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Nanomechanical clinical coagulation diagnostics and monitoring of therapies†

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Clinical coagulation diagnostics often requires multiple tests. Coagulation times are a first indication of an abnormal coagulation process, such as a coagulation factor deficiency. To determine the specific deficient factor, additional immuno- and/or enzyme assays are necessary. Currently, every clinical laboratory has to normalize their assays (international normalized ratio, INR), and therefore, certain variability within the clinical analytics exists. We report a novel strategy for a quick, reliable and quantitative diagnosis of blood coagulation diseases (e.g. haemophilia) and for monitoring factor replacement and anticoagulant therapies (e.g. heparin treatment). We exploit nano-oscillations of microcantilevers for real-time measurements of the evolving blood plasma clot strength (viscosity). The sensors are oscillated at multiple high resonance mode numbers, in order to minimise the oscillation amplitude (a few nanometers), to provide direct internal control and to increase the quality factor. Along with the activated thromboplastin time (aPTT) and prothrombin time (PT) other parameters important for thrombosis diagnostics can be obtained, including the final clot strength and the fibrinolysis time. We demonstrate the dependence of the parameters on factor deficiencies and we diagnose a specific factor deficiency through an integrated and quantitative in situ immunoassay. This approach does not require continuous calibration since it delivers an absolute quantity (clot strength). The low sample volume required (a few µl) and the ability to measure different parameters within the same test (PT, aPTT and global coagulation assay) make the presented technique a versatile point-of-care device for clinical coagulation diagnostics.

