Microrheological Coagulation Assay Exploiting Micromechanical Resonators

Francesco Padovani, James Duffy, and Martin Hegner* CRANN, School of Physics, Trinity College Dublin, Dublin 2, Ireland

Abstract: Rheological measurements in biological liquids yield insights into homeostasis and provide information on important molecular processes that affect fluidity. We present a fully automated cantilever-based method for highly precise and sensitive measurements of microliter sample volumes of human blood plasma coagulation (0.009 cP for viscosity range 0.5−3 cP and 0.0012 g/cm³ for density range 0.9−1.1 g/cm³). Microcantilever arrays are driven by a piezoelectric element, and resonance frequencies and quality factors of sensors that change over time are evaluated. A highly accurate approximation of the hydrodynamic function is introduced that correlates resonance frequency and quality factor of cantilever beams immersed in a fluid to the viscosity and density of that fluid. The theoretical model was validated using glycerol reference solutions. We present a surface functionalization protocol that allows minimization of unspecific protein adsorption onto cantilevers. Adsorption leads to measurement distortions and incorrect estimation of the fluid parameters (viscosity and density). Two hydrophilic terminated self-assembled monolayers (SAMs) sensor surfaces are compared to a hydrophobic terminated SAM coating. As expected, the hydrophobic modified surfaces induced the highest mass adsorption and could promote conformational changes of the proteins and subsequent abnormal biological activity. Finally, the activated partial thromboplastin time (aPTT) coagulation assay was performed, and the viscosity, density, and coagulation rate of human blood plasma were measured along with the standard coagulation time. The method could extend and improve current coagulation testing.

Viscosity and density measurements of biological fluids such as whole blood and blood plasma provide important insights on biological processes that regulate health and disease. In particular during blood coagulation the viscosity of plasma increases due to fibrinogen polymerization. Blood coagulation is the result of a complex series of biological reactions that leads to blood clot formation. The coagulation process is unique, but there are two distinctions: Hemostasis and Thrombosis. Hemostasis is coagulation that occurs in a physiological setting and results in sealing a break in the circulatory system. Thrombosis is coagulation occurring in a pathological context that leads to localized intravascular clotting and potentially to an occlusion of a vessel or embolus formation. Central to the coagulation cascade is the enzyme thrombin. Conversion of fibrinogen into fibrin by thrombin creates a filamentous protein network. Two commonly used tests to evaluate coagulation disorders are the aPTT and the prothrombin time (PT). The PT test and the aPTT test evaluate the extrinsic and intrinsic pathways of the coagulation cascade, respectively. In clinical settings either mechanical or optical systems measure international normalized ratio (INR) levels, which are the standard unit for reporting the end point of clotting time. In both clinical tests the measured variable is blood clotting time; thus, blood clot physical properties such as density and viscosity are not measured.

There are different techniques7,8 to measure viscosity and density of a solution, but they require milliliter samples. Many of these techniques are time-consuming and not suitable for medical environments. Other surface analytical assays have been used to measure coagulation parameters such as quartz crystal microbalance (QCM),10 surface plasmon resonance (SPR),11 and bulk acoustic wave sensors.12 Mostly these methods are single-sensor techniques and do not separate the effects of the mass adsorption onto the sensor from solution bulk viscosity and density. Recent work by Cakmak et al.13 shows a microelectromechanical system (MEMS) technique for measurements of aPTT and PT. The method presented did not evaluate physical properties of the blood clot and did not include a functionalization procedure that can minimize unspecific protein adsorption onto the measurement sensors. We believe that there is a demand for assays that can reliably track viscosity and density changes during plasma coagulation in addition to the coagulation time. This information could improve coagulation tests that are conducted presurgery and for anticoagulant therapy by providing absolute physical parameters to the user. Abnormal blood viscosity has been reported to...

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be involved in long-standing retinal vein occlusion, cerebral thromboembolic events, and ischemic heart disease. Despite this evidence, blood viscosity is a parameter that is not commonly evaluated. Currently each lab has to run its own standardization, and therefore certain variability within the clinical analytics exists.

Cantilever sensors in dynamic mode proved to be an excellent candidate for rheological measurements of liquids using small volumes. We use an array of 8 microcantilevers to measure viscosity and density changes in real time during human blood plasma coagulation. This method also provides the aPTT measurement. An approximation to the exact analytical solution of the hydrodynamic load is presented, and the measurement device has been calibrated using glycerol reference solutions. Utilizing the availability of in situ differential analysis, we have established a functionalization procedure for the sensor surfaces that minimizes the mass adsorption that is inevitable when the sensor is in contact with blood plasma proteins. Mass adsorption onto the hydrophobic surfaces induces conformational changes of proteins, which result in subsequent abnormal activity measurements. It also leads to erroneous calculations of the physical properties of the liquid surrounding the vibrating structure. Finally, we evaluated the performance of the platform using a standardized aPTT test. Along with the coagulation time, three additional parameters could be extracted: density, viscosity, and coagulation rate. The presented assay allows coagulation tests following clinical guidelines and the measured aPTT (~47 s) was within the clinical range. The volume achieved for the tests was 20 μL. The measurement chamber has a volume of 4 μL; therefore, the required total volume of plasma could be further reduced with microfluidic optimization.

