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Investigation of Prothrombin Time in Human Whole-Blood Samples with a Quartz Crystal Biosensor

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Monitoring of blood coagulation and fibrinolysis is an important issue in treatment of patients with cardiovascular problems and in surgery when blood gets into contact with artificial surfaces. In this work a new method for measuring the coagulation time (prothrombin time, PT) of human whole-blood samples based on a quartz crystal microbalance (QCM) biosensor is presented. The 10 MHz sensors used in this work respond with a frequency shift to changes in viscosity during blood clot formation. For driving and for readout of the quartz, both a network analyzer and an oscillator circuit were utilized. The sensor surfaces were specifically coated with a thin polyethylene layer. We found that both frequency analysis methods are suitable to measure exact prothrombin times in a very good conformity with a mechanical coagulometer as a reference. The anticoagulant effect of heparin on the prothrombin time was exemplarily shown as well as the reverse effect of the heparin antagonist polybrene. The change of the viscoelastic properties during blood coagulation, reflected by the ratio of frequency and dissipation shifts, is discussed for different dilutions of the whole-blood samples. In conclusion, QCM is a distinguished biosensor technique to determine prothrombin time and to monitor heparin therapy in whole-blood samples. Due to the excellent potential of miniaturization and the availability of direct digital signals, the method is predestinated for incorporation and integration into other devices and is thus opening the field of application for inline coagulation diagnostic in extracorporeal blood circuits.

Under normal circumstances, the hemostatic system is kept at a balance between the tendency of clot formation and the dissolution of blood clots (fibrinolysis). In pathological situations, during surgical treatments, and during dialysis, this balance is, often purposefully, disturbed. This makes it necessary to quickly evaluate and, ideally, constantly monitor the hemostatic parameters.

For instance, extracorporeal circulation of blood is necessary in a number of surgical procedures employing cardiopulmonary bypass as well as for patients requiring dialysis treatment, most often on a regular schedule. The extracorporeal circuit is regularly used in conjunction with anticoagulants such as heparin, to avoid complications arising from the coagulation of blood. In this and many other therapeutic situations, the level of heparin administration needs to be carefully controlled, to keep a sufficient level of anticoagulation while not increasing the risk of excessive bleeding. This makes it necessary to closely monitor the hemostatic status of the patients by measuring their coagulation parameters in short intervals. Coagulation times in different assays are usually being measured by evaluating the change in viscosity during the coagulation process after addition of an activator mechanically or by optical methods.[1] Moreover, the analytical devices currently on the market necessitate the manual transfer of blood samples from the patient to a separate analytical device. The current overall procedure lacks automation and does not permit continuous sampling and measuring. To improve this situation, an automated system for continuous monitoring would need an inline biosensor directly within the extracorporeal circuit.

Biosensors based on different principles like optical,[2] magnetoelastic,[3] or acoustic sensors[4,5] have already been used for research of blood coagulation processes. But in contrast to the optical method of surface plasmon resonance,[6,7] magnetoelastic sensors[8] and acoustic sensors like the quartz crystal microbalance (QCM) or QCM-D,[9,10] respectively, are able to give information about the changes in viscosity during coagulation.

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Therefore, a QCM biosensor was modified appropriately to permit the quick, reliable, reproducible, and automated measurement of blood coagulation times.

A QCM sensor as used in this study is an electromechanical transducer composed of a piezoelectric AT-cut α-quartz sandwiched between two parallel, flat gold electrodes. When an alternating voltage is applied by connecting the two electrodes to an oscillator circuit, a mechanical wave is generated in the interior of the quartz. The resonance frequency of this wave is characteristic for the geometry of the quartz specimen. An acoustic load like a thin (bio) layer attached to the upper surface or a wetting liquid results in a frequency shift and/or in a dampening shift. Therefore, quartz resonators are sensitive detectors for even minute mass changes[11] and, beyond that, changes in viscosity in the adjacent medium.[12] Parasitic effects like temperature or pressure changes lead also to a QCM sensor signal and should be suppressed. In line with the limitations given by the relation between gold electrode diameter and quartz crystal thickness, QCM sensor technology offers a high potential for miniaturization.[13] In principle, further miniaturization could be achieved using other acoustic sensor transducers. For instance, surface acoustic wave (SAW) sensors possess a very high miniaturization potential, but electronics to drive those sensors are considerably more complex.