Introduction

Hemostasis is a physiological process that leads to sealing of a vasculature break. It must be well regulated, fast and localized. Coagulation is the result of a complex sequence of biochemical reactions, called the coagulation cascade (Fig. 1a). After the clot has been formed, coagulation must be stopped and, after healing, the clot has to be dissolved. From a clinical point of view, the coagulation cascade is divided into three pathways: intrinsic (contact activation), extrinsic and common. The final active product of the common pathway is the protein thrombin. Thrombin cleaves fibrinogen into fibrin that is further polymerized and crosslinked by factor XIII. Once the clot reaches healthy endothelial tissue, the anticoagulation cascade is triggered, coagulation is stopped and later the fibrin clot is dissolved. Any unregulated activity in this sequence of reactions would lead to a pathological disorder. Fast, accurate and reliable determination of multiple coagulation parameters is vital to a correct diagnosis of blood coagulation disorders. Two of the most common coagulation assays performed regularly in a hospital environment are the prothrombin time (PT, induced by an excess of tissue factor, extrinsic and common pathways) and the activated partial thromboplastin time (aPTT, induced by surface activation, intrinsic and common pathways). These two assays measure the time required for the onset of fibrinogen proteolysis that is followed by the formation of a fibrin network. Clot formation is usually detected by increased impedance or turbidity. Any prolongation of one of these two times corresponds to a factor deficiency or inhibition to one (or more) of the corresponding pathway’s proteins. Both assays are extremely useful but have some drawbacks and always require further testing. First of all, they are not factor specific and they are sensitive only if the factor activity is lower than 50%. Secondly, they stop at the moment of “thrombin burst” and they do not assess the polymerization rate nor the factor XIII crosslinking activity and subsequent fibrinolysis. Thirdly, the initial blood plasma viscosity, the clot strength and its ability to hold blood flow pressure are not measured. Lastly, both assays do not evaluate bleeding or thrombosis risk and do not monitor acute bleeding. These drawbacks demand for the development of novel strategies that can improve the clinical diagnosis process. These so-called global hemostasis assays have gained attention in recent years. Global hemostasis
aims to measure the viscoelastic properties of the blood clot in order to assess the global clot strength once it is formed. Two of the most adopted global assays are thromboelastography (TEG)\(^3\) and rotational thromboelastography (ROTEM).\(^4,5\) The goal of these assays is to analyse in real time the process of clot formation, clot stability,\(^6\) and clot resolution (fibrinolysis).\(^6-10\) Other global assays are thrombin generation (TG)\(^11\) and overall hemostasis potential (OHP).\(^12\) All of these assays suffer from a wide variation in results, they are not factor specific, and the output values are not absolute, but related to the particular technique employed. Furthermore, they usually require a large sample volume (300 µl to millilitres). Novel approaches that exploit micro- and nanotechnology have been developed in recent years, such as quartz crystal microbalance (QCM)\(^13,14\) and surface plasmon resonance (SPR).\(^15\) These strategies greatly reduce the amount of the sample needed. However, with QCM it is not possible to distinguish between protein adsorption and liquid viscoelasticity changes, reducing the potential to determine the overall clot strength. SPR on the other hand does not suffer from this drawback but the measured variables are not absolute and only the PT was previously measured.\(^16\) We previously reported a novel strategy to measure aPTT exploiting microcantilever-based resonators.\(^17\) Here we show the ability of this strategy to diagnose different blood coagulation disorders, using a smaller amount of the sample (a few microlitres). We present a new mathematical model to extrapolate PT and aPTT along with global hemostasis parameters (blood plasma viscosity, clot formation, stability and dissolution) and factor specificity in one single assay. The measured global variable is the viscosity of human blood plasma changing during coagulation and clot dissolution. As the viscosity is measured in centipoise (1 cP = 1 mPa s), the presented assay has high potential for standardization. Our approach employs the evaluation of the nanomechanical response of suspended micro-resonators (immersed in fluid) whose oscillation frequency and quality factor are dependent on the density and the viscosity of the liquid surrounding the structure.\(^18\) In recent years, micro-resonators have been successfully used as biosensors to detect biomarkers,\(^19,20\) to monitor single microbial cell growth,\(^21\) to characterize sperm motility,\(^22\) to study the thermodynamics of biomolecule surface transformations\(^23,24\) and to measure the viscosity of small sample volumes\(^25-27\) (in the order of a few µl). However, measurements of biologically relevant liquids that contain proteins require special care. Proteins tend to stick and unspecifically adsorb onto solid surfaces and they might undergo conformational changes and subsequent loss of activity.\(^28\) In our recent work,\(^17\) we optimised the resonating micromechanical sensor surfaces exposed to human blood plasma to make them biocompatible and to minimise unspecific protein adsorption. We showed that tracking of multiple modes at high resonant frequencies (hundreds of kHz) increased the quality factor and minimised the total acoustic energy released in the surrounding fluid. The oscillation amplitude at high mode numbers is in fact in the order of a few nanometres (see the ESI of ref. 17). The tracking of multiple modes also provides an internal control, because tracking more than one peak allows for enhanced evaluation when the peaks shift synchronously.\(^29\) Here we report the capability of the assay to measure specific factor deficiencies and their effects on clot strength. Furthermore, we monitor the coagulation development over time in different blood related disorders. The clot stability, clot dissolution (fibrinolysis) and the effects of anticoagulant drugs on the clot formation process are analysed as well. All these parameters will expand and improve clinical coagulation diagnostics and would enable the monitoring of therapies for coagulation disorders. The small size of the microfluidic
chamber (4 µl) allows for further miniaturisation and enables point-of-care testing.

