRP) and washed platelets in platelet additive solution (PAS), i.e., in protein-rich and protein-poor conditions, respectively.

Fig. 1 shows the QCM-D frequency and dissipation versus time curves (Fig. 1, upper panels) for the activation of platelets attached to the TiO_2-coated QCM-D sensor upon addition of TRAP-6 (64 μM). Quite different QCM responses were observed for the protein-rich (Fig. 1A) and the protein-poor (Fig. 1B) conditions. For platelets in the protein-rich condition, decreasing frequency and dissipation signals, accompanied by a visible aggregation, are observed after injection of TRAP-6 (Fig. 1A and Table 1). It is interesting to note that both of the QCM-D signals, the frequency and the dissipation, decreased, although commonly, a decrease in frequency (mass load) is commonly accompanied by an increase in dissipation (increase in viscosity) (up to a certain layer thickness).

For platelets under the protein-poor condition, no aggregation could be observed by light microscopy, despite a clear response in the QCM-D signal (Fig. 1B). However, compared to the QCM-D signal observed in protein-rich conditions, the response is more complex (Fig. 1B, upper panel). After stabilization of the signal, the net shifts in frequency and dissipation are both positive (Fig. 1B, upper panel and Table 1). Again, we want to point to the unusual behavior of the QCM-D signal, i.e., both dissipation and frequency shifts increased (by comparing the values before and after activation).

In both of the described cases, the QCM-D signals are taken as indicatives for the activation of platelets confined to the TiO_2 surface.

In order to verify platelet activation, in particular in case of protein-poor condition, where no aggregation was observed, complementary experiments were performed separately using fluorescence microscopy (Fig. 2). Two fluorescently labeled antibodies were used: anti-CD61 that binds to glycoprotein IIIa to identify

Table 1
Mean values and the corresponding standard deviation of the frequency shift Δf and dissipation shift ΔD for the set of four independent experiments shown in Figs. 1 and 3.

	Protein-rich		Protein-poor	
	Δf/Hz	$\Delta D/10^{-6}$	Δf/Hz	$\Delta D/10^{-6}$
Plasma proteins	−(113 ± 6)	+(28 ± 2)	–	–
Platelets	−(13 ± 3)	+(6 ± 2)	−(335 ± 43)	+(272 ± 30)
TRAP-6	−(19 ± 2)	−(5 ± 2)	+(19 ± 6)	+(75 ± 18)

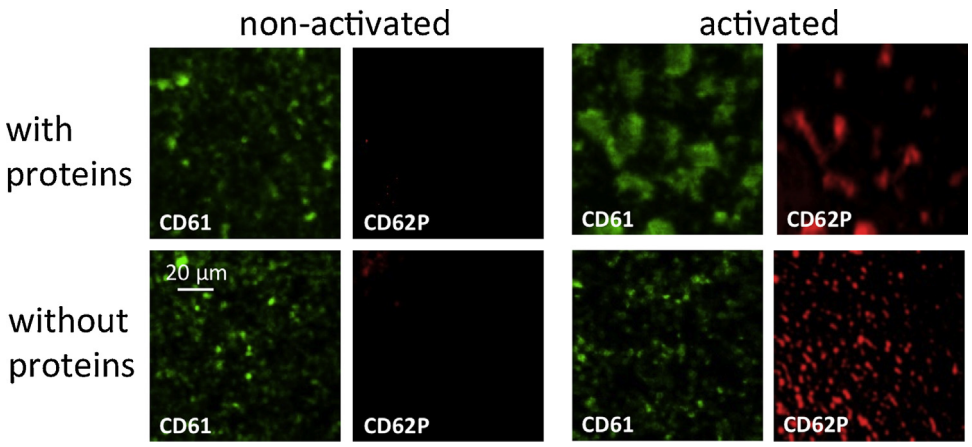

Fig. 2. Fluorescence microscope images of non-activated and activated platelets on TiO_2 coated sensors under protein-rich and protein-poor conditions. CD61 (green) is a marker for platelet glycoprotein IIIa whereas CD62P is used for the detection of P-selectin expressed on activated platelets. (For interpretation of the reference to colour in this figure legend, the reader is referred to the web version of this article.)