Special coatings on the electrode surface allow the selective binding of different target molecules from an analyte solution. By this way, a number of specific applications have been designed for the detection and identification of blood groups,[14−16] different cells, bacteria, viruses, antibodies, and even small molecules[17−19] and DNA.[20]

In this study, the coating of the quartz sensor surface was adapted and optimized to permit the detection of fibrin buildup during the coagulation process. Although our frequency curves of activated samples could in principle be kinetically examined in this paper, we concentrate on measuring standard clinical parameters which also are measured with state-of-the-art devices in clinical practice.

Furthermore, a different setup of quartz excitation and data readout was employed which allows the simultaneous measurement of frequency changes and of dissipation coefficients whereby further conclusions can be drawn on the rheological properties of the analyte medium.

EXPERIMENTAL SECTION

Chemicals. Polyethylene (PE) (427799, $M_w \sim 35\,000$ g mol^{-1}), decahydronaphthalene (D251), sodium chloride, sulfuric acid, tris(2-hydroxyethyl)amine hydrochloride (Tris), and hexadimethrine bromide (polybrene) were purchased from Sigma-Aldrich (Germany). Hydrogen peroxide was obtained from Merck (Germany). Blood coagulation was initiated by Thromborel S (OUHP29) from Dade Behring (Marburg, Germany). Heparin sodium iv was purchased from Ratiopharm (Germany).

QCM Sensors and Sensor Coating. AT-cut 10 MHz quartz crystals with a diameter of 8 mm, a thickness of 166 μm, and special gold electrode design were purchased from KVG Quartz Crystal Technology (Neckarbischofsheim, Germany). Prior to sensor coating the gold surfaces were cleaned for 1 min with acetone, rinsed with Millipore Milli-Q water, and dried under a stream of nitrogen. To yield hydrophilic surfaces, a second cleaning step was done using piranha solution, a 3:1 mixture of concentrated sulfuric acid and 30% hydrogen peroxide, for 1 min followed by excessive rinsing with Milli-Q water and drying under a stream of nitrogen.

After that the sensor surfaces were spin-coated (spin-coater Spin150-v3, Semiconductor Production Systems, Ingolstadt, Germany) with PE as described elsewhere.[5] Briefly, 20 μL of a 1% solution of PE in decaline was coated onto the gold surfaces at a rotation speed of 3000 rpm for 120 s. To evaporate residuals of solvent, the coated sensors were incubated at 70 °C for 15 min. Prior to measurement, the sensors were allowed to cool to room temperature. Polyethylene was selected as a coating material based on published data[5] and comparatively evaluated against cross-linked polymer materials (data not shown).

To minimize unspecific adsorption of proteins during the measurement and to provide the surface with "anchor points" for coagulation directly at the surface, quartz sensors were pretreated with human whole blood of the appropriate donor for a time period of 10 min at room temperature.

QCM Apparatus. A homemade fully automated QCM sensor platform called "FidgeType FgT1" was used to monitor the blood coagulation process. A special advantage of this two-channel sensor platform with automated flow injection, thermocontrolled sensor unit, and camera observation of the sensor surface[13] is the innovative and tension-free mounting of the sensor.[14,21] Two types of measurement systems were used: The first one, based on an oscillating circuit, will only give information about the frequency shift taking place while mass deposition or changes of viscosity of adjacent liquids occurs. The other measurement system consists of a network analyzer (R3765CG, Advantest, Munich, Germany) which allows observing changes in both frequency and dissipation[22,23] and hence of rheological properties of adjacent layers or liquids.

