A Straightforward Detection of HIT Type II via QCM-D

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Abstract
Heparin Induced Thrombocytopenia (HIT) is an undesired antibody based immune reaction against complexes of platelet factor 4 and heparin. HIT occurs in patients receiving heparin as anticoagulant over several days and is connected to the risk of life threatening thrombosis through intravasal platelet aggregation. HIT antibodies are routinely detected by Enzyme Linked Immunosorbent Assay (ELISA). However, not all antibodies lead to platelet aggregation and therefore, to clinical problems. The clinical relevance of a HIT ELISA positive serum can be confirmed by functional tests for platelet activation, like the heparin induced platelet activation (HIPA) test. However, these tests are tedious and cumbersome. Therefore, we present a straightforward approach by applying quartz crystal microbalance technology for functional assays for diagnosis of HIT type II. We utilized the qCell T (quartz crystal microbalance with dissipation (QCM-D)) platform of 3T analytik, Tuttlingen, Germany to demonstrate platelet aggregation measurements induced by clinically relevant HIT positive type II sera of patients. During the measurements changes in frequency and dissipation were recorded. This revealed statistically significant differences between high and low dose measurements in both, PRP and washed platelets. Platelet adhesion to the sensor surfaces was visualized by scanning electron microscopy (SEM). QCM-D data was in good accordance to SEM images. The results presented here promising that in the future specially adapted QCM-D sensors could be a serious straightforward alternative to currently used functional HIT assays.

1 Introduction
HIT is an undesired immunological reaction to the therapeutic use of heparin, potentially leading to thrombosis instead of the desired anticoagulant effects.

In detail, the harmful effects of HIT comprise thrombocytopenia and the risk of thromboembolic complications in arteries and veins. Typically, HIT occurs within 4 to 15 days after the start of heparin medication with platelet drops to less than 50 % of the initial numbers.

Under therapy with heparin, complexes are formed between the positively charged platelet factor 4 (PF4) and negatively charged heparin. Some of the patients respond to the formation of antibodies against the heparin/PF4 complex. Provided that the antibodies belong to the IgG class, these patients are candidates for developing the clinically devastating picture of HIT. On a molecular level, specific IgG antibodies bind to the PF4/heparin complex forming PF4/heparin/IgG immune complexes, which bind to the FcγRIIa receptors on platelets. The consequences are platelet activation, aggregation and reduced platelet counts. In interaction with plasmatic pro coagulative stimulation, this potentially leads to thrombus formation and embolism. Thus the intended beneficial anticoagulant effect of heparin is converted into its very harmful opposite. Since thrombosis and decreases in platelet counts are general symptoms, which can also occur in a variety of other diseases, specific tests for HIT are essential for adequate treatment.

While ELISA tests have a high negative predictive value, a positive ELISA is not necessarily connected with the occurrence of clinical HIT symptoms.
A clearly higher positive predictive value for the occurrence of clinical HIT symptoms is achieved by functional tests, which actually measure platelet aggregation or a surrogate marker.

Current functional assays include HIPA tests (Heparin Induced Platelet Aggregation) and tests for serotonin release.

In HIPA tests heparin is usually added in a low concentration 0.1 IU/ml (close to usual therapeutic concentrations) and a high concentration (100 IU/ml).

For stoichiometric reasons of complex formation, low heparin doses together with HIT positive sera can cause platelet aggregation, while high heparin doses do not.

Although functional HIT diagnostics have entered clinical application, these tests are ambitious and require high expertise. Patient related HIPA diagnosis should not be performed in laboratories with limited experience in this test. Alternative methods are lacking.