all platelets, and anti-CD62P that binds to P-selectin to identify activated platelets. Experiments were carried out similarly to the QCM-D experiments, except that the sensors were modified *ex-situ* in petri dishes. Attached platelets were observed under both conditions, corresponding to platelets in plasma containing proteins and platelets in protein-free PAS (Fig. 2, left). Binding of anti-CD62P was observed after activation with TRAP-6 under both conditions (Fig. 2, right). Similarly to the light microscopy results above, aggregation of platelets in the case of protein-coated surfaces was found after activation with TRAP-6 (Fig. 2, top, right). These observations confirm that the observed QCM-D signal after addition of TRAP-6 can be correlated to the activation of the surface-confined platelets.

Taking a closer look at the monitored QCM-D signals during platelet activation, we observe that the signals with and without plasma proteins are very different. However, both signals indicate changes in the viscoelastic properties of the platelets during activation. In our study, we observed a decrease in frequency accompanied with a decrease in the presence of plasma proteins whilst the opposite was observed in the absence of plasma proteins. Furthermore, aggregation was only observed in presence of proteins. One reason for the observed difference might be the lack of proteins like fibrinogen acting as a "crosslinker" during platelet aggregation.[17] However, performing control experiments where TRAP-6 was added in solution containing plasma proteins essentially the same QCM-D signal together with the absence of visible aggregation was observed (data not shown). Thus, we suggest that the observed different behaviors in protein-rich and protein-poor is due to a difference in the nature of the layer linking the platelets to the substrate.[18] To us, it is reasonable to assume that the interfacial layer linking the platelets to the substrate is different in protein-poor and protein-rich environments. In order to elucidate the linking of the platelets to the underlying substrate, we examined the attachment of platelets to TiO_2-coated sensor surfaces under protein-rich and protein-poor conditions.

3.1.1. *Platelet adhesion to TiO_2 surfaces*

Fig. 3A shows the QCM-D signals–frequency and dissipation versus time curves–recorded upon adhesion of platelets under protein-rich conditions to TiO_2–coated sensor surfaces (i.e., platelet rich plasma (PRP)). The sensor surface was in a first step saturated with plasma proteins by applying platelet poor plasma (PPP) (Fig. 3A, upper panel) giving rise to a significant drop in the resonance frequency accompanied by a distinct dissipation shift (Table 1) indicating the adsorption of a viscous layer of proteins on top of the TiO_2 surface. After stabilization of the signal, platelets (PRP) were injected over the surface that had been saturated with the plasma proteins. Essentially, no changes in the frequency and the dissipation shifts compared to the distinct shifts for proteins adsorption were observed. Despite the small signals observed by QCM-D, images taken with the light microscope at 45 min clearly show the presence of platelets (Fig. 3A).

In contrast to the small QCM-D signal observed for PRP, we observed large signals in both frequency and dissipation upon adsorption of washed platelets to the sensor surface under protein-poor conditions (Fig. 3B and Table 1). Interestingly, the frequency and dissipation shifts decrease and increase, respectively, continued when the platelet solution was changed back to PAS, i.e., when removing platelets from bulk solution, indicating structural rearrangements in the layer on the sensor surface which where independent of the presence of platelets in bulk solution. Images taken of the sensor surface at 55 min clearly show the presence of platelets (Fig. 3B) similarly to images taken for platelets adsorbed from PRP (Fig. 3A).

Thus, in brief, when comparing the light microscopy images of platelets on TiO_2 surfaces, we did not observe a clear difference between the protein-rich and the protein-poor conditions, but in the acoustical signal we observed drastic differences between the two conditions.