MATERIALS AND METHODS

Unless otherwise noted, chemicals were purchased from Sigma-Aldrich, Ireland. Glycerol solutions ranging between 5% and 40% have been prepared by mixing glycerol (≥99%) with nanopure water. Three heterobifunctional organochemical compounds that form SAMs on gold surfaces have been used in the experiments: 1-octadecanethiol for monolayers exposing a hydrophobic (CH₃)₉ terminus; (11-mercaptopoundecyl)tetra-(ethylene glycol) for a hydrophilic, neutral terminus (PEG); and 11-mercaptop-1-undecanol for a hydrophilic, neutral terminus (OH). Human serum albumin (HSA, lyophilized powder) was reconstituted in 1 mM HEPES (≥99.5%) buffer at pH 6.8. Thrombin (from human plasma, lyophilized powder) was reconstituted in 0.1% HSA solution to a concentration of 350 NIH units/mL and diluted down to 70 NIH units/mL when needed. In all of the experiments, thrombin concentration has been kept constant at a physiological concentration of 70 NIH units/mL. Fibrinogen (lyophilized powder, from human plasma) was reconstituted with 0.9% NaCl in 1 mM HEPES buffer, pH 6.8, at a final concentration of 10 mg/mL and diluted down to 2.5 mg/mL when needed. Human blood plasma controls and aPTT kits (HemoSIL Normal Control Assayed and HemoSIL aPTT-SP liquid, Instrumentation Laboratory, U.S.A.) were purchased from Brennan & Co, Dublin, Ireland. Human blood plasma was reconstituted in 1 mL of nanopure water and gently swirled for 2 min to ensure full reconstitution. The aPTT kit contains a 25 mM calcium chloride (CaCl₂) solution and a colloidal silica dispersion with synthetic phospholipids, buffer, and preservatives (ready to use).

Experimental Setup. For details about the fully automated experimental setup, we refer the reader to ref 29. Briefly, an array of 8 cantilevers is mounted into a 4 μL measurement chamber that allows liquid exchange through 4 syringe pumps and a microdispensing valve. The cantilevers were oscillated by a piezoelectric stack mounted underneath the array, and their vibration is recorded using an optical beam detection system. The amplitude and phase spectra of the oscillations were evaluated from the recorded data using a custom LabVIEW software interface. Upgrades on the previously presented device include the following: (1) digitizer with higher resolution (National Instruments PCI-S105, 60 MS/s, 12-bit) to better resolve individual voltage levels of the position sensitive detector (PSD) response, (2) expanded and improved temperature-controlled enclosure, and (3) four remotely controlled syringe pumps housed inside the temperature-controlled enclosure. The normalized differential signal of the PSD is acquired at 10 MS/s, and each driving signal frequency is applied for 1 ms. For detailed information on resonance frequency and quality factor evaluation, see Supporting Information.

Theoretical Background. To evaluate both density and viscosity of the liquid surrounding the vibrating cantilever, both quality factor and resonance frequency were experimentally evaluated. The resonance frequency \( f_{\text{res}} \), and the quality factor \( Q_\alpha \), (at mode number \( n \)) are correlated to the hydrodynamic load as:

\[
f_{\text{res}} = C_{\text{cal}} f_0 \sqrt{\frac{k}{m_l + \Gamma_1^f(Re, \kappa_c)m_l}}
\]

\[
Q_\alpha = C_{\text{cal}}, Q \sqrt{\frac{m_l + \Gamma_1^f(Re, \kappa_c)m_l}{\Gamma_1^f(Re, \kappa_c)m_l}}
\]

where \( \beta_s = C_s^2/2\pi\sqrt{3} \) with \( C_s = 1.875, 4.694, 7.854, 11, ... \), \( k = 3EI/l^3 \) is the cantilever spring constant, which is calculated from the Young’s modulus \( E \) and the length \( l \) of the cantilever; while \( I = bh^3/12 \) is the moment of inertia of a rectangular beam with \( b \) and \( h \), respectively, being the width and thickness of the cantilever beam. The parameters \( m_l \) and \( m_l \) are, respectively, the mass of the cantilever and a “virtual mass” of inviscid liquid comoved by the cantilever when \( \kappa_c = 0.24 \). Both masses are evaluated from the density of the cantilever \( \rho_c \) and the density of the liquid \( \rho_l = \rho_b \rho_i \) and \( m_l = \pi \rho c b^2 l^4/4 \).

