INTRODUCTION

Coagulation monitoring plays a key role in the management of coagulopathic diseases. Traditional approaches include induction of in vitro clotting of plasma (prothrombin time, activated partial thromboplastin time, and fibrinogen), functional assays based on the use of chromogenic substrates (anti-activated factor X assays), and studies of platelet function and count. In the late 1940s, Hartert introduced thromboelastography (TEG) to provide a time-dependent assessment of whole blood coagulation. The device, which has remained almost completely unchanged since its original
introduction in 1948, measures the torsional motion of a cylindrical pin immersed in blood. The blood is contained in a cup forced to execute oscillatory rotations of approximately ±5°. As the blood clots, the rotational motion of the cup is transmitted to the pin, causing it to undergo torsional oscillations. The sensitivity of TEG to hypocoagulation and hypercoagulation defects associated with acute and chronic illness and surgery explains its longevity as a standard diagnostic tool in hospital laboratories.

TEG (or its direct competitors rotational thromboelastometry and, to a lesser extent, Sonoclot) remains the gold standard for whole blood coagulation monitoring. However, TEG suffers from certain well-documented drawbacks. Chief among these is the need for contact containment and manipulation of the blood sample. Blood contact with container walls has a strong effect on initiation of the coagulation cascade, even in the presence of calcium chloride (CaCl₂), resulting in sensitivity of coagulation tests to the types of material and surface treatment used for the containment cup. These effects also lead to widely variable clot rigidity and variations in the quality of clot adhesion to the cup walls.

Several alternative techniques to TEG have emerged. By eliminating the pin, Ungerstedt et al measured freely decaying oscillations of the sample-containing cup (free oscillation rheometry). Viola et al and Xu et al used the acoustic radiation force (ARF) to locally strain the sample contained in a cuvette, and directly measured the resulting displacement strain; such a technique imparts stress in the sample contained in a cuvette, and directly measured the rotational motion of the cup is transmitted to the pin, causing it to undergo torsional oscillations. The sensitivity of TEG to hypocoagulation and hypercoagulation defects associated with acute and chronic illness and surgery explains its longevity as a standard diagnostic tool in hospital laboratories.

Essentials
- Contact containment in thromboelastography affects blood coagulation measurements.
- Acoustic tweezing drop oscillation rheometry avoids contact containment.
- Drop oscillation rheometry compares favorably with current clinical standard.
- Drop oscillation rheometry lowers coagulation time and sample volume requirements.

Hosseinzadeh et al added an optical scattering diagnostic in order to measure the viscosity of normal and sickled blood. By optically measuring the dynamic shape oscillations of blood drops at small strain amplitudes, they employed longstanding theoretical formulations of drop oscillations to infer surface tension and viscosity.

In this article, we report the development of dynamic drop oscillation rheometry (DOR), which is capable of measuring the viscoelastic characteristics of a developing clot in plasma and whole blood. As a proof of concept, we measured the viscoelastic properties of polyacrylamide/bisacrylamide gel (as a non-biological gel) and compared the results with those obtained with a rotational cone/plate rheometer. In order to assess the sensitivity of DOR to constituents affecting coagulation, we studied the effects of the presence of fibrinogen and of platelet, calcium ion and red blood cell concentrations on blood coagulation. A comparison study was also performed by applying DOR and TEG to the same samples. The results indicate that DOR can be used to monitor whole blood coagulation in clinical settings.

2 | MATERIALS AND METHODS

2.1 | Preparation of blood samples

Discarded and deidentified citrated whole blood samples collected for specialized coagulation experiments were used for this study under a Human Study Protocol approved by the Institutional Review Board of Boston Children's Hospital, with a waiver of consent. TEG and DOR assays were performed simultaneously within 2 hours after sample collection. Citrated blood samples were incubated with CaCl₂ (0.02 mol/L; ratio identical to TEG 340/20) at 37°C for 2 minutes prior to the assay. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained by centrifuging whole blood at 175 × g and 1575 × g, respectively.