Blood Sample Preparation. Citrated whole-blood samples were received from healthy donors at the Center for Clinical Transfusion Medicine at the University Hospital of Tuebingen (ZKT). The blood samples were heated to 37 °C and gently agitated prior to coagulation tests which were performed within 6 h after blood withdrawal. To yield coagulation times between

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Table 1. Overview of the Different Parameters of the Performed Blood Coagulation Experiments

	blood		thromborel			
experiment	dilution (Tris buffer)	volume [μL]	dilution (Tris buffer)	volume [μL]	flow rate [mL/min][a]	measurement type
I	1:2	200	1:2	200	0.8	oscillator circuit
II	1:2	200	1:2	200	0.8	network analyzer
III	1:2	200	1:2	200	0.8	oscillator circuit
IV	1:3	200	1:3	200	0.8	network analyzer
V	1:4	200	1:4	200	0.8	network analyzer
VI	1:5	200	1:5	200	0.4	network analyzer
VII	1:5	200	1:5	200	0.4	oscillator circuit

[a] Necessary pump flow rate to inject the coagulation-activated blood sample into the measurement chamber in time.

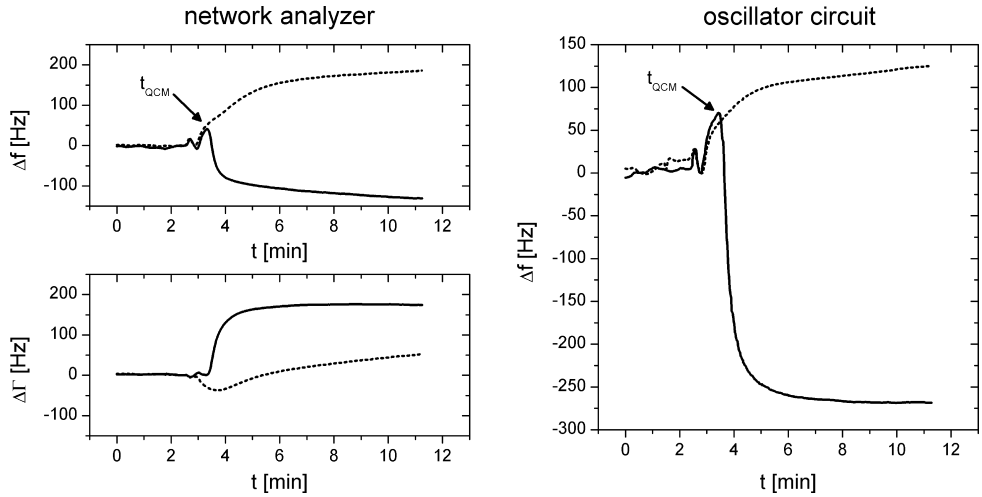

Figure 1. Measurement of the coagulation process (solid) and measurement of a control without coagulation (dotted) in 1:3 diluted whole-blood samples, using a network analyzer (left) or an oscillator circuit (right).

20 and 60 s, whole-blood samples were diluted in Tris buffer (50 mM Tris, 125 mM NaCl, pH 7.4) as indicated in Table 1. To investigate the anticoagulant effect of heparin, heparin was added to the whole-blood sample in the appropriate concentration 5 min before start of the measurement.

Measurement Procedure. Applying the biosensor platform "FidgeType FgT1", modified prothrombin time (PT) tests were carried out on PE-coated quartz sensors at a temperature of 37 °C. The coagulation times of the whole-blood samples obtained with the biosensor were compared to the results from a commercially available coagulometer (Biomatic 2000, Sarstedt, Germany and MC4plus, ABW Medizin and Technik, Germany). Subsequent to preincubation of the sensor with human whole blood as indicated in the QCM Sensors and Sensor Coating section, the sensor was rinsed with Milli-Q water, dried, and mounted into the measurement chamber in order to achieve a stable baseline. The different measurement setups for determination of coagulation time without the influence of heparin are listed in Table 1.

After recording a stable baseline at the appropriate flow rate of Tris buffer, the pump was stopped for 2 s in order to mix the blood sample with the activator thromborel. The activated sample was then transferred onto the sensor with the flow rates indicated in Table 1. Once the sensor was covered with the blood sample, the clot formation was obtained under stop flow conditions. To exclude all other effects than those due to clot formation, a control measurement with an appropriate volume of buffer solution instead of coagulation activator was performed for each blood dilution.

RESULTS AND DISCUSSION

Comparison of Control and Coagulation Measurement and Determination of Coagulation Times Using the QCM Sensor. Figure 1 shows representative QCM curves for coagulation and control measurements performed as described above using a network analyzer and an oscillator circuit, respectively.