The Reynold’s number \( Re = 2\pi \rho c b^2 l^3/\eta \) expresses the importance of inertial forces in the liquid relative to viscous forces (\( \eta \) is the viscosity of the liquid). The calibration factors \( C_{\text{cal}} \) and \( C_{\text{cal}}, Q \) are introduced to account for internal losses in the cantilever structure and uncertainties in the spring constant of the cantilever. These factors are determined through a calibration step where a liquid with known viscosity and density is introduced into the measurement chamber. In all the experiments presented, we indicate the specific density and viscosity for the calibration step. The hydrodynamic load is expressed through the hydrodynamic function \( \Gamma_{\text{f}}(Re, \kappa_c) = \Gamma_1^f(Re, \kappa_c) + i \Gamma_1^f(Re, \kappa_c) \) where the superscript \( f \) refers to the flexural mode while \( r \) and \( i \) refer to real and imaginary parts, respectively (\( i = \sqrt{-1} \)). The hydrodynamic function depends on the Reynold’s number and on the normalized mode number \( \kappa_c = C_b b/l \). We introduce an approximation to the analytical solution of the hydrodynamic function that is valid over a
Figure 1. Hydrodynamic function plot for different Reynold’s numbers and different normalized mode numbers \( \kappa \). Plot (a) is for the real part and (b) is for the imaginary part. For the imaginary part, only 3 normalized mode numbers are plotted to enhance visibility. The real part is shown for all \( \kappa \) values. The exact analytical solution of the hydrodynamic function was computed numerically using Mathematica 10, and the values obtained converged at the sixth digit. Convergence of the solution requires a square matrix size of \( M = 52 \), as discussed in ref 33. Substituting eqs 3 and 4 into eqs 1 and 2, we obtain a dependency of the resonance frequency \( f_{RR} \) and the quality factor \( Q_n \) on the Reynold’s number. Expressing the Reynold’s number as a function of the liquid’s density \( \rho_l \) and viscosity \( \eta_l \), we numerically computed both liquid parameters.

**Cantilever Preparation.** We used arrays consisting of 8 microcantilevers (IBM Zurich Research Laboratory, Switzerland) with length, width, and thickness of 500, 100, and 1 \( \mu m \), respectively. They were cleaned with subsequent immersions in three different solvents: (1) 10 min acetone (CHROMASOLV), (2) 5 min ethanol (CHROMASOLV), (3) 1 min nanopure water, and (4) 5 min acetone. The solvent cleaning was followed by plasma cleaning (Diener Electronic GmbH PICO Barrel Asher, 0.3 mbar oxygen (O₂), 160 W, 40 kHz for 3 min) and a final immersion in ethanol. The arrays were then coated with 3 nm of titanium (deposition rate 0.2 Å/s) on both sides and with 23 and 33 nm of gold (deposition rate 0.5 Å/s) on the top and bottom sides, respectively, using electron beam evaporator (Temescal FC-2000, U.S.A.). After metal coating, the arrays were stored under argon to prevent chemical degradation of the gold layers. Before use the array was cleaned again with the above solvent cleaning sequence followed by a UV cleaning step to activate the gold layers, and it was then functionalized with 1 mM solutions in ethanol of different SAMs for 60 min using a capillary technique. The above concentration and incubation time provide optimal coverage and ensure good quality of the SAM. Finally the array is rinsed with ethanol and stored in 1 mM HEPES buffer until use.

**Glycerol Solution Experiments.** Reference solutions were prepared by mixing different concentrations of glycerol (v/v) with nanopure water. The concentration range was 5–40%, which corresponds to a viscosity range of 1–3 cP and a density range of 1–1.1 g/cm³. Table S2 (Supporting Information) reports the expected viscosities and densities of solutions with different concentrations of glycerol. These ranges cover the viscosity and density range of many biological fluids, including human blood plasma. The temperature for these experiments was kept constant at 23 \( \pm 0.01 \) °C. The array was first immersed in water (viscosity 0.9532 cP, density 0.9977 g/cm³), and then the data acquisition began. Then 500 \( \mu L \) of reference solutions were flushed automatically through the chamber at 100 \( \mu L/min \). The flow was then stopped, and for each solution \( \sim 100 \) spectra (amplitude and phase) were acquired per cantilever in 20 min. The average resonance frequency and quality factor were then used for the computation of the viscosity and density of each solution.

**SAMs Performance Experiments.** The array of 8 sensors was selectively functionalized; three cantilevers were functionalized with OH-terminated SAM, three with PEG-terminated SAM, and two with CH₃-terminated SAM. PEG and OH SAMs have been reported to resist protein adsorption, while CH₃ provides a hydrophobic surface that increases adsorption of most of the proteins. A selective functionalization allows the evaluation of different SAMs’ resistance to protein adsorption using the same array. The array was stabilized at 37 °C in water (viscosity 0.6913 cP, density 0.9933 g/cm³; see ref 41) for 10 min; then 100 \( \mu L \) of 1 mM HEPES buffer (pH 6.8) was flushed at 10 \( \mu L/min \) and left in the chamber for 15 min. Note that these experiments were performed at higher
temperature than previous experiments; therefore, the viscosity and density of water is lower. Subsequently, 500 μL of fibrinogen was flushed in at 50 μL/min, and finally, after another 10 min, 25 μL of fibrinogen and thrombin solution was injected at 250 μL/min. The relatively high injection speed is required for a fast exchange (6 s) of the liquid; otherwise, the reaction between thrombin and fibrinogen would occur before entering the measurement chamber. The mixing occurred automatically for 10 μL before the chamber, and this volume has to be pushed 15 μL further to enter the chamber (see design in ref 29). To ensure optimal mixing of the solution, different volumes and injection speeds were tested with two different colored solutions, and the final color (a mix of the two) was recorded and analyzed with image-analysis software (see video in ref 29). During the whole experiment, amplitude and phase spectra were recorded every ∼20 s.