QCM is one of the most attractive transducer for pharmaceuticals and clinical studies. Therefore, we here present the QCM-D technology as an innovative approach toward functional HIT diagnostics (in the following designated QCM-HIPA). Recently, several reports have been documented for capability of QCM sensors for blood coagulation, while HIT platelet aggregation measurements have been demonstrated by using surface imprinting approach. Following up on these studies, we determined to use QCM-D technology (qCell T, 3T analytik, Tuttlingen Germany) for testing blood samples from HIT patients with typical clinical symptoms and a strongly positive, commercial ELISA test for HIT. Sera with strongly positive HIT ELISA results have an increased probability of containing functionally relevant platelet activating antibodies. The QCM-HIPA was carried out with PRP and with washed platelets. The sensors used for the test were either uncoated or pre incubated with platelets. Working with platelets and sensors principally requires special care to avoid false positive signals from unspecific platelet adhesion, without hampering the detection of specific signals from platelet aggregation.

The findings exhibited that QCM-HIPA was qualified for the determination of functionally active HIT antibodies. We see this as a step forward towards a QCM-D based point of care method (POC) for fast detection of these highly destructive antibodies. The real time character of QCM-HIPA bears further potential for kinetic analysis of the HIT antibody-induced aggregation process.

The measurements were carried out with platelet rich plasma (PRP) and with washed platelets, since it has been suggested that functional platelet assays like HIPA are more sensitive when using washed platelets. Besides increased sensitivity and reproducibility, the use of washed platelets is also supposed to diminish the problems associated with fibrinogen mediated platelet adhesion to the sensor surface. Hence there was no need for coating of the sensor surface.

In order to avoid undue activation of platelets we used a Sepharose column based size exclusion chromatography for washing as suggested by Vollmar et al. This isolation procedure is known to be gentler on the platelets than centrifugation based protocols. Subsequently, we used flow cytometric analysis of PAC-1 antibody binding to prove that the platelets were not activated by the separation procedure.

Additionally to the QCM-D measurements platelet aggregation at the sensor surface was visualized by scanning electron microscopy (SEM) after measurements.

2 Materials and methods

2.1 Chemicals and blood sample preparation

The project was approved by the ethical committee of the university hospital of Tuebingen. Blood collection was performed by the Institute for Clinical and Experimental Transfusion Medicine of the University Hospital of Tuebingen. The participants provided their written consent to participate in this study. The patients were clinically diagnosed HIT positive (data not shown here), thus selected by the ethical committee of university hospital of Tuebingen for this project. Fresh human whole blood from healthy blood donors was collected in appropriate syringes containing 1.0 ml of 0.106 mol l\(^{-1}\) citrate solution (10.0 ml 9NC S-Monovette, Sarstedt, Nümbrecht-Rommelsdorf, Germany). Clinically recommended needle was used to draw blood to minimize in vitro platelet activation. The collected blood was centrifuged at 150 x g for 15 min at room temperature for separating platelet rich plasma (PRP). After the PRP was carefully removed from the syringes, the remaining blood was centrifuged again for a further 10 min at 2500 x g for separating platelet poor plasma (PPP). PRP was measured with a cell counter (Abbott Cell-Dyn 3500, Chicago, Illinois, USA) and platelet counts were adjusted at 300.000 PLT per ml by mixing platelet rich and platelet poor plasma.

For measurements with washed platelets PRP was prepared as described above. Next the platelets were isolated from PRP with size exclusion chromatography using a Sepharose column (Sephadex 2B, product number 2b300, Sigma Aldrich, Steinheim, Germany). The isolated platelets were resuspended in HEPES-buffer (145 mM NaCl, 10 mM Hepes, 10 mM glucose, 0.2 mM Na\(_2\)HPO\(_4\), 5 mM KCl, 2 mM MgCl\(_2\), 0.3 % w/v bovine serum albumin, pH 7.4). After platelet isolation, platelets were counted and stored for 30 min at room temperature.

Hussian et al. A Straightforward Detection of HIT Type II via QCM-D
Prior to the actual HIT measurements PRP and washed platelets were tested for platelet functionality by control measurements with an optical platelet aggregometer PAP4 (Mölab point of care, Langenfeld, Germany). In case of HIT measurements with PRP, the measurements were carried out with 180 µl PRP and 20 µl ADP (Mölab point of care) in concentrations of 2 x 10⁻⁴ M resulting in final concentrations of 2 x 10⁻⁵ M. For measurements with washed platelets, the aggregometer measurements were performed with collagen from horse tendon, 100 µg/ml, (No 0203009, Mölab point of care) using 225 µl of washed platelets and 30 µl of collagen.