There was an obvious difference between the coagulation and control measurement curves. The short baseline phase with constant buffer flow was followed by a small peak in frequency, due to the flow changes during mixing and injection phase as mentioned above in the Experimental Section. Although the frequency of the control measurement with no occurring coagulation process increased exponentially and then leveled off to a plateau phase, a decrease in frequency and an increase in dissipation, followed by a stable plateau, were obtained when blood coagulation took place. The resulting negative frequency shifts were in the range of 100−400 Hz, depending on the dilution factor of the blood sample. Measurements performed with an oscillator circuit resemble closely the curve shape and results gained using a network analyzer.

The commonly used coagulometric methods simply display a time value marking the beginning of coagulation. To compare the results gained from the QCM measurement with those of a standard coagulometer, the time value of a significant point of the frequency curve has to be picked. Therefore, we optimized the setup as described in order to obtain a characteristic point in the frequency curve. We found that this point is the local

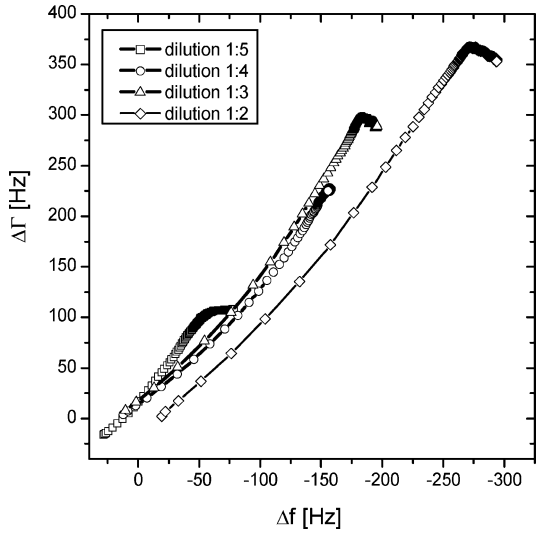

Figure 2. Plot of change in dissipation vs change in frequency during ongoing coagulation process of human whole-blood samples at different dilutions.

maximum of the frequency curve as shown in Figure 1 and is given by $df/dt\ (t_0) = 0$ and $d^2f/dt^2\ (t_0) < 0$. The resulting time value was defined as coagulation point obtained with the QCM sensor (t_{QCM}) and then compared with the data of a coagulometer. Monitoring of dissipation changes using the network analyzer is very useful for understanding the processes during blood coagulation. Principally, the combined evaluation of frequency and dissipation changes would allow the calculation of the basic layer properties using specially adapted simulation algorithms. A program for the simulation of cell binding on protein layers has been developed in our research group[14,24] and is currently being adapted to the simulation of the present problem. But particularly with regard to the procedure of routine analysis in a medical apparatus, recording frequency only by an oscillator circuit is sufficient. In addition to the lower costs, this device requires little space compared to a network analyzer. As shown in detail elsewhere,[14] the frequency shifts of both the network analyzer and our self-made oscillator circuit are quite similar. We observed this behavior also by measuring the frequency changes of an ongoing coagulation. So we consider the oscillator circuit as an appropriate tool for the determination of the clotting time point t_{QCM} in this application.

Changes in Frequency and Dissipation during Coagulation of Whole Blood in Different Dilutions. Figure 2 shows the shift in frequency Δf plotted versus the shift in dissipation $\Delta\Gamma$ (half of half-width of the Lorentz curve, in Hz) of the indicated dilutions of whole blood during the coagulation process. The results of the different dilutions are quite similar concerning the curve shape. The more the whole-blood sample is diluted, the more the absolute response decreases both in frequency and dissipation. All curves show at the end of the coagulation process a bend in direction of frequency with no further increase in dissipation.

For purely viscous liquids, called Newtonian liquids, Δf and $\Delta\Gamma$ have the same absolute value with opposing sign,[14] and hence the curve is a bisecting line. As can be seen in Figure 3, the behavior of the blood sample in the process of coagulation resembles very closely the behavior of a Newtonian liquid.

