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The application of large amplitude oscillatory stress in a study of fully formed fibrin clots

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The application of large amplitude oscillatory stress in a study of fully formed fibrin clots

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The suitability of controlled stress large amplitude oscillatory shear (LAOStress) for the characterisation of the nonlinear viscoelastic properties of fully formed fibrin clots is investigated. Capturing the rich nonlinear viscoelastic behaviour of the fibrin network is important for understanding the structural behaviour of clots formed in blood vessels which are exposed to a wide range of shear stresses. We report, for the first time, that artefacts due to ringing exist in both the sample stress and strain waveforms of a LAOStress measurement which will lead to errors in the calculation of nonlinear viscoelastic properties. The process of smoothing the waveforms eliminates these artefacts whilst retaining essential rheological information. Furthermore, we demonstrate the potential of LAOStress for characterising the nonlinear viscoelastic properties of fibrin clots in response to incremental increases of applied stress up to the point of fracture. Alternating LAOStress and small amplitude oscillatory shear measurements provide detailed information of reversible and irreversible structural changes of the fibrin clot as a consequence of elevated levels of stress. We relate these findings to previous studies involving large scale deformations of fibrin clots. The LAOStress technique may provide useful information to help understand why some blood clots formed in vessels are stable (such as in deep vein thrombosis) and others break off (leading to a life threatening pulmonary embolism). https://doi.org/10.1063/1.4999991

I. INTRODUCTION

Characterisation of the nonlinear rheological properties of biopolymer gels has important implications for understanding their structure-function relationships under large stress and strain regimes. A salient example is a fibrin gel that provides the structural backbone of a blood clot and serves to perform a haemostatic function by preventing the loss of blood at a site of injury to a vessel.[1] A fibrin gel is formed following the conversion of fibrinogen to fibrin by the action of the catalytic enzyme thrombin and subsequent polymerisation of fibrin to produce a three-dimensional gel network of branching fibres.[1] The fibres are formed from protofibrils that may aggregate both laterally and longitudinally. Conditions that favour lateral aggregation produce thicker fibres with relatively low degree of branching, while conditions that inhibit lateral aggregation yield thin fibres with more branching.[1] The evolving fibrin network entraps cells such as red blood cells, white blood cells, and platelets and is reinforced by Factor XIII mediated covalent cross-linking to produce a more mechanically and chemically stable fully formed fibrin clot.[2–4] In disease, the undesirable formation of fibrin clots can block blood vessels, thus preventing blood supply to tissues. Blockages in the veins are termed a Venous Thromboembolism (VTE) and are responsible for a significant global disease burden.[5] Clots formed in the deep veins (i.e., a deep vein thrombosis) can embolise (i.e., break off), travel in the bloodstream, and lodge in the pulmonary arteries leading to a lack of blood supply to the lungs (i.e., a pulmonary embolism).[6,7]

In vivo, shear forces due to the flow of blood may induce fracture or breakage of the blood clot and is a potential mechanism for embolism.[8,9] The wall shear stress in veins can span several magnitudes depending on the size, branching, and number of vessels and can be elevated in disease states.[10] Microfluidic channel approaches studying clot growth have shown that, in a "pressure-relief mode" whereby the flow of blood is allowed to divert to other channels, the shear stress increases as the flow squeezes past the growing thrombus and then rapidly decreases at approximately 80% of full occlusion.[9] Thus, it is important to consider the effects of both the vast range and progressive increase in stress experienced by fully formed clots. Marked changes in the structure and rheological properties of the clot can arise as a consequence of shear stress through several different mechanisms including strain-stiffening of the fibrin network,[11] strain-stiffening of individual fibrin fibres,[12,13] protein unfolding of stretched fibres,[14] unfolding of fibrin monomers,[15] yielding of branching points,[16] slippage of protofibrils within fibres,[17] and breakage of individual fibrin fibres.[18,19] The propensity of the formed blood clot to macroscopically fracture may be influenced by all of the aforementioned mechanisms.