In the case of washed platelets, i.e., in the absence of plasma proteins, the observed frequency signal for the attachment of platelets onto the TiO_2 surface is as expected if platelets are assumed to be "spherical" viscoelastic particles. In other words, the QCM-D directly senses the adhering platelets as an increase of mass and a corresponding decrease of the resonant frequency. However, for the protein-rich condition, i.e., with plasma proteins, the QCM-D signal is perhaps unexpectedly low (keeping in mind that we clearly observed platelets attaching to the sensor surface with microscopy and that we were able to monitor the subsequent activation). In order to understand this finding, we have to take into account that the QCM-D sensor surface was saturated with a protein layer prior to platelet adhesion. In other words, platelets "land" on a thick and soft protein layer or cushion, potentially prone to structural rearrangements, and not directly on the TiO_2 surface. This protein cushion needs to be taken into account when interpreting the observed QCM-D signal, there are different possible scenarios: (i) the protein layer is simply too thick, i.e., it is thicker than the sensing depth of the QCM-D technique which will thus not be sensitive to the platelets; (ii) the additional mass added to the protein layer occurs in a regime where the QCM-D technique is insensitive as a consequence of the thickness and viscoelastic properties

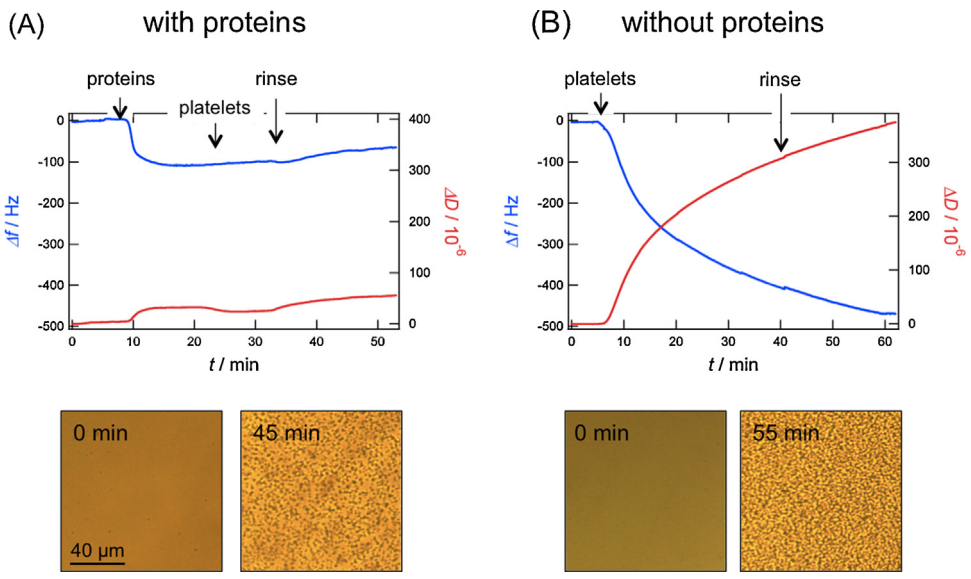

Fig. 3. QCM-D frequency and dissipation versus time curves (upper panels) and light microscopy (lower panels) of platelets adhering on TiO_2 coated surface in (A) a protein-rich environment and (B) a protein-poor environment. Light microscopy images were taken during the QCM-D experiment, before injection of platelets and in the end of the experiment.

of the sensor adlayer (see e.g., Voinova et al. [5,6]); (iii) there is a rearrangement in the protein-water-platelet-layer during platelet adhesion cancelling the effect of platelet adhesion so that the net-loading of the QCM-D sensor does not change; (iv) the nature of the (mechanical) bond between the platelets is too "weak" to significantly affect the QCM-D signal, although the platelets are still within the sensing depth of the QCM-D. The first scenario is not likely since the calculated thickness of the protein layer is around 40 nm (estimated using a viscoelastic model, see Section 2) and the sensing depth of the fundamental frequency of the QCM-D in water is 250 nm, thus more than 6 times the thickness of the protein layer. Therefore, any additional material, such as platelets, adsorbed to the protein layer should be sensed. The second argument seems not to apply either. Simulations based on the Voigt model for viscoelastic elements have shown that either the frequency or the dissipation is insensitive to the additional mass at a certain regime, but never both at the same time [6]. In our case, we observed no signal neither for the frequency, nor for the dissipation in the protein-rich environment, which let us exclude this argument. The third argument raised above, a rearrangement of proteins and coupled water during platelet adsorption, is not likely to be the main reason for the observed differences since we observed frequency and dissipation shifts of platelets adhering to TiO_2 surfaces without proteins to be 10 times higher than the shifts observed for the adhesion of plasma proteins. Thus, if platelets would displace proteins, there should be a clear net shift in both frequency and dissipation. Instead, we suggest the last scenario to be the most likely one, i.e., where a difference in the layer linking between the platelets to the substrate explain the observed difference in the QCM-D signal. This would be in agreement with previous findings by Johannsmann et al. showing that the QCM-D signal observed upon adsorption of nanoparticles is not dependent on the core properties of these particles but rather on the geometry of their attachment to the surface [18]. Since platelets do not adhere to most plasma proteins, but are known to adhere to TiO_2, [19] it seems likely that the binding to TiO_2 is stronger than to TiO_2 covered with plasma proteins (Fig. 4). It is not easy to predict but one may assume the QCM-D signal to be higher if the crystal had been precoated with a defined protein that allows for strong attachment of the platelets.