Status of cell activation due to the platelet isolation procedure was assessed by flow cytometric analysis (FACS) of PAC-1 binding.

The PAC-1 antibody binds to an epitope on the platelets' glycoprotein IIb/IIIa receptors, which are only found on activated platelets. Therefore, binding of fluorescence labeled PAC-1 antibodies can be used to evaluate platelet activation.17

For our experiments, PRP and washed platelets were diluted with tyrode buffer in a 1:50 v/v dilution and subsequently incubated with a fluorescein isothiocyanate (FITC) labeled, antihuman PAC-1 antibody (No 340507, BD Biosciences, Franklin Lakes, NJ, USA). Next, each sample was aliquoted into two samples one of which was left untreated while the other one was stimulated with ADP to test for maximum platelet activation. This was followed by 30 min of incubation at 37 °C. After that the samples were fixated with CellFix (BD CellFix, BD Biosciences, Franklin Lakes, NJ, USA) and measured with FACS (Becton Dickinson, Franklin Lakes, NJ, USA).

2.2 Treatment of HIT Sera

Prior to HIT measurements patient sera were incubated for 45 min at 56 °C in order to inactivate complement proteins that otherwise may lead to unspecific activation of the donors' platelets.18

2.3 ELISA test procedure

HIT specific antibodies were determined using an IgG specific ELISA test (PF4 IgG assay, Gen-Probe, LIFECODES®, San Diego, CA, USA). In this test ELISA plate immobilized polyvinyl sulfonate (PVS) conjugated platelet factor 4 (PF4) is used in the detection of IgG antibodies associated with type II HIT. The PF4:PVS complex mimics the epitope formed by undesired PF4/heparin interactions in HIT patients, and is used to bind the patients HIT specific antibodies. Binding of HIT antibodies was detected by incubation with IgG specific alkaline phosphatase conjugated secondary antibodies and subsequent incubation with p-nitrophenyl phosphate. By reaction with alkaline phosphatase, this chromogenic substrate develops a soluble yellow reaction product that can be measured at 405 nm. The absorption measurements were carried out with an appropriate absorbance reader (Biotek elx 800 NB, Biotek, Vermont, USA). Results of ELISA tests are shown in table 1.

Table 1: ELISA test results for HIT specific IgG antibodies. Different HIT samples from 4 different donors with the according optical density (OD value)

<table>
<thead>
<tr>
<th>HIT Sample No</th>
<th>OD value</th>
</tr>
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<tbody>
<tr>
<td>Donar 1</td>
<td>3.500</td>
</tr>
<tr>
<td>Donar 1</td>
<td>&gt;4.000</td>
</tr>
<tr>
<td>Donar 1</td>
<td>3.313</td>
</tr>
<tr>
<td>Donar 1</td>
<td>2.702</td>
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</tbody>
</table>

2.4 QCM-D Instrument

For the experiments the QCM-D instrument qCell T (3T analytik, Tuttlingen, Germany) was used. The sensor system is based on an AT-cut 10 MHz quartz crystal with 8 mm diameter. The instrument measures dissipation in addition to frequency and is thermo controllable with high precision in a wide temperature range. The sensors are actuated through an oscillating circuit by applying an alternating voltage. With this setup it is possible to measure viscosity changes in adjacent liquids and mass deposition to the sensor surface. By the study of frequency and dissipation curves it is possible to gain insights into the viscoelastic properties of attached layers.19 The measuring chamber itself with a volume of 30 µl is crafted from stainless steel with an inert PTFE insert. Tension free mounting of the quartz resonator is allowed by a patented holding.20 A computer controlled roller pump with adjustable flow rates transfers the sample from the thermostated 1.5 ml reaction cup to the measuring chamber. For the measurements this thermo controlled sensor unit was kept set at a constant temperature of 37°C.