There have been several methods used to characterise the rheological properties of fibrin clots under relatively large

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[a)] Author to whom correspondence should be addressed: k.m.hawkins@swansea.ac.uk stresses and strains. These include conventional rheological approaches involving oscillatory techniques under conditions that provoke strain-stiffening behaviour of the network and the subsequent reporting of storage moduli.[11,20] However, it is important to recognise that, under such conditions, the resulting waveform is non-sinusoidal and thus does not pertain to the theory of linear viscoelasticity.[21] Other workers have exploited inertio-elastic oscillations in controlled stress rheometry to provide a measurement of tangent moduli.[22] Several studies have reported the application of a pre-stress followed by the superposition of small amplitude oscillatory shear stress to probe the rheological properties in terms of differential moduli.[15,23,24] Using this approach, Piechocka *et al.*[15] identified distinct regimes of elastic response. Large Amplitude Oscillatory Shear (LAOS) has been used in a controlled strain manner to perform repeated large strain loadings on fibrin clots and the data were presented using Lissajous-Bowditch curves.[17,25] Using repeated cyclic strain loadings, Münster *et al.*[17] showed the ability of un-crosslinked fibrin clots to adapt to stress through the slippage of protofibrils. Moreover, the LAOS studies of van Kempen *et al.*[25] revealed several nonlinear features of fibrin clots, including time-dependent softening, intra-cycle strain stiffening, and increased viscous dissipation with increased strain, illustrating the usefulness of LAOS in capturing rich nonlinear behaviour.

Controlled stress LAOS (LAOStress) is arguably a more suitable technique than controlled strain LAOS for studying the nonlinear properties of fully formed fibrin clots. LAOStress is physiologically relevant insofar that it affords progressive increases in stress to be imposed on the sample which can be related to the hemodynamic forces experienced by clots *in vivo*. LAOStress has been previously used to characterise the nonlinear rheology of polymer solutions and gels.[26,27] In the present study, we investigate the suitability of LAOStress for studying the nonlinear behaviour of fibrin clots up to the point of macroscopic fracture. Furthermore, we combine alternating LAOStress and linear viscoelastic measurements in order to study reversible and irreversible changes in fibrin clot microstructure as a consequence of different levels of stress.

II. EXPERIMENTAL

A. Materials

Purified, plasminogen depleted human fibrinogen at least 95% clottable and human thrombin were obtained from Enzyme Research Laboratories, UK. The fibrinogen was made up to a stock solution of 44.54 mg/ml by the addition of Tris-buffer saline, TBS (20 mM Tris, pH 7.4% and 0.9% NaCl, Sigma Aldrich, UK) and left to fully dissolve in a water bath at 37 °C. Thrombin was dissolved in distilled water and made up to a stock solution of 500 NIH unit/ml. These stock solutions were aliquoted and frozen at −80 °C until required. A 1M stock solution of $CaCl_2$ (Sigma Aldrich, UK) was also stored at 4 °C. The appropriate volumes of TBS, fibrinogen, and $CaCl_2$ were mixed together to make a (final) concentration of 4 mg/ml fibrinogen and 0.005M $CaCl_2$. Clotting was initiated by adding the required volume of thrombin to produce a (final) concentration of 0.1 NIH/ml. After appropriate mixing, the sample was transferred onto the lower plate of the rheometer.

B. Rheometry

Rheological measurements were conducted using an AR-G2 controlled stress rheometer (TA Instruments, UK) fitted with a cone and plate (40 mm cone, 0.0351 rad) measuring system. The lower plate was maintained at a temperature of 24 °C for all measurements. In order to reduce effects of evaporation, the sample was sealed with a thin layer of low viscosity (10 mPa s) silicone oil. The sample was allowed to clot between the rheometer plates for a period equivalent to 40 gel times, after which the network is fully formed.[28,29] LAOStress measurements were performed on the fully formed clot by applying continuous stress waveforms at a stress amplitude in the nonlinear viscoelastic range of the sample and a frequency of 1 Hz until the sample reached a steady state. Following the acquisition of LAOStress data, the sample was probed by small amplitude oscillatory stress measurements at a stress level of 5 Pa and a frequency of 1 Hz until it reached a steady state in order to provide a value of linear elastic compliance J'. Adherence of the Small Amplitude Oscillatory Shear (SAOS) measurements to the linear viscoelastic range of the sample was confirmed by the absence of a third harmonic response. The process of alternating LAOStress measurements at incrementally increasing stress amplitudes and SAOS measurements was performed until the sample macroscopically fractured leading the upper rheometer plate to overspeed. A criterion was imposed throughout all experiments that steady state was reached once the difference in the strain response between consecutive waveforms was less than 2%. This criterion ensured that point symmetry was observed in the Lissajous-Bowditch curves obtained from the LAOStress tests and that irreversible damage following relaxation of the sample was probed by SAOS. The sample stress and strain response waveform datasets were obtained by the rheometer software (TRIOS) in a transient data collection mode at the software's default sampling rate of 978 Hz.