Polyacrylamide/bisacrylamide (2% v/v) gel was prepared by adding 1.25 mL of 40% acrylamide solution (BioRad) to 32 µL of 2% Bis solution (BioRad). The polymerization was initiated by adding and gently mixing 100 µL of ammonium persulfate (Sigma-Aldrich) and 20 µL of tetramethylethylene diamine (TEMED) (Sigma-Aldrich).
2.2 | Acoustic levitation

The experiments were performed with a single-axis acoustic levitator, single-axis levitator powered by a half-wave Langevin stack actuated by piezoceramic transducers, with a nominal frequency of 29 kHz. By adjustment of the stack to its resonance, a standing wave was formed in the air gap between the stack and a flat reflector placed a full wavelength away. The levitator was placed inside a housing that provided a measure of environmental control. The housing was equipped with a thermocouple monitoring the inside temperature during the experiment. Furthermore, in order to prevent scab formation on the surfaces of levitated drops, all blood assays were performed in minimum 90% humidity. Isotonic saline (phosphate-buffered saline) was used as a humidification material, to prevent osmolality changes in the levitated blood drop. A drop was deployed manually near one of the pressure nodes with a syringe and needle. An expanded He-Ne laser beam was incident on the drop, and a photodetector situated directly in the forward scattering sensed the resultant light intensity. To decrease sensitivity to ambient light, a narrow 632.8-nm optical bandpass filter was placed in front of the photodiode. To further increase the signal-to-noise ratio, a vertical slit of width 300 μm was placed in front of the optical bandpass filter. Shape oscillations of the levitated drop were excited by amplitude-modulating the transducer drive signal. The output voltage of the photodiode was linearly proportional to the time-varying area of the drop. Digital camera images were used to measure drop size. Figure 1 shows a blood drop levitated in the acoustic field and a schematic of the experiment setup. A more detailed description of the apparatus and method is given in may be found in Ansari Hosseinzadeh et al\textsuperscript{25,29} and Ansari Hosseinzadeh and Holt.\textsuperscript{25,29}

2.3 | Theoretical modeling

In order to infer material properties, we applied the single degree of freedom harmonic oscillator model to the shape oscillations of drops. As the harmonic oscillator is a linear second-order ordinary differential equation, we obtained two generic oscillator parameters, i.e., resonance frequency (ω_0) and damping ratio (ζ); n represents the mode number of the oscillation. In the DOR method, a drop was initially excited into steady-state quadrupole oscillations, whereupon the modulation was turned off and the drop transient response (x) was recorded. By fitting the experimental decay curve to the analytical decay in Equation 1, we obtained best-fit estimates of ω_0 and ζ as:

\[
x = A e^{-\zeta \omega_0 t} \cos(\omega_0 \sqrt{1 - \zeta^2} t)
\] (1)

To relate the rheologic properties to the measured resonance frequency and damping ratio, we employed the approach of Temperton et al\textsuperscript{28} who considered the dispersion of capillary waves on the surface of a semi-infinite viscoelastic medium. The semi-infinite medium assumption is justified here because the radius of the droplets was typically two orders of magnitude larger than the amplitude of vibration. For the range of droplet radii studied (1-1.5 mm), the oscillation amplitudes were typically observed to be <0.01 mm.\textsuperscript{29} We were not able to see this small oscillation amplitude with a camera. Only a laser scattering technique is sensitive to this oscillation.

The capillary-wave dispersion relationship in Equation 2 is adopted from Pleiner et al\textsuperscript{31} with our harmonic oscillator notation for the quadrupole oscillation mode n = 2:

\[
p\omega_0^2(1 + i\zeta)^2 = 2i(1 + i\zeta)(G'' - iG')k^2 + \sigma k^3
\] (2)

where \( k = \frac{2\pi}{R} \) and \( R \) and \( \sigma \) are drop equivalent radius and surface tension, respectively. The surface tension of our blood samples was...
determined in situ by use of our shape oscillation technique on the liquid blood drop when it is first deployed, with an eight-sample average of 54 ± 0.9 mN/m. From Equation 2, we can obtain the components of the complex shear modulus \( G = G' + iG'' \), or more familiarly known as the storage and loss moduli \( G' \) and \( G'' \) as:

\[
G' = \frac{\rho_0^2 R^2}{2n^2} \left( 1 - \frac{\alpha n^3}{\rho_0 R^2 (1 + \zeta_n^2)} \right)
\]

\[
G'' = \frac{\rho_0^2 R^2}{2n^2} \left( 1 + \frac{\alpha n^3}{\rho_0 R^2 (1 + \zeta_n^2)} \right)
\]

For the second mode of oscillation \((n = 2)\), dynamic viscosity can be determined from the loss modulus \( G'' \) as follows:

\[
\nu = \frac{G''}{\omega_2}
\]