**Experimental section**

**Materials**

The hetero-bifunctional compound (11-mercaptoundecyl)tetra(ethylene glycol) for a hydrophilic, neutral terminus (PEG) surface functionalization, 1,4-dioxane (99.8%), ethanolamine (≥98%), thrombin, human serum albumin (HSA), fibrinogen (from human plasma, lyophilized powder), and tissue plasminogen activator (tPA) were purchased from Sigma Aldrich, Ireland. Dithiobis(succinimidyl undecanoate) (DSU) was purchased from Dojindo Molecular Technologies, Japan. Pepsin/hydrochloric acid for the removal of proteins was purchased from VWR Ireland. Fibrinogen was reconstituted in 0.9% NaCl solution at 250 IU ml\(^{-1}\) and diluted down to the required concentration when needed. Thrombin was reconstituted in 0.1% HSA at 350 NIH\(^{10}\) unit per ml and then diluted down to 70 NIH unit per ml prior to the experiment. Tissue plasminogen activator was reconstituted in nanopure water at 1 mg ml\(^{-1}\) and diluted down to 350 ng ml\(^{-1}\) when utilized. HemoSIL® human blood plasma control, factor VIII and IX deficient plasma, aPTT kits, and PT reagents (RecombiPlasTin 2G) were all purchased from Brennan&Co, Ireland. Factor XIII deficient plasma was purchased from Quadracht Diagnostics, United Kingdom, and factor XIII concentrate (fibrogammin, CSL Behring) from Allphar Ltd, Ireland. Plasma controls were reconstituted in 1 ml of nanopure water, prior to the experiment. PT and aPTT kits were ready to use. Fibrogammin was reconstituted in water or 0.9% NaCl solution at 250 IU ml\(^{-1}\) and diluted down to 2 IU ml\(^{-1}\). One millilitre of the resulting solution was used to reconstitute FXIII deficient plasma or 2.5 mg of fibrinogen. Antibodies (GMA-012 anti-factor VIII and 13F42-F6 anti-factor IX) were purchased at a concentration of 6.7 µM (IgG1, 150 kDa, 1 mg ml\(^{-1}\)) from Abcam, United Kingdom.

**Experimental device**

The experimental device has been described fully in our previous work (see ref. 17 and 31). Arrays of 8 microcantilevers (IBM Zurich Research Laboratory, Switzerland) with a length, width and thickness of 500 µm, 100 µm and 1 µm, respectively, were cleaned, coated and functionalized with two different functionalization procedures. For viscosity measurements without antibodies, we minimized protein adsorption with a PEG terminated monolayer using a recently reported protocol.\(^{17}\) For the immobilization of antibodies onto gold surfaces, we used the N-hydroxysuccinimide (NHS) ester linker method.\(^{32-34}\) First, the array was immersed in 1 mM dithiobis(1-succinimidyl undecanoate) (DSU) in 1,4-dioxane for 30 minutes. The chip was then thoroughly rinsed first with 1,4-dioxane, secondly with acetone and then with 20 mM HEPES buffer at pH 6.8. This step creates a NHS-terminated SAM that covalently binds to a primary amine group of the antibodies.\(^{34}\) The microcantilevers were then functionalized selectively by immersing in 6.7 µM solutions of two different antibodies for 1 hour in individual micro-capillaries.\(^{32}\) GMA-012 anti-factor VIII antibody solution contains 1% mannitol, 0.15 M NaCl and 10 mM sodium phosphate at pH 7.4, while 13F42-F6 anti-factor IX antibody contains 0.7% sodium phosphate, 0.03% EDTA and 0.58% sodium chloride at pH 6.6.

**Results and discussion**

**Determination of coagulation parameters**

Accurate and repeatable evaluation of coagulation times such as PT and aPTT is crucial in blood tests in clinical environments. To achieve these requirements, we exploited suspended microresonators that oscillate at high speeds (Fig. 1b). The damping (measured by the quality factor) and the speed (resonance frequency) of the oscillations are correlated with the viscosity and density of the liquid surrounding the structure.\(^{17}\) When coagulation is triggered, the viscosity increases due to the formation of the fibrin network (see Fig. 1a). The coagulation assays PT and aPTT measure the time required for the onset of fibrinogen proteolysis associated with a sudden viscosity increase (fibrin polymer network). We propose a mathematical model that can describe the clot strength development over time. The equation that describes the model is the following:

$$\eta(t) = A + \frac{B}{1 + e^{(t-t_0)}} + c e^{(t-t_1)}$$

where \(\eta(t)\) is the change in the plasma viscosity during coagulation and \(A, B, t_0, t_1, k, c\) are fitting parameters. This model includes a baseline that corresponds to the plasma viscosity (parameter A, Fig. 1c), the final clot strength (given by \(A + B, \) Fig. 1c), two slopes that correspond to the initial and final coagulation rates (parameters \(k\) and \(c\), Fig. 1c) and two coagulation times (parameters \(t_0\) and \(t_1\)). Note that eqn (1) represents a new and more powerful method than the one presented in our previous work.\(^{17}\) Our previous method was based on a simple linear fit of the sudden viscosity change.
occurring straight after reagent injection. Therefore, it relies on the assumption that the viscosity changes linearly over time. While the assumption proved to be acceptable, we believe that an improved description of the overall process was required. The newly presented mathematical model (eqn (1)) describes the whole coagulation process, from the initial plasma viscosity to the final clot strength. Furthermore, it does not extrapolate only the coagulation time, but also five other clinically relevant parameters (A, B, k, c and t0). Finally, it suffers from less uncertainty in the determination of the sudden viscosity change starting point. The viscosity change shows three different stages: (a) a slow start, (b) a sudden increase and (c) a clot strength change rate decreasing before reaching a stable viscosity value. While the previous model described only stage b, the new model contains a mathematical description for all these stages, increasing the accuracy in the determination of the coagulation times. In order to prove the validity of this model, we tested three different plasma samples that have been designed to have a specific PT or aPTT when tested with commercially available devices (see Table S1†) called normal control, low abnormal and high abnormal controls. When the model described above is fitted to the viscosity data, the parameter t0 corresponds to the aPTT (see Fig. 1c) or PT (see Fig. S1†). Note that all the parameters extracted have a clinical relevance, but they are not measured in standard PT or aPTT tests. In particular, A (plasma viscosity) could be directly correlated with hyperviscosity,15 A + B (clot strength) was found to be inversely proportional to the control abnormality (the higher the abnormality, the lower the clot strength), while for k, c and t0, a clinical connection to a disease state has to be established. All the parameters are fundamental for an accurate and adequate fitting process. As described in our previous work,17 higher viscosity values correspond to lower quality factors. Lower quality factors increase the uncertainty in the fitting process, increasing the noise around both the resonance frequency and quality factor. For normal control plasma (the highest viscosity value), the signal-to-noise ratio is lower than all other abnormalities presented (see Fig. 1 and 3). The coagulation times extracted from the viscosity curves are within the expected range (see Table S1†). The test can be repeated up to three times using the same array of sensors after a pepsin/hydrochloric acid cleaning procedure (see Fig. S2†). Note that plasma proteins lose their activity over time (plasma can be considered stable for 4 hours after reconstitution), resulting in a lower initial plasma viscosity and lower final clot strength after a few hours (see Fig. S2†).

**Fibrinogen disorders**

Two different hereditary categories of plasma fibrinogen defects exist: a quantitative fibrinogen deficiency called afibrinogenemia or hypofibrinogenemia and a qualitative fibrinogen deficiency called dysfibrinogenemia or hypodysfibrinogenemia. Bleeding severity can range from mild to severe.2 Both deficiencies may prolong the PT and aPTT, but only if the fibrinogen concentration is below 1 mg ml−1 (healthy physiological fibrinogen concentration range is 1.5–4 mg ml−1). Dysfibrinogenemia is usually asymptomatic, but it may be associated with thrombosis.36 Thrombotic events have also been reported in patients with afibrinogenemia.37,38 To evaluate the effects of fibrinogen on the final clot strength, we first tested different fibrinogen concentrations mixed with a constant concentration of thrombin solutions (70 NIH30 units per ml). Fig. 2 shows the final clot viscosity of the mixture at 37 °C in the fibrinogen concentration range 0.5–3.5 mg ml−1. Note that the viscosity of water at 37 °C is about 0.7 cP. As expected, higher fibrinogen concentrations increase the final clot strength. However, the fibrin network formed is not cross-linked due to the absence of factor XIII and even with high fibrinogen concentrations the clot strength was considerably lower than a normal plasma clot. Next, we measured how the clot strength develops over time when an aPTT reagent (see Materials and methods) is added to two different plasma samples: a low fibrinogen plasma control (fibrinogen concentration below the physiological range) and a low abnormal plasma control with the addition of 2.5 mg ml−1 of fibrinogen (Fig. 3).