However, at the end of the coagulation process the shape of the curve is different. While the frequency increases, the dissipation is mainly constant or decreases slightly. This behavior is typical for an increase of mass deposition on the upper quartz surface induced by a thin rigid layer as well as for an increase of rigidity of the attached multiple layer system. We assume that it is a superposition of both processes. The behavior of an attached rigid mass layer can easily be described with the Sauerbrey model.[11] The quartz responds to deposition of a thin rigid layer with a frequency shift negatively proportional to the mass of the deposited layer. For the description of a change in rigidity more complex considerations based on the transmission line model of Mason[14,25,26] are needed. Further considerations and a more detailed discussion including the interpretation of phase and damping spectra will be published in a subsequent paper. However, the curve shape during the process of coagulation of human whole blood and the distinctive bend at the end can be rationalized as follows.

The blood sample is activated by homogenously distributed "Thromborel S" containing calcium ions and thromboplastin. In the extrinsic pathway of coagulation, thromboplastin ("tissue factor") activates factor VII, which in turn leads to an activation of factor X. Together with activated factor V, calcium ions and phospholipids-activated factor X forms the so-called prothombinase complex. Finally, this complex catalyzes the conversion of prothrombin to thrombin. Thrombin as central protein in the coagulation cascade cleaves fibrinogen to fibrin. The fibrin monomers eventually polymerize into fibers. As a whole, the homogenously distributed activator "Thromborel S" leads to likewise spatially homogenously distributed polymerization centers. Due to the buildup and growth of polymerization clusters the viscosity of the liquid increases. Therefore, during the first phase the liquid behaves like a Newtonian liquid, and we found an approximately linear correlation between Δf and $\Delta\Gamma$.

We assume that during the first phase blood constituents that do not respond with a mass effect and which present binding sites for fibrin fibers bind unspecific on the PE layer. Hence, at a certain point parts of the fibrin polymer fibers establish bindings and deposit onto the sensor surface. The established bindings lead to both an increase in coupled mass and an increase in the rigidity of the viscoelastic initial PE layer.[27,28] Fibrin clot retraction[29] results in a 10 times lower clot volume and therefore to a more closely meshed clot and fibrin network. These effects result in an increase in clot rigidity that is now fractionally coupled to the quartz surface and thus account for the bend in the $\Delta f/\Delta\Gamma$ curves. For a better understanding of the processes and which process is mainly responsible for the distinctive bend, detailed analyses are in process as mentioned above.

Our results can be compared to earlier studies of blood coagulation with sensoric methods. To observe coagulation of

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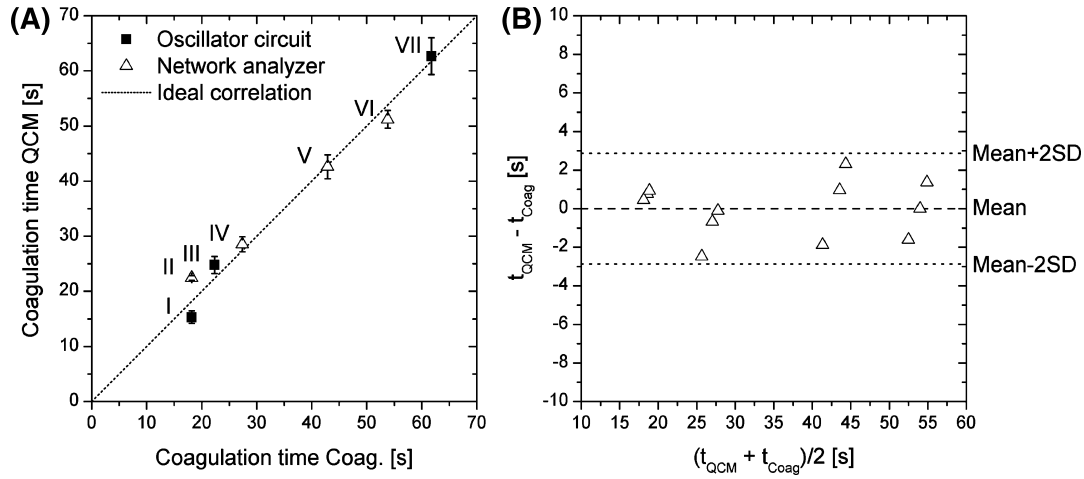