The above interpretation of the different nature of linking of the platelets to the underlying substrate also helps to explain the different behaviors observed upon activation of surface-confined platelets in protein-rich vs. protein-poor conditions. In the protein-rich environment, platelets are weakly connected to the surface via the proteins, meaning that the binding to the proteins as such is weak or, that the interactions between the proteins in the protein layer closest to the sensor surface are weak. Thus, upon activation,

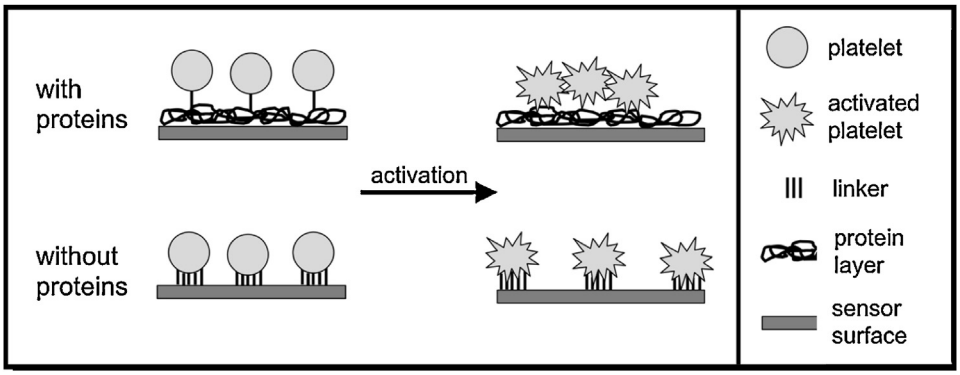

Fig. 4. Schematic illustration of the suggested scenario of platelet adhesion and activation on TiO_2 coated sensor surfaces.

the protein cushion between the platelets and the surface allows the platelets to "move" over the surface and to aggregate. In the protein-poor environment, the platelets are firmly bound to the substrate, and cannot "move" over the surface (Fig. 3) and as a consequence, they cannot aggregate. The fact that platelets are firmly attached to the surface and thus are unable to aggregate upon activation provides the ability to study platelet activation separated from platelet aggregation. This is a great advantage compared to most activation assays where platelets are in bulk solution and aggregation is inevitable upon activation.

4. Conclusion

Using QCM-D in combination with light microscopy, we were able to monitor activation of surface-confined platelets on TiO_2-coated surfaces in real-time. The results of our study clearly show that the QCM-D signal depends on the nature of the interfacial layer between the platelets and the underlying substrate. This finding together with ongoing studies along these lines using different surface modifications (e.g. extracellular matrix mimics and lipid membranes) will bring us closer to a physical model for the observed QCM-D signals in cell studies. We also want to highlight the major advantage of the combination of real-time monitoring of surface-confined platelets using QCM-D in combination with light microscopy compared to most common assays used for platelet studies, it is not only more convenient, it will also allow for more detailed understanding of the platelet-surface interactions, and furthermore it allows to study platelet activation separated from platelet aggregation. All together this will pave the way towards more advanced testing of the blood-compatibility of future medical devices.

Acknowledgements

Financial support was received from the Swedish Research Council (VR), the Linnaeus program SUPRA and the European Union Seventh Framework Program (FP7/2007-2013) under grant agreement no NMP4-SL2009-229292 ("Find & Bind").

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