Human Blood Plasma Coagulation Experiments. To study the coagulation of human blood plasma, we followed the standard aPTT clinical protocol. In an aPTT test citric acid is added to the blood plasma sample to prevent spontaneous coagulation. The lyophilized human blood plasma already contains citric acid. Reconstitution is ensured by gentle swirling for 2 min after addition of 1 mL of nanopure water. Next a 25 mM calcium chloride solution (CaCl₂) is added to the blood plasma together with a phospholipid suspension that contains silica particles. The excess of calcium is required to counteract the anticoagulant activity of citric acid, while the silica (negatively charged surface) acts as an activator, inducing contact activation.42 To perform the procedure described above, 100 μL of human blood was stabilized in 1 mM HEPES buffer and automated the same aPTT protocol. The cantilever array approach is presented. The method enables small volumes and injection speeds to be tested with two different SAMs surfaces.

RESULTS AND DISCUSSION
A microrheological measurement of the viscosity and density of human blood plasma during coagulation using a microcantilever array approach is presented. The method enables small volumes (20 μL) to be used and achieves high sensitivity (0.009 cP for the viscosity and 0.0012 g/cm³ for the density, 3 sigmas) along with a full characterization of biologically relevant fluids as long as they behave as Newtonian fluids. Three different types of experiments are presented: (1) the validation of the theoretical model using solutions with known density and viscosity, (2) fibrinogen polymerization triggered by thrombin, and (3) clinical aPTT coagulation test.

Theoretical Model Validation Using Glycerol Reference Solutions. To test the validity of the theoretical model for the calculation of density and viscosity of a liquid, we used solutions with different concentrations of glycerol. Figure 2 shows a comparison between the expected values and the measured values of both density and viscosity. The accuracy of the measurement is very much dependent on the quality factor. The SHO model is valid for Qn ≫ 1.24 Liquid viscosity is directly proportional to dissipative effects. With increasing dissipative effects (hydrodynamic function imaginary part), the quality factor decreases. Deviations from reference values are in the range 0.2–11% for the viscosity and 0.1–2.2% for the density. To take into account these deviations that depend on the quality factor, we have used the results (Figure 2) as a calibration curve for the measurements involving fibrinogen polymerization and blood plasma coagulation. Other work evaluated the rheology of liquids21,22 using cantilevers vibrating at the first two oscillation modes. Such an approach can be greatly enhanced when measuring at higher oscillation modes.19,24 Driving beams clamped at one end directly with a piezo electric element underneath the array at higher modes lead to an increase in the quality factor from ∼2 at first mode to ∼25 at the 10th mode. While increasing the percentage of glycerol from 0% to 40% (with steps; see Figure 2), the quality factor drops accordingly from the highest value of 24.26 (0%) to the lowest value of 13.5 (40% glycerol). The measured values of quality factors still surpass the previously reported values31 by 14-fold.