C. Analysis of LAOStress waveforms

A quantitative framework for the analysis of LAOStress data in terms of deducing the nonlinear compliances has been described by Dimitriou *et al.*[26] and Läuger and Stettin.[27] This includes the minimum-stress elastic compliance J'_M and the large-stress elastic compliance J'_L (see Fig. 1) which can be calculated from Lissajous-Bowditch curves of strain versus stress.[26]

For an imposed sinusoidal stress, $\sigma(t) = \sigma_0 \cos \omega t$, the minimum-stress elastic compliance, J'_M, can be defined by

$$J'_M \equiv \left.\frac{d\gamma}{d\sigma}\right|_{\sigma=0} = \sum\nolimits_{n\,odd} (-1)^{(n-1)/2} n J'_n = \sum\nolimits_{n\,odd} (-1)^{(n-1)/2} n c_n \tag{1}$$

and the large-stress elastic compliance, J'_L, can be defined by

$$J'_L \equiv \left.\frac{\gamma}{\sigma}\right|_{\sigma=\sigma_0} = \sum\nolimits_{n\,odd} J'_n = \sum\nolimits_{n\,odd} c_n, \tag{2}$$

where σ_0 is the applied stress amplitude, γ is the resulting strain, c_n is the nth order Chebyshev coefficient, in which the

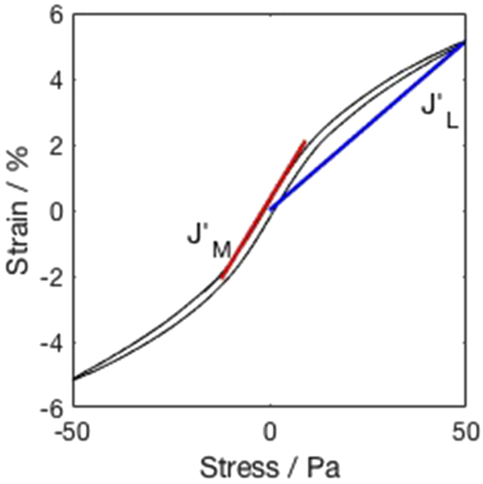

FIG. 1. A Lissajous-Bowditch curve obtained from a LAOStress experiment and an illustration of the calculation of nonlinear compliances, the minimum-stress elastic compliance J'_M (red) and the large-stress elastic compliance J'_L (blue).

letter "c" represents the compliance nonlinearities, and J'_n is the *n*th compliance.[26]

Figure 1 shows an illustration of a Lissajous-Bowditch curve of stress versus strain data obtained from a LAOStress measurement. J'_M is calculated from the gradient of the tangent at zero stress. J'_L is calculated from the gradient of a straight line connecting the origin to the point of maximum stress. The calculations were performed using MATLAB (Version 9.2, MathWorks, Inc.) after importing data from TRIOS.

III. RESULTS AND DISCUSSION

A. LAOStress data processing

Figure 2 displays an example of sample stress waveforms (*upper*) and strain response waveforms (*lower*) at an applied stress level of (a) 100 Pa (slightly into the nonlinear viscoelastic range of the sample) and two higher levels of applied stress (b) 500 Pa and (c) 1500 Pa. The dimensionless inertia number, *In*, proposed by Dimitriou *et al.*[26] was calculated for all LAOStress measurements and $In \ll 0.05$ throughout, indicating that instrument inertia alone did not significantly affect the total torque signal. However, at increasing levels of applied stress, the sample stress and strain response waveforms contain artefacts due to a source of ringing. To our knowledge, this is the first observation of ringing phenomena in a LAOS measurement. This ringing is possibly attributable to a resonance caused by a coupling of inertial and elastic forces at relatively large levels of applied stress.[30]

In an attempt to eliminate the effects of ringing, the sample stress and strain response waveform datasets (obtained from TRIOS) were smoothed to a cubic spline function (specifically a piecewise cubic polynomial computed from a smoothing parameter p = 0.999 992 52) using the Curve Fitting Toolbox in MATLAB. The datasets were then reconstructed from the smoothing function by sampling at a rate identical to the original dataset (978 Hz). Figure 3 displays an example of the resulting smoothed waveforms. The process of smoothing eliminates any noticeable effects of ringing. However, in order to ensure that nonlinearities due to the sample response (which manifest as odd harmonics) are not affected by the process of smoothing, the datasets were subjected to Fourier analysis and the resulting frequency spectra were compared. To this end, the strain and stress datasets were trimmed to contain 15 000 data points which contain more than 15 complete cycles. The datasets were windowed using a Hanning window function and zero padded with 5000 data points in order to adjust the resolution of the frequency spectrum to 0.05 Hz. The datasets were analysed in MATLAB using a discrete Fourier transform.