Figure 3. (A) Plot of coagulation time values acquired on the QCM sensor vs coagulation time values measured on a commercially available coagulometer. Whole-blood dilutions are as indicated in Table 1. (B) Bland−Altman plot of linear calibrated data measured by a network analyzer to compare the two methods. Mean ± two standard deviations are indicated in the figure.

horse, rat, and human whole blood in real time, Puckett et al.[3] first used magnetoelastic sensors, made of ferromagnetic amorphous alloys. Muramatsu et al.[26] mentioned the determination of blood coagulation factors as an example of the application of viscosity sensing by a QCM. Their theoretical considerations and the experimental results presented in this work are in very good accordance. Not only the work of Vikinge et al.[4] but also of Andersson et al.[5] dealt with the study of blood and plasma coagulation by using a modification of a QCM, the so-called QCM-D technique. This technique also permits the determination of changes in frequency and dissipation simultaneously. The curves presented here for different dilutions of whole blood show the same parallel curve linearity as the QCM-D curves measured by Vikinge et al., who used different concentrations of thromboplastin and calcium to activate undiluted whole blood. Therefore, the use of the same dilution both for the whole-blood sample and coagulation activator results in the same ratio of fibrinogen and thromboplastin/calcium. This, in turn, causes comparable reaction kinetics of the different dilutions which resulted in nearly parallel $\Delta f/\Delta\Gamma$ curves. The more the blood sample is diluted, the more the clot density decreases because of a less compact fibrin network. This effect is displayed by lower absolute values of frequency and dissipation with higher dilution factors.

Prothrombin Time Measured by the QCM Sensor in Relation to Coagulometer Values. To validate this new measurement technique, blood samples of one healthy donor in a defined dilution were measured three times using both the QCM technique and established mechanical coagulometric method. This procedure was performed four times, using blood samples of different donors in different dilutions as shown in Table 1. In order to demonstrate that not only an expensive network analyzer can be used for investigation of coagulation times, these measurements were also carried out using the cheaper oscillator circuit. For this purpose, two blood samples of two different donors (donors I and III, Figure 3) were studied at lower dilution resulting in low coagulation times and one further donor (donor VII, Figure 3) in higher dilution resulting in longer coagulation times (Figure 3).

Figure 3A shows the PT acquired by the traditional coagulometric method versus PT determined by QCM technology. Higher dilutions of the whole-blood sample cause longer times of clot formation, which are in the range of 20−60 s. Optimal correlation between the two measurement techniques is indicated in Figure 3 by the dotted bisector. With higher time values, the standard deviation of the QCM measurements increases. The more the blood sample is diluted, the more the viscosity of the sample on the sensor is in the range of physiological buffer. Hence, the change in viscosity occurring at the coagulation process is lower. This results in higher standard deviations for more diluted blood samples.

A well-established procedure for the comparison of two different measurement techniques is, as well in science as in clinical research, the method by Bland and Altman.[30,31] In this special scatter diagram, the differences of the values of the two methods are plotted against the mean of the values. For an easier interpretation, there are three lines shown: the mean of the differences, and the mean plus/minus two times the standard deviation, which declare that about 95% of the values are within two standard deviations. This diagram gives an optical estimation of the fluctuation range of the accordance. Furthermore one can see if one of the methods delivers systematically higher or lower values and if the variation of the methods depends on the absolute value. So this type of diagram is mainly used to compare new measurement techniques with a well-approved technique. The raw data of measurement performed by a network analyzer as shown in Figure 3A were calibrated using a linear calibration function. As recommended by Bland and Altman, the differences of these calibrated values of the two methods were plotted against the mean of the corresponding values (Figure 3B). One can see that all data points lie in the given range of mean ± 2SD. In addition, the data in Figure 3B show that none of the methods delivers higher or lower values, as a matter of principle. The deviation from symmetrical distribution in the case of lower coagulation times may be due to the manual injection technique and the sampling frequency of 0.67 Hz. On longer clotting times the distribution is symmetrical around the mean which is consistent with a normal distribution.