Fibrinogen Polymerization: Protein Adsorption on Different SAMs Surfaces. Fibrinogen polymerization is triggered by the protein thrombin. When thrombin and fibrinogen are mixed, the latter is cleaved into fibrin, which creates a polymer-like structure, and the liquid viscosity increases. Cantilever-based measurements require the sensors to be fully immersed in the solution. Fibrinogen can be spontaneously adsorbed onto gold surfaces43,44 increasing the
cantilever’s overall mass. Unspecific mass adsorption should be minimized; otherwise, the convolution of the individual physical properties (mass increase versus bulk fluid property changes) would lead to an incorrect estimation of the viscosity and density properties. Furthermore, for subsequent testing of biological fluids, it is necessary that the sensor is rendered biocompatible. The performances of three different SAMs and nonfunctionalized gold-coated surfaces (called simply gold) have been tested in terms of relative protein adsorption. We have investigated two SAMs that prevent protein adsorption (PEG- and OH-terminated SAMs) and a hydrophobic terminated SAM (CH$_3$). Figure 3 shows resonance frequency shift, quality factor, and viscosity and density change (after calibration) for the three different SAMs and gold during fibrinogen polymerization. After initial stabilization in water, 1 mM HEPES buffer is flushed through the chamber followed by an injection of fibrinogen (2.5 mg/mL). Due to the different densities of buffer and the fibrinogen solution compared to water, the resonance frequency decreases while the quality factor remains constant. The buffer used for fibrinogen polymerization and human blood plasma coagulation experiments was always 1 mM HEPES buffer at pH 6.8. We have measured the density and viscosity of the buffer, and we have used it as calibration solution in the following experiments for the calculation of the calibration parameters $C_{cal}$ and $C_{cal,Q}$ (see eqs 1 and 2). The calculated density and viscosity of 1 mM HEPES buffer at 37 °C were 0.9949 ± 0.0004 g/cm$^3$ and 0.692 ± 0.003 cP, respectively. After another 10 min, thrombin (70 NIH units/mL) and fibrinogen (2.5 mg/mL) 1:1 mixture was injected. This requires 6 s; thus, it is too short to be visible on the plot. Resonance frequencies and quality factors during the reaction between thrombin and fibrinogen were recorded for the last 10 min (green plain area). By calculating density and viscosity of the polymerized fibrin solution, we obtained different density and viscosity values between hydrophilic and hydrophobic coatings of the sensor. In particular with a hydrophobic surface, the resonance frequency and quality factor shifted to lower values and to higher values, respectively, compared to a hydrophilic surface. This further decrease must be caused by nonspecific protein adsorption on the hydrophilic surfaces that can lead to a conformational change of the proteins. If we suppose uniform mass adsorption on both sides of the cantilever, the adsorbed mass can be evaluated as an increase of the cantilever’s overall mass. Uniform mass adsorption results in an increase of the quality factor (see eq 2, where $m_i$ becomes $m_i + \Delta m$ after mass adsorption) while the resonance frequency decreases. Resonance frequency also decreases if the mass is not uniformly distributed and concentrated in particular locations. In Figure 3 after thrombin injection the resonance frequency for OH-coated sensors shifted to lower values while PEG-coated sensors while the quality factor remained in the same range (~24). This resonance frequency difference indicates a mass adsorption onto the OH-coated sensors while a similar quality factor indicates that this mass adsorption is not uniform. Nonuniform mass adsorption suggests that clusters of fibrin are adsorbed onto the OH-coated surfaces. These clusters are far enough apart that they do not interact with each other to form a uniform mass. The gold-coated, nonfunctionalized surfaces present higher uniform mass adsorption of fibrinogen than hydrophobic surfaces. Adsorbed fibrinogen might undergo a conformational change, and subsequent abnormal activity was measured by a sudden decrease in both quality factor and resonance frequency when
no other solutions were injected (see minute 38 in Figure 3). A simultaneous change of both resonance frequency and quality factor indicates that the firmly adsorbed fibrinogen attracts other molecules from the surrounding solution. These molecules are loosely bound to the surface and to each other, causing a local, abnormal increase in viscosity. When the conversion of fibrinogen into fibrin was triggered by thrombin, further decreases in both resonance frequency and quality factor were measured. The formed clot showed stability for the next 10 min. The stability of the fibrin mesh suggests that the previously loosely bound fibrinogen did not change its conformation and maintained its normal activity. As expected, the gold-coated, nonfunctionalized surfaces trigger an irreversible protein mass adsorption compared to hydrophilic, protein-resistant surfaces.

Figure 4 shows the difference in mass adsorption between the hydrophobic and the hydrophilic surfaces. The final mass of polymerized fibrin adsorbed onto the hydrophobic surfaces is on the order of ~20 ng, which corresponds to a layer with thickness of ~160 nm (assuming protein density to be 1.22 g/cm\(^3\); see ref 49). To correctly calculate density and viscosity of a solution that contains proteins, a hydrophobic coating and nonfunctionalized, gold-coated surfaces have to be avoided. The performances of PEG- and OH-terminated SAMs compared to CH\(_3\)-terminated SAM were tested further by subsequent injections of fibrinogen and thrombin mixtures. While on the PEG- and OH-coated surfaces no further quality factor and resonance frequency decrease were observable, the CH\(_3\)-coated sensors showed a continuous shift, indicating further unspecific mass adsorption (data not shown). Surfaces that resist protein adsorption have to present the following characteristics: (a) hydrophilic, (b) include hydrogen-bond acceptors, (c) neutral, and (d) do not have hydrogen-bond donors. Surfaces functionalized with PEG are therefore optimal for rheological measurements of biological liquids.