Figure 4 shows the frequency spectra of the sample stress and strain response waveforms for the original (i.e., unprocessed) data and smoothed data at three different levels of applied stress (a) 100 Pa, (b) 500 Pa, and (c) 1500 Pa. The frequency spectra of the strain response are dominated by the fundamental (imposed) frequency and contain odd harmonics, confirming that the measurement is within the nonlinear viscoelastic regime.[21] The magnitude of the even harmonics

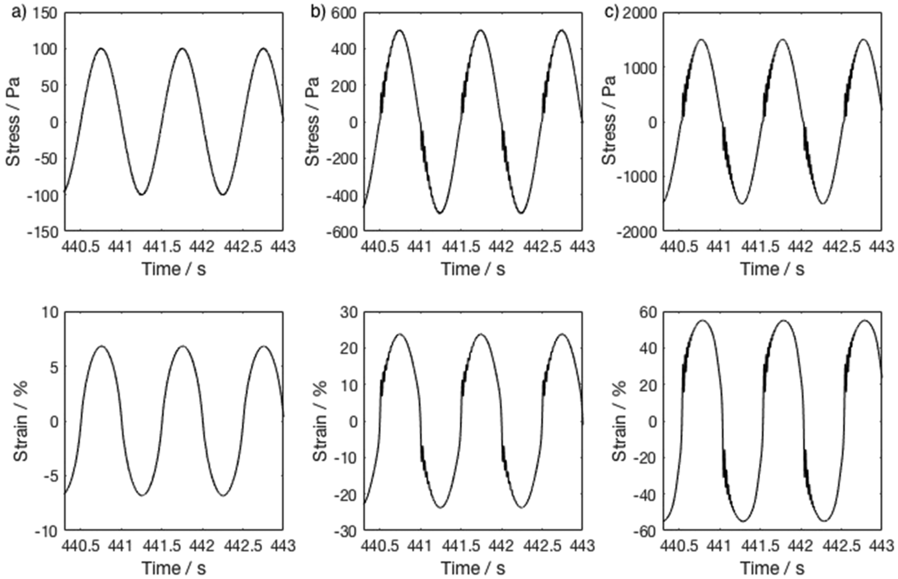

FIG. 2. Sample stress (upper) and strain response (lower) waveforms obtained from the rheometer (TRIOS) software at input stress levels of (a) 100 Pa, (b) 500 Pa, and (c) 1500 Pa. These waveforms consist of discrete data points at a sampling rate of 978 Hz.

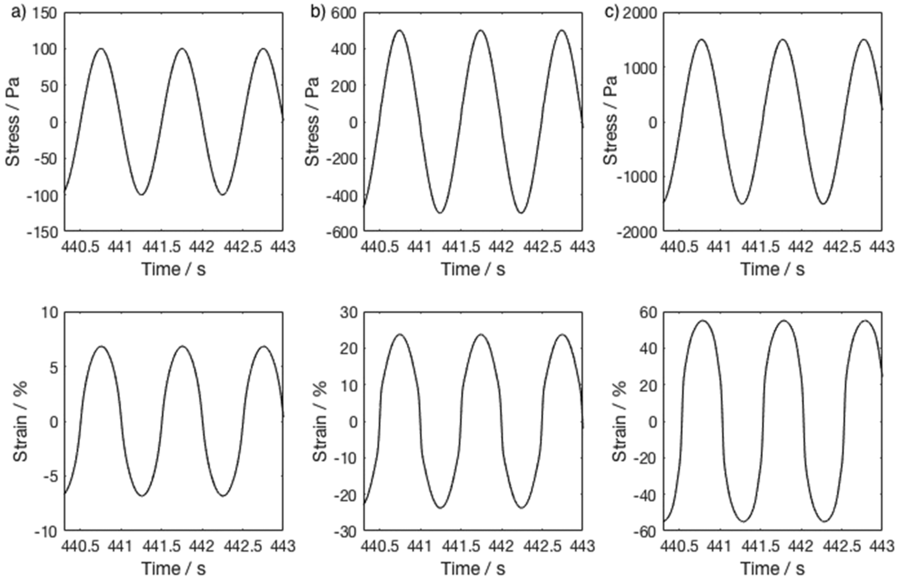

FIG. 3. Smoothed sample stress (upper) and strain response (lower) waveforms (corresponding to the waveforms shown in Fig. 2) at input stress levels of (a) 100 Pa, (b) 500 Pa, and (c) 1500 Pa. These waveforms consist of discrete data points at a sampling rate of 978 Hz.