In the first case, the aPTT is prolonged and the final clot strength was significantly low. In the second case, the aPTT remains comparable to a low abnormal plasma control, but the final clot strength was in the same range (∼3 cP) of a normal plasma control. This result indicates that there are still other elements that cause the prolonged aPTT (low abnormal plasma has many factor concentrations in the low abnormal range), even though the normal concentration of clottable fibrinogen has been restored. A low clot strength could indicate a fibrinogen defect. To diagnose it, the clot strength should shift to the normal range when fibrinogen is added to the sample prior to coagulation.

![Fig. 2. Thrombin and fibrinogen final clot viscosity at different fibrinogen concentrations. Physiological concentration of fibrinogen in blood plasma is 2–4 mg ml−1. Thrombin was kept constant at a concentration of 70 NIH units per ml. Viscosity is a measure of the clot strength and it is directly proportional to fibrinogen concentration. Uncertainty in the measure is in the range 0.02–0.08 cP.](image-url)
In mild haemophilia, the aPTT might be normal. To diagnose a specific factor deficiency, the first approach is normally a mixing test. The aPTT in fact should be restored to normal values when the factor deficient plasma is mixed with 50% of normal control plasma. Further testings (e.g. immunoassay) are then required to determine the specific factor deficiency and its activity. Notably, the final clot strength for the three levels of haemophilia was always significantly lower (~1.5 cP) than a normal plasma clot strength (~3.2 cP) (Fig. 4a). The aPTT was almost entirely restored with 30% factor activity (by addition of 30% normal control plasma). However, the clot strength was still low. The specific factor deficiency and its activity were addressed by functionalizing the surfaces of the resonators with different antibodies. Dithiobis(1-succinimidylundecanoeanoate) (DSU) was used as a linker between the gold surfaces of the sensors and the antibodies (see Materials and methods). To include a passive control surface, some DSU functionalized surfaces that were not functionalized with antibodies were exposed to ethanolamine. Ethanolamine passivates and quenches the DSU surface.32 Three sensors were functionalized with factor VIII antibodies (anti-FVIII), three with factor IX antibodies (anti-FIX), and two with quenched DSU (qDSU). An aPTT test with factor IX deficient plasma was then performed. The mass adsorbed onto the sensors was calculated according to ref. 29. During the first exposure to plasma, anti-FVIII surfaces adsorbed a significantly higher mass compared to both anti-FIX and qDSU surfaces (Fig. 4b). The difference between anti-FVIII and anti-FIX provides the specific factor VIII adsorption from the plasma sample. Note that factor VIII circulates in plasma bound to the Von-Willebrand factor (vWF). After triggering the coagulation, the signal-to-noise ratio (SNR) of the anti-FVIII sensors during the stable clot phase is significantly lower (higher SNR-1) than anti-FIX and qDSU sensors. This lower SNR indicates a continuous mass adsorption–desorption caused by motion-release of the high molecular weight Von-Willebrand factor. The specific factor IX deficiency was therefore directly confirmed through a differential measurement (FVIII–FIX). Note that the aPTT and the final clot strength were measured within the same experiment (Fig. 4a). The final clot strength was in a high abnormal range and the aPTT was significantly prolonged. A second possible approach to diagnose a factor deficiency is to add a specific factor to a deficient plasma and test whether the coagulation times and the final clot strength are restored or not. We tested factor XIII deficient plasma and we proved that despite a normal PT the final clot strength is significantly lower than normal control plasma (Fig. 4c). After the addition of 2 IU ml\(^{-1}\) of factor XIII (as per clinical guidelines), the final clot viscosity was restored back to the normal range. For the effects of factor XIII added to thrombin and fibrinogen solutions, see Fig. S3.†