**Human Blood Plasma Coagulation.** The performance of the sensor array device as described above has been compared to one of the most commonly used coagulation assay in clinical laboratories, the aPTT test. The measured variable in a standard aPTT test is coagulation time, and no other information is evaluated. Currently there are different methods used to measure the aPTT time, and they include the following: (a) amperometric (electrochemical) measurement, (b) optical detection of mechanical motion, and (c) optical readout of light scattering. The volumes of plasma required vary depending on whether the instrument is portable or not. In these methods the minimum volume required is ~20 μL (portable, fingerstick) and ~1 mL (nonportable). By measuring resonance frequencies and quality factors of cantilevers (PEG-coated) changing over time, we can determine not only the aPTT time but also the instantaneous coagulation rate plus the density and viscosity of the final clot, therefore providing additional coagulation parameters. Figure 5 shows (a) frequency and quality factor change and (b) the calculated density and viscosity change during blood plasma coagulation. The resonance frequency and the quality factor shift to lower values when human blood plasma is injected into the chamber because plasma is both denser and more viscous than buffer. After oscillating the sensors for 10 min in plasma, all the reagents required for the aPTT test that trigger the coagulation cascade are injected into the chamber within 6 s, and a further decrease of both resonance frequency and quality factor is detected. Note that the given few nanometer displacements of the microcantilever, the total energy released into the surrounding liquid is negligible. Details about calculation of the displacements and comparison of energy levels with other clot-disrupting techniques are provided in the Supporting Information. As mentioned above from these clinical assays, it is possible to extract more parameters than aPTT testing such as (1) the viscosity and the density of both blood plasma and final clot, (2) the aPTT time, and (3) the coagulation rate (measured in cP/s). These parameters provide important information about the overall process of the blood plasma coagulation, and they can be used to assess whether it is occurring normally or abnormally. At present each clinical laboratory has to determine and normalize the performances of their devices using an internal control (normal coagulation) in order to evaluate the INR. The INR is then used as a target to reach when the patient is undergoing anticoagulation therapy. By providing absolute values (density, viscosity, and rate of coagulation), there is no need for an internal control and the anticoagulant therapy can be further tuned toward personalized diagnostics. Other work has extensively reviewed self-monitoring as an effective option for anticoagulation tests, and it has been concluded that self-monitoring is feasible only for a portion of patients. We believe that this portion could be increased by measuring absolute values along with the usual PT, aPTT, and INR. Figure 6 shows detailed analysis of the parameters that can be extrapolated from measuring the viscosity change during coagulation. The measured viscosity of plasma is 0.967 ± 0.003 cP (physiological range). When the coagulation cascade is triggered, viscosity starts to increase at a rate of 0.015 cP/s and then stabilizes at 1.832 ± 0.003 cP. The aPTT time is calculated at 47.2 s, which is in the typical...
The analysis has been performed on the viscosity values because they are more representative of the coagulation process.

**CONCLUSIONS**

We introduced a method that exploits vibrating microcantilevers to measure the coagulation time of human blood plasma along with viscosity, density, and coagulation rate. These parameters are monitored in real time, with high precision, and in small volumes. The sensitivity is 0.009 cP for a viscosity range 0.5–3 cP and 0.0012 g/cm³ for density range 0.9–1.1 g/cm³. To the best of our knowledge, this is the highest viscosity sensitivity value reported with micromechanical resonators in the low-viscosity range. The device has a fluid volume chamber of 4 μL, and the volume required for complete volume exchange within the measurement chamber is 20 μL. The procedure is fully automated. Manual pipetting or dispensing of the liquids is not required, increasing repeatability and minimizing operator errors. The sensors have been functionalized with SAMs that minimize mass adsorption and subsequent protein conformational change, avoiding possible abnormal activity and providing biocompatible protein-resistant surfaces. The exact analytical solution of the hydrodynamic function provided by ref 24 has been approximated with a maximum deviation of 0.6% over a wide range of Reynold’s numbers ($10^{-4}$–$10^5$). The device has been tested and the theoretical model validated with glycerol reference solutions and then utilized to monitor human blood plasma coagulation in real time. The small volumes achieved, the speed, and the reliability of the analysis make the presented device ideal for the micro rheological measurements of coagulation parameters in diagnostics.

**ASSOCIATED CONTENT**

*Supporting Information*

The Supporting Information is available free of charge on the ACS Publications website or DOI: 10.1021/acs.analchem.6b03347.

Resonance frequencies and quality factors evaluation, shear stress calculation and comparison of energy levels between the microcantilever method and ultrasound-assisted lipoplasty (UAL) used for clot disruption; tabulated fitting parameters for the hydrodynamic approximation, viscosity, and density parameters of various glycerol solutions (PDF)
REFERENCES

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Supporting Information for:

Microrheological Coagulation Assay
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Francesco Padovani¹, James Duffy¹ and Martin Hegner¹*

¹CRANN, School of Physics, Trinity College Dublin, Dublin, Ireland.

*Corresponding Author

E-mail: martin.hegner@tcd.ie

Abstract
This Supporting Information provides details on resonance frequencies and quality factors evaluation, shear stress calculation and comparison of energy levels between the microcantilever method and Ultrasound-assisted lipoplasty (UAL) used for clot disruption. Tabulated fitting parameters for the hydrodynamic approximation, viscosity and density parameters of various glycerol solutions that are cited in the main text are provided at the end of this document.
Resonance frequency and quality factor evaluation.