was negligible confirming that the sample was non-transient and had reached a steady state.[31] It is noticeable that some harmonic distortion in the sample stress waveform is apparent, including at the lowest stress shown where there was no ringing encountered. These nonlinearities will be transmitted to, and replicated in, the corresponding strain response. However, the magnitude of the odd harmonics in the strain response is much greater than the sample stress indicating that the rich nonlinearity of the sample is being captured. Importantly, the frequency spectra of the smoothed waveforms are identical to their original waveforms providing evidence that the process of smoothing does not eliminate the essential information originating from the nonlinear response of the sample. Henceforth, we use the smoothed stress and strain waveforms as the basis of the Lissajous-Bowditch curves and calculation of nonlinear compliances.

Figure 5 shows a comparison of Lissajous-Bowditch curves constructed from original and smoothed datasets. At relatively low levels of stress [Fig. 5(a)], the Lissajous-Bowditch curves appear identical. Figures 5(b) and 5(c) show that the ringing encountered in the original waveforms introduces secondary loops in the corresponding Lissajous-Bowditch curves. These secondary loops are eliminated by the process of smoothing. Furthermore, at these relatively large stress levels, inspection of the original and smoothed curves shows profound differences around the region pertaining to the minimum stress. Thus, any calculated values of J'_M are highly sensitive to ringing artefacts.

B. Nonlinear and linear viscoelastic properties

Figure 6 shows the results of the alternating LAOStress and SAOS measurements. Figure 6(b) displays the magnitude of the strain response to the applied stress versus time profile shown in Fig. 6(a). The time period for which each stress is applied depends on the duration of the measurement to reach a steady state. Figure 6(c) shows that values of the nonlinear

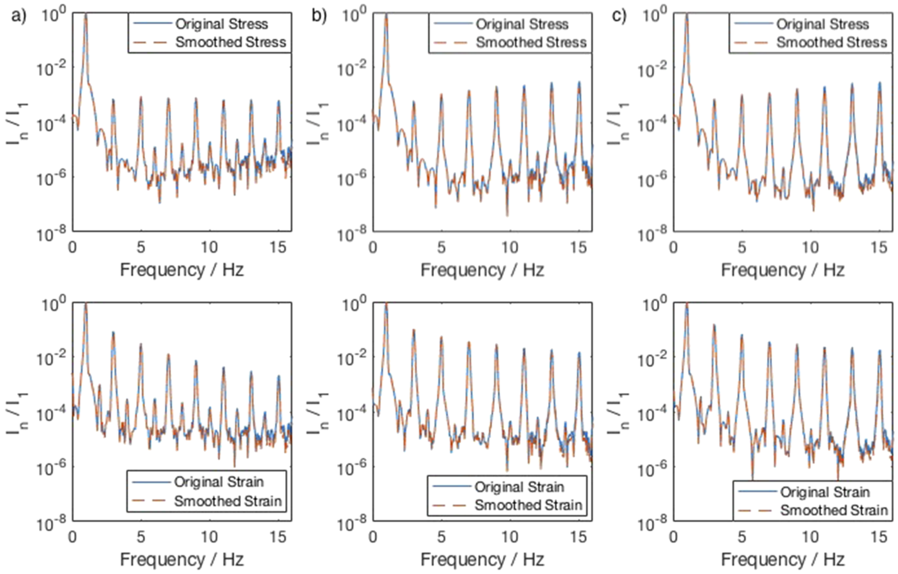

FIG. 4. Comparisons of frequency spectra obtained from discrete Fourier transforms of the original (blue) and smoothed (red) datasets for the sample stress waveform (upper) and strain response waveforms (lower) at input stress levels of (a) 100 Pa, (b) 500 Pa, and (c) 1500 Pa. The magnitudes of the spectra (I_n) are normalised to the magnitude of the fundamental harmonic response (I_1). The fundamental (imposed) frequency is 1 Hz throughout.