2.5 Sensor cleaning and preparation

QCM crystals sensors were cleaned for 1 min with acetone for the removal of organic residues. Subsequently, the sensors were rinsed with double distilled water and dried under a stream of nitrogen. In a second cleaning step the sensors were cleaned for 1 min in piranha solution, a 3 : 1 mixture of concentrated sulfuric acid and 30 % hydrogenperoxide. Afterwards, the sensors were again rinsed with double distilled water and dried under a stream of nitrogen. Sensors used for measurements with washed platelets were not modified any further and used for measurements directly after the cleaning procedure.

For measurements with PRP, the sensors were incubated with PRP to prevent unspecific platelet adhesion.

UK J Pharm & Biosci, 2015: 3(6); 20

Hussian et al. A Straightforward Detection of HIT Type II via QCM-D
90 µl of PRP was stimulated with 10 µl of 10^{-4} mol/l ADP (Mölab point of care, Langenfeld, Germany) and added to the sensor surface. Afterwards the sensors were incubated for 30 min at a temperature of 37°C in a humid chamber. For better distribution of cells across the sensor surface, the PRP covered sensors were mounted on a spin coater (Spin 150-v3, Semiconductor production systems, Ingolstadt, Germany) and coated for 2 min at 3000 rpm.

2.6 HIT-Measurements on the QCM-D sensor platform

Next the sensors were mounted into the measuring device and stable baselines at flow rates of 100 µl/min were recorded. Measurements with PRP were carried out with Tris/NaCl buffer (125 mM NaCl, 50 mM Tris, pH 7.4), for measurements with washed platelets HEPES buffer was used (145 mM NaCl, 10 mM Hepes, 10 mM glucose, 0.2 mM Na_{2}HPO_{4}, 5 mM KCl, 2 mM MgCl_{2}, 0.3 % w/v bovine serum albumin, pH 7.4).

For HIT-measurements on the QCM-D sensor platform 75 µl of PRP or washed platelet suspension were mixed with 20 µl of HIT serum and incubated for 2 minutes at 37°C. Next, 10 µl of unfractionated heparin (5000 IU/ml, Heparin-Natrium-25.000, Ratiopharm, Ulm, Germany) in either low concentrations (0.1 IU/ml) or high concentrations (100 IU/ml) were added to the samples for starting the HIT aggregation. After mixing, the samples were pumped to the measuring chamber of the QCM-D at a flow rate of 100 µl/min. After the sample reached the measuring chamber the following reactions were measured at static conditions without any pump flow.

2.7 Scanning electron microscopy (SEM)

Scanning electron microscopic images were used to correlate the sensor signal to actual platelet aggregation on the sensor surface. After the measurement, the sensors were removed from the QCM-D and carefully rinsed with buffer to remove non-adhering platelets. Next the sensors were incubated overnight at 4°C in 2 % glutaraldehyde-PBS solution (140 mM NaCl, 10 mM Na_{2}HPO_{4}, 2,7 mM KCl, 1,8 mM KH_{2}PO_{4}, pH 7.4). After that the quartzes were washed again with PBS to remove residual glutaraldehyde. Next the remaining water was removed from the samples using 40 % to 100 % of ethanol (Merck, Darmstadt, Germany) in ascending concentrations. Finally, all samples were critical point dried (CPD), sputtered with gold palladium, and then analyzed using SEM (scanning electron microscopy, Evo Ls 10, Carl Zeiss, Oberkochen, Germany).

2.8 Statistics

Frequency and dissipation shifts were expressed as the arithmetic mean (M) values with standard deviation (SD). Statistical analysis was performed using the software BIAS for Windows Version 9.06 (Epsilon Verlag, Frankfurt, Germany). Data were tested for normal distribution by Kolmogorov–Smirnov test. Homogeneity of variances was tested by Bartlett’s test and multiple comparisons with Scheffe’s method. Differences between groups were calculated by univariate analysis of variance. Values of p < 0.05 were considered as significant.