3 | RESULTS

3.1 | Polyacrylamide gel assay as a benchmark

Unlike simple synthetic polymer gels, many biological materials, including gels composed of fibrin (the key protein involved in blood clotting), have non-linear elasticity characterized by high compliance at small strains and strain stiffening at larger deformations, which appears to be essential for their function as hemostatic plugs.\(^{32,33}\)

Although the principal mechanism for this non-linear elasticity is unknown, it has been conjectured that stiff fibers are easier to bend than to stretch, and therefore, as strain increases and the filaments align more in the strain direction, a transition occurs from bending to stretching modes, which leads to increased stiffness at increased strains.\(^{32}\)

In order to avoid this strain-dependent rheology, we performed an assay that uses polyacrylamide/bisacrylamide (2%) gel polymerized with ammonium persulfate and TEMED. The Bis concentration in solution was chosen so that we were just able to deploy a spheroidal drop with a syringe-needle combination. Higher Bis concentrations could not be used with this technique. By use of the experimentally measured drop shape frequency and damping, \( G' \) and \( G'' \) were calculated. Figure 2 shows rotational rheometer results for \( G' \) and \( G'' \) of the gel plotted as a function of the percentage strain \( \gamma \) at 10 rad/s. As expected, the gel showed no strain dependence over three orders of magnitude of strain. At a single frequency (that of free decay), DOR results were within 2 Pa of those obtained with the rotational rheometer. Temperton et al.\(^{38}\) also showed good agreement between the results of rotational rheometry and DOR for polyacrylamide acrylate in the range of \( G' \sim 10 \mathrm{Pa} \).

3.2 | Whole blood coagulation: DOR response depends on platelets, fibrinogen, and calcium ions

The role of platelets in hemostasis has been well established.\(^{34,35}\) In order to assess the dependence of DOR on platelets, we compared the coagulation profiles of PRP and PPP. The results shown in Figure 3A demonstrate that PRP has a higher clot stiffness than PPP, in agreement with previous studies.\(^{36}\) Heat inactivation (30 minutes at 56°C)\(^{37}\) of normal control plasma destroys fibrinogen and heat-labile coagulation factors. As shown in Figure 3A, this treatment results in the inability to initiate a measurable storage modulus in the sample. To determine platelet dependence in a plasma sample, samples were reconstituted by combining PPP with PRP to achieve a range of platelets between 0 and 256 (×10^3/μL). Platelet count was determined with a Sysmex XN Hematology analyzer. Figure 3B shows that clot stiffness \( (G') \) increased with increasing platelet count. For platelet counts below approximately \( 64 \times 10^3/\mu L \), coagulation did not occur, whereas it did occur for platelet counts higher than \( 64 \times 10^3/\mu L \). Note that the increase in \( G' \) shows that the elastic component of the 10-minute-old plasma is not negligible even if there is no observed solid clot present.

The effect of CaCl\(_2\) (used to recalciﬁcate sodium citrated blood and plasma) on DOR coagulation measurements was also studied. Figure 4A shows that CaCl\(_2\) at 0.58 mmol/L resulted in a small but measurable modulus magnitude \(|G| \sim 50 \mathrm{Pa}\) in a whole blood sample. However, visual inspection showed no gelation. Increasing, CaCl\(_2\) at 0.96 mmol/L initiated coagulation (according to visual inspection), and resulted in a higher modulus magnitude \(|G| \sim 150 \mathrm{Pa}\) at 20 minutes. Interestingly, increasing the CaCl\(_2\) concentration beyond 0.96 mmol/L had no discernible effect on the modulus. A similar trend was seen for PRP (Figure 4B). These results are in agreement with Brass et al.\(^{38}\) who reported that calcium decreases the time required for fibrin formation from fibrinogen by markedly accelerating the phase of fibrin monomer polymerization.

3.3 | Whole blood coagulation: DOR is sensitive to hematocrit

Red blood cells (RBCs) are known to constitute a key component of clot formation, but their effect on in vitro coagulation is not fully
understood. Whereas a few studies have shown that an increase in the hematocrit (Hct) results in increases in coagulation rate and clot stiffness,\textsuperscript{39–42} others have reported an opposite effect.\textsuperscript{43,44}

One hypothesis to explain these contradictory results places the blame on contact activation during TEG measurement.\textsuperscript{43} Using DOR, we studied the effect of the Hct on blood coagulation in a non-contact tenvironment. We added washed RBCs to PRP and PPP, and adjusted the Hct to 45% to mimic the original Hct of the autologous blood. Figure 4B shows that a CaCl\textsubscript{2} concentration of 0.54 mmol/L was not adequate to initiate coagulation in either PRP or PPP. Strikingly, Figure 5A shows that, when RBCs were present in the sample, CaCl\textsubscript{2} at 0.54 mmol/L was sufficient to initiate coagulation in both PRP and PPP.