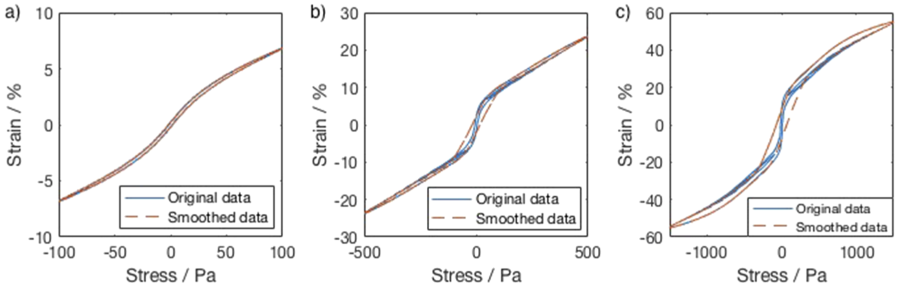

FIG. 5. Comparisons of Lissajous-Bowditch curves using original (blue) and smoothed (red) datasets for input stress levels of (a) 100 Pa, (b) 500 Pa, and (c) 1500 Pa.

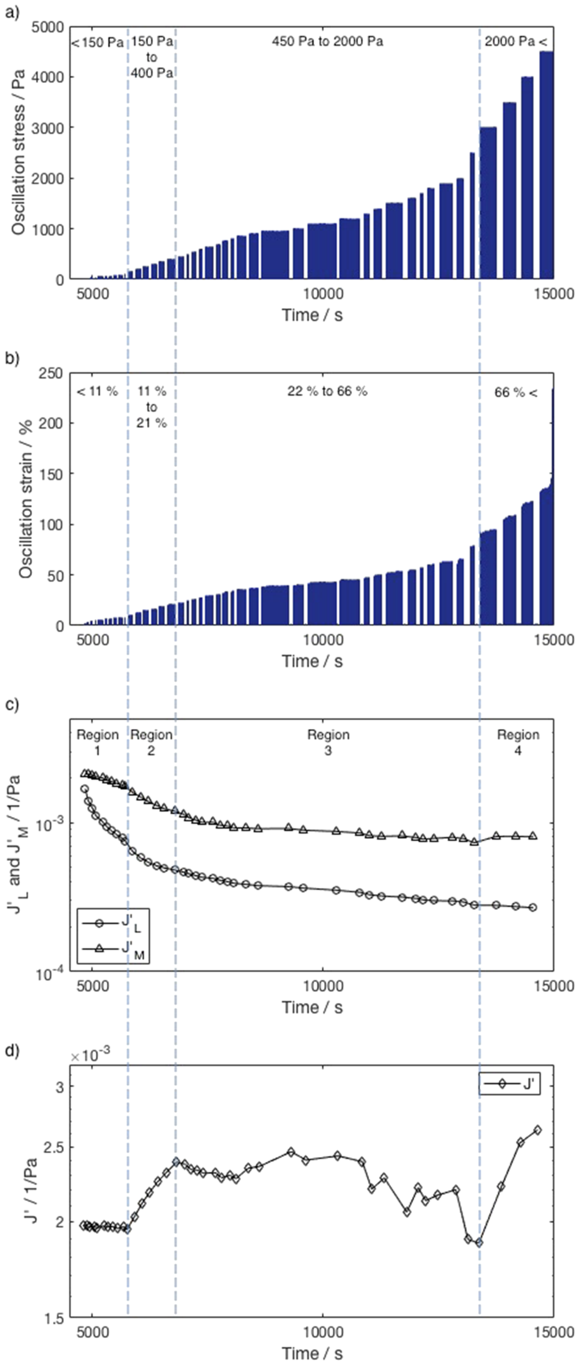

FIG. 6. Results of alternating SAOS and LAOStress measurements; (a) the applied stress versus time profile, (b) the resulting strain response versus time profile, (c) large-stress and minimum-stress elastic compliances (as measured by LAOStress), and (d) linear elastic compliance (as measured by SAOS). The results reveal several nonlinear viscoelastic regions (numbered 1–4, as illustrated by the dashed lines).

compliances J'_L and J'_M gradually decrease with increasing levels of stress and strain indicating stress stiffening of the sample. This stress stiffening is also captured in individual LAOStress measurements at discrete levels of stress by a dominance of J'_M over J'_L over the entire range of stress levels studied. This behaviour is expected given that individual fibrin fibres' strain stiffens and is highly extensible.[12,13,15] Our findings are similar to the LAOS controlled strain studies of van Kempen *et al.*[25] that reported intra-cycle stiffening and softening during continuous application of large strain deformations to fibrin clots. However, an advantage of LAOStress measurements is that the fibrin clot is allowed to reach the steady state under a fixed level of oscillatory stress. This is more physiologically relevant insofar that the stress is imposed on the clot.[9,10]