3 Results and Discussions

3.1 Evaluation of platelet pre activation

After the isolation procedure platelet, activation was evaluated by FACS analysis. It was confirmed that after the isolation procedure the isolated platelets had similar GPIIb/IIIa activation levels than control PRP directly after blood collection and subsequent centrifugation. When stimulated with ADP, PAC-1 binding of Sepharose column isolated platelets showed that after isolation, the platelets still retained their full potential for activation (Data not shown).

3.2 QCM-D measurements of HIT induced aggregation

HIT sera from all donors were tested in high concentrations (100 IU/ml) and low concentrations of heparin (0.1 IU/ml).

While the low concentration should lead to typical HIT aggregation, the high concentrations serve as negative control (high dose inhibition)

As additional negative controls we also tested HIT negative sera in presence of 0.1 IU/ml heparin.

All sera were tested with PRP on PRP pre coated sensors and additionally with washed platelets on uncoated sensors. Changes in resonance frequency and dissipation were recorded. The results of the HIT measurements are shown in figure 1A for measurements with PRP and in figure 1B for measurements with washed platelets. In the graphs changes in frequency and dissipations are plotted against time.

3.3 Results of frequency and dissipation measurements

Changes in frequency and dissipation were recorded from all measurements and arithmetic means and standard deviations were calculated.

3.3.1 PRP measurements 0.1 IU/ml heparin

In the PRP measurements with 0.1 IU/ml heparin the initial (first) frequency shift was 375 Hz ± 28 Hz, followed by a further decrease to 1311 Hz ± 243 Hz. Initial (first) dissipation shifts were about 341 Hz ± 119 Hz followed by a second shift in dissipation to a mean of 2409 Hz ± 632 Hz.

3.3.2 PRP measurements with 100 IU/ml heparin

UK J Pharm & Biosci, 2015: 3(6); 21
Hussian et al. A Straightforward Detection of HIT Type II via QCM-D

411 Hz ± 189 Hz. Initial dissipation shifts had a mean of about 255 Hz ± 135 Hz. The maximum shift in dissipation was about 540 Hz ± 436 Hz in mean.

Figure 1A: HIT Aggregation measurements with PRP. Changes in frequency (Δf), solid, and changes in dissipation (ΔΓ), dashed, are plotted versus time. Measurements were performed with low heparin concentrations (0.1 IU/ml) and high heparin concentrations (100 IU/ml)

3.3.3 Measurements with washed platelets 0.1 IU/ml heparin

In measurements using washed platelets and 0.1 IU/ml heparin an initial frequency shift of 66 Hz ± 28 Hz was detected followed by a further decrease of 1050 Hz ± 111 Hz. Initial damping (dissipation) shifts were about 88 Hz ± 12 Hz followed by a second shift of 1931 Hz ± 416 Hz.

3.3.4 Measurements with washed platelets 100 IU/ml heparin

Measurements with washed platelets and 100 IU/ml heparin revealed an initial frequency shift of 85 Hz ± 17 Hz. In the following measurement only minor frequency changes were detectable, with an maximum decrease in frequency of 124 Hz ± 52 Hz. Initial dissipation shifts were about 80 Hz ± 26 Hz in the end of the measurement the maximum shift in dissipation was about 104 Hz ± 90 Hz.

3.3.5 Control measurements with HIT negative sera

Corresponding to measurements with HIT positive sera, the measurements were carried out with PRP and washed platelets. In the measurements with washed platelets, the maximum frequency shifts were about 140 Hz ± 61 Hz. Maximum shifts in dissipation were about 97 Hz ± 41 Hz.

The parallel measurements with PRP on PRP pre coated sensors revealed frequency changes of 332 Hz ± 86 Hz and changes in dissipation were about 234 Hz ± 148 Hz.