According to the simple harmonic oscillator model \(^1\) the transient solution of the cantilever response diminishes exponentially with time constant \(\tau = 2Q/\omega_0\) where \(Q\) is the quality factor and \(\omega_0\) is the radial resonance frequency. In all of the following experiments the quality factor was in the range 10-30 and the resonance frequency was in the range 300-400 kHz which results in \(\tau < 0.05\) ms. This time constant is much lower than the driving time (1 ms for each frequency in the spectrum), therefore the steady state solution is reached. The amplitude and phase of the response signal were evaluated for each driven frequency. The total time required for the acquisition of a single spectrum can be adjusted by changing the number of driven frequencies. For fast tracking of the resonance frequencies and quality factors a total of 1000 frequencies were chosen, resulting in an overall driving time of 1 s for each sensor analysis. The time resolution is therefore in the order of 2-3 s, thus quick changes in viscosity and/or density can be tracked in real-time.

The recorded amplitude and phase spectra were analysed using a custom LabVIEW programme where the Simple Harmonic Oscillator (SHO) model is fitted to the data through non-linear least square fitting. The well-known SHO equations correlate amplitude and phase spectra to resonance frequency \(f_{R,n}\) and quality factor \(Q_n\) as follows:

\[
A_0(f) = \frac{A_d f_{R,n}^2}{\sqrt{(f_{R,n}^2 - f^2)^2 + (\frac{f_{R,n} f}{Q_n})^2}} + A_{bl}
\]

\[
\Delta \phi(f) = \tan^{-1} \left( \frac{f_{R,n} f}{Q_n (f_{R,n}^2 - f^2)} \right) + \phi_{bl}
\]

where \(A_d\) and \(f\) are the amplitude and frequency of the driving signal respectively, \(A_0(f)\) is the amplitude spectrum of the cantilever response signal, \(\Delta \phi(f)\) is the phase spectrum of the difference between the driving signal’s phase and the cantilever response signal while \(A_{bl}\) and \(\phi_{bl}\) are the white noise floor amplitude \(^2\) and a phase constant respectively. The phase constant \(\phi_{bl}\) is introduced to consider the offset of the phase. Note that compared to previous work \(^3\) no slope correction parameters are required. These slope parameters would in fact distort the SHO equations and could lead to an incorrect quality factor evaluation. The constants \(A_{bl}\) and \(\phi_{bl}\) increase the fitting accuracy but do not affect quality factor and resonance frequency evaluation.
Shear stress on the microcantilever surface.

The microcantilever mode shape (Figure S1) for the displacement is a function \( u(x, t) \) that can be expressed using the Euler-Bernoulli beam theory as follows:

\[
\begin{align*}
    u(x, t) &= u_1 \left[ \cosh \left( \frac{C_n x}{l} \right) - \cos \left( \frac{C_n x}{l} \right) + B_1 \left( \sin \left( \frac{C_n x}{l} \right) - \sinh \left( \frac{C_n x}{l} \right) \right) \right] \\
    B_1 &= \frac{\cos(C_n) + \cosh(C_n)}{\sin(C_n) + \sinh(C_n)}
\end{align*}
\]  

(3)

where \( l \) is the length of the cantilever. The force generated by the cantilever reaches a maximum in time when \( \sin(\omega t) = 1 \). We can then calculate the acceleration \( \ddot{u}(x, t) \) in this particular \( t = t_{max} \) as:

\[
\ddot{u}(x) = u(x) \cdot \omega^2 \cdot \sin(\omega t_{max})
\]

(4)

The total force can be evaluated with the well-known equation of motion (where the damping term is included in \( F(x, t) \)):

\[
F(x, t) = k \cdot u(x, t) + m \cdot \ddot{u}(x, t)
\]

(5)

The stress (\( u \) direction, see Figure S1) on the surface of the cantilever can be related to the total force through the following relationship:

\[
\frac{dF(x, t)}{dx} \cdot \frac{1}{b} = \sigma(x, t) = \left[ k \cdot \frac{du(x)}{dx} + m \cdot \frac{du(x)}{dx} \cdot \omega^2 \cdot \sin(\omega t) \right] \cdot \frac{1}{b}
\]

(6)

where \( b \) is the width of the cantilever. We can now calculate the shear stress (component of the force that is tangent to the cantilever motion) at \( t = t_0 \) as:

\[
\tau(x) = \sigma(x) \cdot \sin \left( \tan^{-1} \left( \frac{du(x)}{dx} \right) \right)
\]

(7)

**Figure S1** – Cantilever mode shape \( u(x) \) (see equation (3)) and decomposition of the stress vector \( \sigma(x) \) into normal \( \sigma_n(x) \) and tangent \( \tau(x) \) components to the surface of the cantilever.
Equation (7) requires that $u(x)$ is fully determined, thus $u_1$ has to be measured. Figure S2 shows a schematic representation of the optical readout geometry that we used to determine $u(x)$.