Analysis of the nonlinear and linear viscoelastic behaviour of the sample [in Figs. 6(c) and 6(d), respectively] reveals four distinct regions. In region 1 ($\sigma_0 < 150$ Pa), the sample shows increasing tendency to stress stiffen as evidenced by an increase in the difference between J'_M and J'_L with increasing levels of stress. The sample returns to its original linear viscoelastic behaviour following a decrease in applied stress indicating full recovery of the structure. In region 2 ($150 < \sigma_0 < 400$ Pa), the linear elastic compliance gradually increased following the application of increasing levels of LAOStress, indicating irreversible structural rearrangements. This stress-softening, which is also evident by close inspection of the curvature of the oscillatory strain response in the LAOStress experiments, is likely to be due to yielding of branching points leading to a slackening of the network.[16] The behaviour encountered in region 3 ($450 < \sigma_0 < 2000$ Pa) is similar to region 1 insofar that the sample returns to a level of linear elastic compliance that is independent of the stress applied in the LAOStress measurement. This finding is similar to the study of Piechocka *et al.*,[15] which showed that the differential modulus is independent of values of pre-stress within a specific stress regime. In region 3, the structure of the sample has been permanently re-arranged by the stresses encountered in region 2, resulting in a sample that has the ability to resist structural changes when exposed to further increases in stress. Furthermore, in the latter stages of region 3 (i.e., at long times), there is generally a decrease in linear elastic compliance following the application of increasing levels of LAOStress, indicating that effects due to ageing of the sample (possibly due to Factor XIII mediated cross-linking) are greater than any stress induced structural damage. In region 4 ($\sigma_0 > 2000$ Pa), the ability of the sample to resist structural changes at increasing levels of stress diminishes as evidenced by an increase in linear elastic compliance. Further increases in the applied levels of stress lead to fracture of the sample network causing the rheometer upper plate to overspeed.

IV. CONCLUSIONS

We investigated the applicability of LAOStress in studying the nonlinear viscoelastic properties of fully formed fibrin clots. The stress and strain waveforms obtained from the LAOStress measurements contained artefacts due to a source of ringing. This is the first observation of ringing in a LAOS measurement. We showed that the artefacts due to ringing can be effectively eliminated whilst retaining the essential harmonic information related to the nonlinear viscoelastic response of the sample by fitting the original waveforms to a smoothing spline. This has allowed a characterisation of the nonlinear compliances of fibrin clots up to the point of fracture. Combined with supplementary SAOS measurements, various regions of nonlinear viscoelastic behaviour were identified which can be related to previous findings involving large scale deformations of fibrin clots. The quantitative framework of LAOStress provides additional information of fibrin clot nonlinear behaviour, and the approach is more physiologically relevant than LAOS in a controlled strain mode. Further work will involve the application of LAOStress in studies of abnormal clots such as those formed in blood taken from patients with venous thromboembolism.

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[1] J. W. Weisel, "The mechanical properties of fibrin for basic scientists and clinicians," Biophys. Chem. **112**, 267 (2004).

[2] N. A. Kurniawan, J. Grimbergen, J. Koopman, and G. H. Koenderink, "Factor XIII stiffens fibrin clots by causing fiber compaction," J. Thromb. Haemostasis **12**, 1687 (2014).

[3] J. W. Weisel, "Mechanisms of fibrin polymerization and clinical implications," Blood **121**, 1712 (2013).

[4] J. W. Weisel and R. I. Litvinov, "Fibrin Formation, Structure and Properties," Subcell. Biochem. **82**, 405 (2017).

[5] ISTH Steering Committee for World Thrombosis Day, "Thrombosis: a major contributor to the global disease burden," J. Thromb. Haemostasis **12**, 1580 (2014).

[6] J. E. Dalen, "Pulmonary embolism: what have we learned since Virchow? Natural history, pathophysiology, and diagnosis," Chest **122**, 1440 (2002).

[7] K. R. Machlus, M. M. Aleman, and A. S. Wolberg, "Update on venous thromboembolism: risk factors, mechanisms, and treatments," Arterioscler., Thromb., Vasc. Biol. **31**, 476 (2011).

[8] W. W. Roberts, O. Kramer, R. W. Rosser, F. H. Nestler, and J. D. Ferry, "Rheology of fibrin clots. I. Dynamic viscoelastic properties and fluid permeation," Biophys. Chem. **1**, 152 (1974).

[9] T. V. Colace, R. W. Muthard, and S. L. Diamond, "Thrombus growth and embolism on tissue factor-bearing collagen surfaces under flow: role of thrombin with and without fibrin," Arterioscler., Thromb., Vasc. Biol. **32**, 1466 (2012).