3.4 Comparison between measurements with high and low heparin concentrations

Ideally HIT aggregation should only occur in measurements with low heparin concentration. Measurements with high heparin concentration therefore should display no aggregation response and serve as negative controls. Both, the frequency and dissipation curves of high and low heparin concentrations show an initial shift due to adhesion of platelets and viscosity changes. Following to this shift the frequency and dissipation curves of low heparin concentration show a further shift due to HIT aggregation. No significant second shift was observed in high heparin concentration. Thus, a significant difference between high and low heparin concentration was recorded for both, washed and unwashed platelets. Results with washed platelets showed, however, a stronger distinction between positives and control samples (Fig 1A and 1B).

3.5 Analysis of frequency and dissipation shifts

The differences in maximum frequency and dissipation shifts between high and low heparin measurements were statistically significant in both, PRP measurements (p < 0.01) as shown in figure 2 and measurements with washed platelets (p < 0.001) illustrated in figure 3.

The comparison between low dose measurements of HIT negative and HIT positive sera also identified significant differences in frequency and dissipation data. The differences were significant for both PRP measurements and measurements using washed platelets (p < 0.001). Results are shown in (Fig. 4).
Figure 1B: HIT Aggregation measurements with washed platelets. Changes in frequency (Δf), solid, and changes in dissipation (ΔΓ), dashed, are plotted versus time. Measurements were performed with low heparin concentrations (0.1 IU/ml) and high heparin concentrations (100 IU/ml).

Figure 2: Frequency A and dissipation B changes in QCM-HIPA measurements with PRP. For all groups n = 4. Values are expressed as arithmetic mean from n measurements ± standard deviation. Differences between groups were calculated by univariate analysis of variance. Values of p < 0.05 were considered significant, values of p < 0.01 were considered as highly significant and marked with **

In addition to the information given by frequency and dissipation changes alone, the combined analysis of both provides a lot more information about biophysical processes that occur on the sensor surface.

The ratio between frequency and dissipation shift gives information about whether the signal represents a pure viscosity change (Δf=ΔΓ), a rigid mass deposition (ΔΓ=0 Hz) or a deposition of a viscoelastic layer (ΔΓ>Δf) to the sensor surface. Attached cells (platelets) represent a viscoelastic layer covered with the sample liquid (Plasma).

An illustration of dissipation versus frequency plot is shown in figure 5. Here it is evident that the dissipation versus frequency plots of all aggregation measurements lie above the bisecting line (the line through origin represents a pure viscosity change) indicating that the elasticity contributes to a larger extent than the pure rigid mass deposition. Meaning that the ratio between dissipation and frequency is higher than 1.0.

In measurements with washed platelets, the ratio between maximum change in dissipation and frequency was 1.84. In measurements with PRP this ratio was also 1.84. This data suggests that the sensor...
response in measurements with low heparin can be attributed to cell
adhesion to the sensor surface and besides absolute frequency
changes measurements with PRP and washed platelets are in
accordance to each other. Since the frequency to dissipation ratio is
the same in both types of measurements, plasma protein adsorption
from adhesion mediating proteins like fibrinogen does not seem to

Hussian et al. A Straightforward Detection of HIT Type II via QCM-D
influence this ratio. Therefore, the measurement signal can most
likely be attributed to the aggregation of the cells. This result is in
very good accordance to the scanning electron microscopic images
shown in figure 6 but in contrast to the findings described in where
protein-poor plasma results in no platelet aggregation on TiO$_2$ coated
quartzes.

Figure 3: Frequency A and dissipation B changes in QCM-HIPA measurements with washed platelets. For all groups n = 4. Values are
expressed as arithmetic mean from n measurements ± standard deviation. Differences between groups were calculated by univariate
analysis of variance. Values of p < 0.05 were considered significant, values of p < 0.001 were considered as highly significant and
marked with ***

Figure 4: Comparison of frequency shifts and dissipation shifts between HIT negative and positive sera with PRP (a) and washed
platelets (b) All measurements were carried out with low concentration of heparin (0.1 IU/ml), respectively. For all groups n = 4. Values
are expressed as mean of n measurements ± standard deviation. Differences between groups were calculated by univariate analysis
of variance. Values of p < 0.05 were considered significant, values of p < 0.001 were considered as highly significant and marked with ***
Figure 5: Plot of dissipation shifts versus frequency shifts during HIT mediated platelet aggregation measurements in low concentration of heparin with PRP and washed platelets.