To determine Hct dependence in a PPP sample, samples were reconstituted by combining PPP with a mixture of packed washed RBCs to achieve a range of Hct between 5% and 45%. Figure 5B shows that clot stiffness increased with increasing Hct. For an Hct of <20%, coagulation did not occur, whereas it did occur for an Hct of >30%. Eugster and Reinhart\textsuperscript{42} also showed that no occluding platelet plug was formed in a platelet function analyzer (PFA) at an Hct of ≤20%.

### 3.4 Comparison of DOR and TEG

Having established the sensitivity of DOR to changes in the elements of coagulation, we performed a direct comparison of our results with those obtained with TEG. Figure 6A shows a typical TEG thromboelastogram and the associated measured parameters. 'R-time' is the time to initiation of the assay to clot formation of 2 mm. The K value is the time to clot formation of 2-20 mm. The maximal amplitude (MA) represents the overall clot strength, and is a measure of platelet activity.\textsuperscript{36}

To convert TEG output(s) to |G|, we used a formula described by Hartert,\textsuperscript{5} in which s (mm) is the measured amplitude in the thromboelastogram, and $G_{\text{TEG}}$ (dyne/cm\textsuperscript{2}) is the shear modulus inferred by TEG (Equation [6]).

$$G_{\text{TEG}} = \frac{5000s}{100 − s}$$

From Equation 6, the corresponding $G_{\text{TEG}}$ value for each TEG parameter can be calculated. Thus, R-time is the time taken for...
GTEG to increase by 10 Pa from its initial state. Similarly, for K-time, s = 20 mm corresponds to GTEG = 125 Pa. Finally, substitution of MA for s results in a maximum value, GTEG,Max, which will be different for each sample.

We can directly compare TEG and DOR by equating GTEG and |G|. Figure 6B shows that R-time and K-time were obtained from DOR data by finding the times for the corresponding GTEG values, as shown on the graph. We equated the quantitative values of GTEG, which is a shear elastic modulus, with the magnitude of the complex (shear) modulus |G|. As can be inferred from Figure 6, the storage modulus G', which represents the elastic component of the complex modulus, was an order of magnitude greater than the loss modulus G'' for whole clotting blood, justifying our approximation.

Figure 7 shows the ratio of TEG parameters to DOR parameters (TEG/DOR). Comparison of the parameters shows that the measured R-time from TEG was ~20% higher than that from DOR. This is in agreement with Contreras-Garcia et al, who attributed a delay in the coagulation process to Cyrolite, the material used for the TEG cup. The TEG coagulation rate (K-time) was ~40% higher than that found by DOR. Finally, the clot firmness Gmax was fully three times higher in TEG than in DOR. In the literature, there are some inconsistencies among measurements of maximum clot stiffness. Using the laser speckle rheology method, Tripathy et al45 (for human blood) and Kempen et al46 (for porcine blood) measured a clot stiffness of ~700 Pa, whereas Kaibara et al10 measured a clot stiffness of approximately 200-300 Pa for human blood by using a custom linear shear oscillatory rheometer. We attempted to employ a standard commercial rotational rheometer (HR-2; TA Instruments) for additional measurement of clot firmness, but it did not produce reliable or repeatable results, owing to the need for careful management of the contact and free surface conditions.
When no free surface treatment was employed, a scab formed around the lower plate, yielding unreasonably high values for $G'$ (>20 000 Pa). When mineral oil was employed to prevent evaporation and scab formation, the plate surface contact was compromised, yielding unreasonably low $G'$ values (<100 Pa). We concluded that it was far easier to obtain reliable results for blood with our DOR technique, which at least agreed to within a few pascals with the commercial rheometer for the control polymer shown in Figure 2.