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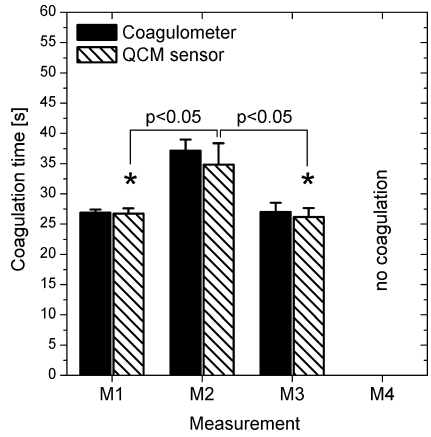

Figure 4. Anticoagulant effect of heparin monitored using a coagulometer in comparison with the QCM sensor. All measurements were performed with 1:2 diluted blood samples: M1, no heparin, no polybrene; M2, 1.0 IU/mL heparin, no polybrene; M3, 1.0 IU/mL heparin, 300 μg/mL polybrene; M4, 4 IU/mL heparin, no polybrene. Statistical evaluations were done using the two-sample Student's t test ($n = 4$).

Not only the bulky and expensive network analyzer but also the small and low-cost oscillator circuit is capable of measuring PT. Further tests using whole-blood probes of different donors in a given dilution are currently in progress and will be published in a subsequent publication. Most commercially available instruments in clinical routine and QCM technology observe the same measurand, the change in viscosity during coagulation process. Furthermore, the parameters of the measurement, like reagents, reagents dilution, ratio, and temperature can easily be set such that results can be directly compared with the results from standard biological or clinical test. Preliminary experiments indicate that pathological blood sample can also be analyzed in a comparable way. Data are not shown in this paper. Further experiments will be necessary.

Influence of Heparin and Heparin Antagonization on Prothrombin Time. To investigate the influence of heparin and its antagonization with polybrene on PT values, four samples of different donors were measured, each with the QCM sensor and coagulometer. Figure 4 shows the clotting time of a 1:2 diluted blood sample (Figure 4, M1). With the addition of 1.0 IU/mL heparin (Figure 4, M2) the clotting time increases, measured by the QCM sensor and coagulometer, respectively. As both different measurement techniques demonstrate in very good accordance, addition of an excess quantity of heparin antagonist polybrene results in a decrease of clotting time (Figure 4, M3) to the value without any addition of heparin. Control measurements with 4 IU/mL heparin added were also performed (Figure 4, M4). Both techniques show the absence of a coagulation process.

Heparin, a highly sulfated glycosaminoglycan, is widely used in clinical routine as anticoagulant drug. As a result of the heparin binding to antithrombin, the enzymatic activity of antithrombin increases. Therefore, the blocking effect of antithrombin to activated coagulation factors, like factor Xa, increases dramatically. Finally, this leads to a reduction of fibrin generation and therefore to an increase in PT values. The quaternary ammonium salt polybrene binds to heparin due to electrostatic interactions and thus antagonizes the anticoagulant effect of heparin. Polybrene is routinely used in biological tests to determine heparin activity in human plasma.[32] It has been used after cardiopulmonary bypass and has been recommended as an alternative neutralizing agent besides protamine.[33]

As shown in the measurements, QCM sensors can be used to monitor heparin and antiheparin effect (polybrene) with respect to PT analysis even in whole-blood samples. In conclusion, this series of QCM measurements reveals the possibility to clinicians to decide on the basis of QCM data if the anticoagulant therapy is still sufficient (M1−M2) or if the heparin concentration in the sample is so high that no further coagulation processes can occur (M4). In the last case, therapeutical use of polybrene can also be monitored using QCM sensors.

CONCLUSIONS

Measurements of coagulation times on FidgeType FgT1 correlate well with measurements of established devices for coagulation measurements. The results obtained by combined evaluation of Δf and $\Delta\Gamma$ using a network analyzer are closely paralleled by results using Δf alone taken from a cost-effective oscillator circuit. The results from heparinized whole-blood samples demonstrate the applicability of FidgeType FgT1 toward the evaluation of heparin status of a patient.

Further studies will be carried out for broadening the database and improving the statistical evaluation of the data. Measurement series will be extended toward using pathological and clinically relevant blood samples.

As a consequence of the possibility to observe viscosity change during blood coagulation, a comprehensive field of further tests of hemostatic status like activated partial prothrombin time (aPTT) or fibrinolysis testing can be performed in a similar manner. This will prepare the way for developing a cost-effective biosensoric analytical system applicable for routine diagnostics of hemostatic status in medical laboratories.

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