Figure 6: Scanning electron microscopic images from QCM-HIPA measurements with washed platelets in low heparin concentrations (a, b) and in high heparin concentrations (c, d), respectively. Images e and f represent PRP pre coated sensors.
3.6 Comparison between measurements with PRP and washed platelets

On comparing PRP measurements to measurements with washed platelets it is remarkable that the measurements with washed platelets reveal lower initial shifts in frequency and dissipation than PRP measurements. This is mostly due to the higher viscosity of the PRP samples compared to the washed platelets samples. Hence low heparin measurements with washed platelets also had lower maximum shifts in frequency and dissipation (see Fig. 2 and Fig. 3).

In regard to the biochemical reactions associated with the sensor signal the minor part of these differences may be attributed to the lack of unspecific protein/platelet and protein/surface interactions in measurements using washed platelets. Since, it is well known that unspecific protein adsorption and fibrinogen platelet interactions trigger platelet adhesion to biomaterials\textsuperscript{23}. Furthermore, the uncoated gold sensors represent a homogenous surface for the HIT mediated platelet aggregation compared to the PRP pre coated surfaces.

Preparation of the sensor surface by pre incubation with PRP is a manual process which is supposed to vary between different samples. Additionally, rinsing with buffer during frequency stabilization prior to the measurements may also lead to washing of loosely adherent platelets from the sensor surface.

Based on experiences from the application of functional platelet assays in clinical HIT diagnosis it has been found that functional HIT assays to utilize washed platelets are more sensitive than PRP based protocols\textsuperscript{24, 25}. Our results are in very good accordance with these results.

For a combination of these reasons the measurements with washed platelets are more consistent.

3.7 Correlation of ELISA results to QCM-D platelet aggregation

In experiments with washed platelets, the time from start of the measurement to the onset of platelet aggregation seems to be in proportional correlation with the optical density (OD) of the corresponding ELISA. In other words, sera with high OD caused earlier aggregation than sera with lower OD (Fig 1B and Table 1).

OD can be seen as mirror of antibody titer. The formation of stoichiometric complexes between heparin, PF4 and HIT antibodies is essential for HIT aggregation. Therefore it implies, if one of the reactants is below the equivalence range, increasing its concentration may accelerate the kinetics of complex formation.  

3.8 SEM images

SEM images of quartz sensors after the measurements are shown in Fig 6. The images are taken in magnifications of 500 x and 2500 x.

Hussian et al. A Straightforward Detection of HIT Type II via QCM-D

3.8.1 SEM images with low heparin concentrations and washed platelets

In measurements with low heparin concentrations and washed platelets, platelet aggregates were homogenously distributed across the whole sensor surface. The individual spots consisted of aggregates formed by a certain quantity of platelets, but did not overlap with neighboring aggregates. SEM images of platelet aggregates are in good correlation to the sensor signal in which higher frequency and dissipation changes indicate a major extended of platelet aggregation. Since there is no fibrinogen in buffer suspended platelets, no fibrin fibers connecting the aggregates could be observed. Instead, the platelets are directly adhering to each other.

3.8.2 SEM images with high heparin concentrations and washed platelets

SEM images of sensor surface after measurements with high heparin concentrations and washed platelets show no attachment of platelets as expected for negative controls. Since the washed platelets’ suspensions include no platelet adhesion mediating proteins, platelets are only loosely attached to the sensor surface, and washed away during SEM preparation procedure. This finding is in accordance with the QCM-D data and may also help to explain why high heparin curves with washed platelets give a clearer picture of negative results compared to measurements with PRP.  