To distinguish both elastic and viscous contributions to blood coagulation, we obtained the loss modulus $G''$ during the clotting cascade. Figure 8 shows the variation of $G''$ vs time during clotting of a whole blood sample. As the blood clotted, the loss modulus increased until the clot was fully formed. Once the fibrin network was fully formed and cross-linked, there was no more fluid flow in the sample, so dissipation as measured by the loss modulus $G''$ started to decrease (Figure 8; at approximately 20–25 minutes), whereas, at the same time, the stiffness as measured by the storage modulus $G'$ began to level off (Figure 6B; also at 20–25 minutes).

3.5 | DOR: normal ranges and detection of abnormal samples

Haemonetics suggests that every clinical laboratory should establish its own normal ranges for each of the TEG parameters. The laboratory of Boston Children's Hospital provided the following values as normal ranges: 5–10 minutes for $R$-time, 1–3 minutes for $K$-time, and 50–70 mm Hg for $G_{\text{max}}$. Using these values to restrict the sample size to a population defined as "normal," we define the mean$_{\text{healthy}}$, and the 95% confidence interval CI$\text{95}_{\text{healthy}}$ as mean ± 2 standard deviations based on the assumption of Gaussian statistics for each metric. Table 1 summarizes our statistical results for the normal subset.

### DISCUSSION

Coagulation metrics can be population-normalized as in Table 1 to demonstrate that DOR, like TEG, can be useful for describing coagulation norms for diagnosis purposes. However, there remain significant differences in the actual mean values of the comparable coagulation parameters $R$-time, $K$-time, and $G_{\text{max}}$. As we discuss above, the contact requirement of TEG (and other similar techniques) is very probably at the heart of the $R$-time difference. However, TEG has always required kaolin to increase the coagulation rate (thus reducing $K$-time) and thus bring the total test time to within 30 minutes. This requirement is directly related to the large sample volume needed by TEG. Finally, the larger maximum stiffness shown by TEG than by DOR may partly be explained by surface contact effects. Surface contact activates platelets, which, in turn, secrete polyphosphate, leading to thicker fibrin fibers that are more resistant to fibrinolysis. This is consistent with Ostomel et al. who found that clot stiffness depends on oxide charge effects.

DOR is a novel, non-contact technique for assessing coagulation of whole blood. In DOR, sample containment and manipulation are accomplished by the acoustic field with no mechanical contact. The measurement results show that clotting as measured by DOR is sensitive to the concentrations of platelets, calcium ions, and RBCs, and the presence of fibrinogen. On comparison with TEG, DOR is able to reproduce the results of TEG, while also providing information about dissipative effects ($G''$) and their evolution during clotting. DOR, like its previously reported static counterpart QATT, requires only a single drop of blood, which is 1/30 of the standard sample size.

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**CONFICT OF INTEREESTS**

R. G. Holt and D. B. Khismatullin have filed a patent and founded a company, Levisons Inc., to commercialize the acoustic tweezing technology. V. A. Hosseinzadeh, C. Brugnara and S. Emani have no conflicts of interest to report.

**AUTHOR CONTRIBUTIONS**

V. A. Hosseinzadeh, C. Brugnara, S. Emani, D. B. Khismatullin and R. G. Holt designed the study and wrote the manuscript. S. Emani provided blood samples and their TEG data. V. A. Hosseinzadeh acquired the DOR data. All authors interpreted the results and revised the manuscript.

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**TABLE 1** The means and 95% confidence intervals (CIs) of normal samples (normal samples fall within the normal ranges provided by Boston Children’s Hospital quoted in the text) (n_Normal = 28)

| TEG | DOR |
|-----|-----|
| **Mean** | **95% CI** | **Outliers (>2 SD)** | **Mean** | **95% CI** | **Outliers (>2 SD)** | **P value** |
| R-time (min) | 6.48 | 4.2–8.76 | 1 | 5.45 | 3.19–7.71 | 1 | 0.0008 |
| K-time (min) | 1.54 | 0.8–2.28 | 2 | 2.77 | 0.23–5.31 | 1 | <0.0001 |
| G_{max} (Pa) | 680.06 | 535.8–824.8 | 2 | 224.68 | 158.5–290.8 | 1 | <0.0001 |
| G” (Pa) | 11.87 | 5.5–18.24 | |

Abbreviations: DOR, drop oscillation rheometry; G, magnitude of the complex modulus; G”, loss modulus; G_{max}, maximum G during the clotting cascade; K-time, time taken from R-time until | G | = 125 Pa; R-time, time required for | G | to increase by 10 Pa from its initial value; SD, standard deviation; TEG, thromboelastography.
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SUPPORTING INFORMATION

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