![Figure S2](image)

**Figure S2** – Geometrical representation of the optical readout for the determination of $u(x)$.

The displacement $\Delta d$ of the laser spot on the PSD reflected from the cantilever surface is geometrically related to the rotation angle $\alpha$ of the tangent to the cantilever surface in the node as follows:

$$
\alpha = \frac{1}{2} \tan^{-1} \left( \frac{\Delta d}{d_{PSD}} \right)
$$

To estimate the maximum amplitude of the oscillation $u_{\text{max}}$ first we need to calculate the position $x_n$ of the node impinged by the laser beam and then from the measurement of $\alpha$ (equation (8)) we can calculate $u_{\text{max}}$ as follows:

$$
\left\{ \frac{du(x)}{dx} \right\} = \frac{C_n}{l} u_1 \left[ \sinh \left( \frac{C_n x_n}{l} \right) + \sin \left( \frac{C_n x_n}{l} \right) + B_1 \left( \cos \left( \frac{C_n x_n}{l} \right) - \cosh \left( \frac{C_n x_n}{l} \right) \right) \right]
$$

From the set of equations (9) we can calculate $u_1$ and $x_n$ and subsequently the maximum displacement $u_{\text{max}}$. Evaluation of $x_n$ requires a numerical method. Numerical values for a cantilever with $l = 500 \mu m$ are summarized in Table S1.

Maximum shear stress $\tau_{\text{max}}$ was calculated by combining equations (6), (7) and (9).

<table>
<thead>
<tr>
<th>d_{PSD} [mm]</th>
<th>$\Delta d$ [µm]</th>
<th>$x_n$ [µm]</th>
<th>$\alpha$ [deg]</th>
<th>$u_1$ [nm]</th>
<th>$u_{\text{max}}$ [nm]</th>
<th>$\tau_{\text{max}}$ [Pa]</th>
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</thead>
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<tr>
<td>50</td>
<td>19.050</td>
<td>328.95</td>
<td>$\sim 0.011$</td>
<td>$\sim 2.3$</td>
<td>$\sim 4.5$</td>
<td>0.52</td>
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</table>

**Table S1** – Numerical values obtained using equations (1) to (9).

The obtained value of 0.52 Pa is $\sim 280$-fold smaller than the critical shear stress for fibrin clots disruption\(^4\). Figure S3 shows the comparison between the measured normalized differential signal and the expected $du(x)/dx$ along the cantilever length ($x$ direction).
Figure S3 – Comparison between the measured and expected derivative of the mode shape $u(x)$ at mode 10. The experimental data points were evaluated combining equations (8) and (9). Each maxima and minima correspond to nodes in $u(x)$. Note that $x=500$ is the tip of the cantilever. To calculate $u_{mm}$ the measured value 0.00381 at the node $x_n=328.95$ was inserted into equation (S9) to obtain $u_1$. According to equation (S3) the maximum displacement is at $x=500$ and is $u_{max} = 2u_1$. These displacements are consistent with typical displacements of piezo-electric stack actuators$^5$.

Ultrasound-assisted lipoplasty versus microcantilever energies.

Previous works have reported clot disruption with ultrasound techniques$^{6-10}$. These methods exploit two different physical phenomena: cavitation and mechanical vibration of a probe. In order to compare these methods to our presented study we can assume the vibrating microcantilever as a structure that generates acoustic waves into the surrounding media. The principle is similar to ultrasound-assisted lipoplasty (UAL)$^{7,10}$. In UAL a probe is placed with the help of a catheter in the vicinity of or directly into a thrombus. The probe is then driven by an ultrasound energy source and probe vibrations eventually cause disassociation of the clot. By comparing frequencies, amplitudes and energies between the microcantilever method and UAL we can determine if the vibrating microcantilever can break the fibrin clot. The microcantilever displacement $u_{max}$ obtained with the above method is in the order of 4-5 nm (mode 10, $\sim$360 kHz). In UAL typical displacements of the probe tip are in the order of 60-100 $\mu$m, while the frequency of the vibrations is 20 kHz. These two parameters provide a quick comparison between total energy levels of the two techniques (microcantilever and UAL). The sound intensity of the acoustic waves generated by the vibrating structures is directly proportional to the square of the frequency and amplitude$^{11}$. While the
microcantilevers resonance frequency is \( \sim 18 \)-fold higher than the UAL probe operating frequency the displacements are \( \sim 16000 \)-fold smaller resulting in a total acoustic intensity almost 6 orders of magnitude smaller with the microcantilever method. Furthermore the typical driving time in a UAL is 30 ms while the microcantilevers array is driven for 1 ms.

**List of tables cited in the main text.**

**Table S2** – Fitting parameters for the approximation of the hydrodynamic function (see equations (3) and (4) in the main text).

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<th>Mode ( n ) (( b/l = 1/5 ))</th>
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<th>G</th>
<th>C</th>
<th>B</th>
<th>a(_2)</th>
<th>a(_3)</th>
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Table S3 – Density (g/cm$^3$) and viscosity (cP) of glycerol solutions at 23°C$^{12}$.

<table>
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<th>Glycerol %</th>
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References

(S1) Bhusan, B. Springer Handbook of Nanotechnology; Springer-Verlag Berlin Heidelberg, 2010.