3.8.3 SEM images PRP with PRP pre coated sensors

Because of the PRP pre incubation process, in measurements with PRP, it was not reasonable to compare SEM images of high dose and low dose measurements, since the PRP incubation of the sensors prior to the measurements leaves the sensor surface covered with adhering platelets. Later, it is not possible to distinguish clearly between platelets deposited during sensor preparation or measurement. The SEM image shows a PRP coated sensor. Compared to the low dose heparin measurements with washed platelets, platelet aggregates on the sensor surface are more overlapping than on measurements with washed platelets which may be attributed to the involvement of Fibrinogen into the aggregates.  

3.9 Recent advances in the automation of functional HIT assays

Safe HIT diagnosis is of mayor importance not only for the patient’s health but also for cost savings. A German study carried out by Wilke et al.\textsuperscript{26} revealed that each clinical case of HIT adds a mean of 9000 Euro of additional costs due to prolonged hospital stay and the need of alternative more costly anticoagulants.

These alternative anticoagulants like argatroban, lepirudin, and danaparoid are also connected with higher bleeding risks than heparin and are more difficult to monitor and to antagonize\textsuperscript{27}.

UK J Pharm & Biosci, 2015: 3(6); 26
The development of new test methods, both for immunological and functional HIT diagnosis are therefore of great interest. During the last years, several efforts have been made to establish rapid assays for both kinds of HIT diagnosis.

Sachs et al.\(^1\) have recently tested a new lateral-flow immunoassay for the detection of HIT antibodies. The tests were performed with HIT sera from different patients and correlated to other established ELISA tests and functional assays. Current functional HIT assays were furthermore used to calibrate the lateral flow assay and define cut off values between platelet activating and non-activating antibodies. As a result: sensitivity for IgG detection was comparable to established ELISA tests.

In another approach of that workgroup a further automated immune assay was tested by Althaus et al.\(^2\). The authors emphasize the potential of higher standardization of laboratory HIT diagnosis that may come with these new assays, but they still acknowledge the necessity of functional assays.

Morel-Kopp et al.\(^3\) developed a functional platelet aggregation based HIT assay in whole blood. They utilized a commercially available Multiplate\(^\text{®}\) analyzer for whole blood impedance aggregometry. The potential HIT sera were prescreened with an ELISA test and subsequently tested with the functional assay.

In a following publication by Solano et al.\(^4\), this new method was compared to the likewise new HITAlert\(^\text{®}\) kit, a flow cytometry based functional HIT assay. The results for this study was that the functional assays had little problems with the diagnosis of HIT sera with high positive ELISA values; however, in samples with medium or low antibody titers a specific diagnosis was more difficult like in many other functional assays. Regardless of these problems, the authors conclude that the new methods may add sensitivity and practicality to existing functional assays like serotonin release assays. Similar conclusions have been made by Garritsen et al.\(^5\) who also tested the new HITAlert\(^\text{®}\) flow cytometric kit.

Modification to the PF4/heparin ELISA and a cell-based functional assay for HIT that outperformed the conventional ELISA recently\(^6\).

4 Conclusions

In our experiments, we followed the guidelines of clinical HIT diagnosis, by testing sera with positive ELISA results with a subsequent functional assay. The current study using 4 sera is a proof of principle that QCM-D can measure platelet aggregation induced by PF4/heparin antibodies.

Utilizing our QCM System as a detection system for the functional HIT assay, we observed that HIT sera from all four donors responded in the expected way, namely positive outcomes with low dose measurements and negative test result with the high heparin

Hussian et al. A Straightforward Detection of HIT Type II via QCM-D doses. Hereby the measurements with washed platelets revealed especially little variation between individual measurements as confirmed by statistics.

QCM system was successfully used for a functional HIT assay. The QCM-D method has proven for its applicability for different types of hemostaseological measurements. In future clinically validated, QCM-D possibly will be applied for HIT diagnosis.

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6 Competing interests

Authors declare no competing interests.

7 Authors’ contributions

All authors equally contributed in the study, writing and revision of the manuscript.

8 References


Hussain et al. A Straightforward Detection of HIT Type II via QCM-D
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UK J Pharm & Biosci, 2015: 3(6); 28