**Thrombosis diagnostics and heparin therapy**

The duration, location and amount of clot formation at the site of injury need to be well regulated to prevent the uncontrolled growth of a hemostatic plug. Any disorder in these
regulations may lead to excessive clot formation and the creation of a thrombus (thrombosis). Fibrinolysis is an important part of the anticoagulation process that in conjunction with anticoagulant proteins leads to clot dissolution. An impaired function of the fibrinolytic system increases the risk of thrombosis.43 Unfortunately, there is still a need for a robust global coagulation assay that can assess the fibrinolytic state.2 One approach that has been recently studied9,10,44 is thromboelastography (TEG), where the clot strength is measured during clot formation and lysis. However, TEG does not provide absolute values (critical standardization) and does not allow to retain PT and aPTT information. Here we propose the use of microresonators to monitor clot formation triggered by PT reagents (tissue factor) and the simultaneous tissue plasminogen activator (tPA) naturally induced fibrinolysis (Fig. 5a). When plasminogen is activated to its active form called plasmin, it cleaves the fibrin network leading to the dissolution of the clot. The parameters that were extracted from these tests are: PT, starting clot strength (given by $C + B$, Fig. 5a), 50% lysis time (given by the time required to reach

![Diagram](image-url)
half the clot strength, Fig. 5a), and the final dissolved clot strength (parameter C, Fig. 5a). After triggering the coagulation, the clot strength was not stable due to simultaneous plasmin activity. The final dissolved clot strength was lower than the initial plasma viscosity. This is caused by the material breakage (plasmin activity) into soft fibrin particles that have no viscosity. Note that all the clot strengths are given in centipoise, which is an absolute unit for viscosity. Thrombosis treatment is often carried out with the use of heparin. Heparin is an antithrombotic drug that binds and activates the coagulation inhibitor antithrombin. We studied the effects of heparin on the development of the clot strength during coagulation by testing different (low and high) heparin concentrations added to normal control plasma samples (Fig. 5b). Notably, heparin can delay clot formation (prolonged aPTT), but once the coagulation starts the final clot strength was in the normal range.

Conclusions

The diagnosis of blood coagulation disorders often requires multiple tests. If a patient has bleeding symptoms, the most common assays are the prothrombin time (PT) and the activated partial thromboplastin time (aPTT). They provide coagulation times such as PT and aPTT. We have demonstrated that it is possible to combine the essential PT and aPTT assays with global hemostasis assays in one single test. We have applied suspended microresonators (in a 4 µl microfluidic chamber) to greatly reduce the amount of blood needed (20 µl, fingerprick), and to achieve the required temporal and clot strength sensitivity. Our novel approach proves to be suitable for a wide range of blood related disorders. First of all, it is possible to study the initial status (plasma viscosity) and the clot strength changes during clot initiation, formation, and lysis (see Fig. 1c). Secondly, we successfully diagnosed a specific factor deficiency such as haemophilia or factor XIII deficiency. Factor XIII deficiency would present normal aPTT and PT, but it is a severe bleeding disorder. It is a fundamental factor that cross-links the fibrin network, therefore increasing the clot strength and stabilizing the hemostatic plug. Thirdly, we have studied tissue plasminogen activator (tPA) assisted fibrinolysis. Fibrinolysis is a fundamental part of the anticoagulation cascade. It prevents abnormal and uncontrolled growth of the blood clot that would eventually lead to thrombosis. Finally, we tested the effects of anticoagulant treatments, such as heparin, on the coagulation times and on the clot strength development. Note that a prolonged aPTT or PT is not uniquely an indication of a factor deficiency. The factor concentration might in fact be normal, but an inhibitor could be present. Almost no studies have provided evidence-based laboratory medicine on how to proceed with inhibitor screening studies. For instance, in haemophilia factor replacement treatment, one of the complications is the development of autoantibodies (inhibitors) that interfere with the coagulation factor. In order to detect this inhibition, we showed that a simple approach could be the addition of the specific factor to
the deficient plasma in vitro. If the coagulation time and/or the clot strength is restored, an inhibition can be excluded (see Fig. 4c). Another approach is the modification of the sensor surfaces to immobilise factor specific antibodies. The factor deficiency can then be diagnosed through differential mass adsorption that quantifies the specific antigen–antibody interaction (immunoassay). We believe that the presented technology will improve coagulation testing in a broad range of blood related disorders, from specific factor deficiency to the assessment of fibrinolysis. The small sensor size (µm size range) provides the possibility to miniaturise this device for point-of-care testing.

Conflicts of interest
There are no conflicts to declare.

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References