[10] M. H. Kroll, J. D. Hellums, L. V. McIntire, A. I. Schafer, and J. L. Moake, "Platelets and Shear Stress," Blood **88**, 1525 (1996).

[11] J. V. Shah and P. A. Janmey, "Strain hardening of fibrin gels and plasma clots," Rheol. Acta **36**, 262 (1997).

[12] N. E. Hudson, J. R. Houser, E. T. O'Brien, R. M. Taylor, R. Superfine, S. T. Lord, and M. R. Falvo, "Stiffening of individual fibrin fibers equitably distributes strain and strengthens networks," Biophys. J. **98**, 1632 (2010).

[13] W. Liu, C. R. Carlisle, E. A. Sparks, and M. Guthold, "The mechanical properties of single fibrin fibers," J. Thromb. Haemostasis **8**, 1030 (2010).

[14] K. P. Purohit, R. I. Litvinov, A. E. X. Brown, D. E. Discher, and J. W. Weisel, "Protein unfolding accounts for the unusual mechanical behavior of fibrin networks," Acta Biomater. **7**, 2374 (2011).

[15] I. K. Piechocka, R. G. Bacabac, M. Potters, F. C. MacKintosh, and G. H. Koenderink, "Structural hierarchy governs fibrin gel mechanics," Biophys. J. **98**, 2281 (2010).

[16] W. Liu, L. Jawerth, E. Sparks, M. Falvo, R. Hantgan, R. Superfine, S. T. Lord, and M. Guthold, "Fibrin fibers have extraordinary extensibility and elasticity," Science **313**, 634 (2006).

[17] S. Münster, L. M. Jawerth, B. A. Leslie, J. I. Weitz, B. Fabry, and D. A. Weitz, "Strain history dependence of the nonlinear stress response of fibrin and collagen networks," Proc. Natl. Acad. Sci. U. S. A. **110**, 12197 (2013).

[18] A. E. Brown, R. I. Litvinov, D. E. Discher, P. K. Purohit, and J. W. Weisel, "Multiscale mechanics of fibrin polymer: gel stretching with protein unfolding and loss of water," Science **325**, 741 (2009).

[19] N. Badiei, A. M. Sowedan, D. J. Curtis, M. R. Brown, M. J. Lawrence, A. I. Campbell, P. A. Evans, J. W. Weisel, I. N. Chernysh, C. Nagaswami, P. R. Williams, and K. Hawkins, "Effects of unidirectional flow shear stresses on the formation, fractal microstructure and rigidity of incipient whole blood clots and fibrin gels," Clin. Hemorheol. Microcirc. **60**, 451 (2015).

[20] P. Riha, X. Wang, R. Liao, and J. F. Stoltz, "Elasticity and fracture strain of whole blood clots," Clin. Hemorheol. Microcirc. **21**, 45 (1999).

[21] A. J. Giacomin and J. M. Dealy, "Large amplitude oscillatory shear," in *Techniques in Rheological Measurement*, edited by A. A. Collyer (Champan and Hall, London, 1993).

[22] N. Y. Yao, R. J. Larsen, and D. A. Weitz, "Probing nonlinear rheology with inertio-elastic oscillations," J. Rheol. **52**, 1013 (2008).

[23] C. P. Broedersz, K. E. Kasza, L. M. Jawerth, S. Munster, D. A. Weitz, and F. C. MacKintosh, "Measurement of nonlinear rheology of cross-linked biopolymer gels," Soft Matter **6**, 4120 (2010).

[24] K. A. Jansen, R. G. Bacabac, I. K. Piechocka, and G. H. Koenderink, "Cells actively stiffen fibrin networks by generating contractile stress," Biophys. J. **105**, 2240 (2013).

[25] T. H. S. van Kempen, G. W. M. Peters, and F. N. van de Vosse, "A constitutive model for the time-dependent, nonlinear stress response of fibrin networks," Biomech. Model. Mechanobiol. **14**, 995 (2015).

[26] C. J. Dimitriou, R. H. Ewoldt, and G. H. McKinley, "Describing and prescribing the constitutive response of yield stress fluids using large amplitude oscillatory shear stress (LAOStress)," J. Rheol. **57**, 27 (2013).

[27] J. Läuger and H. Stettin, "Differences between stress and strain control in the non-linear behavior of complex fluids," Rheol. Acta **49**, 909 (2010).

[28] F. Ferri, M. Greco, G. Arcovito, F. Bassi, M. De Spirito, E. Paganini, and M. Rocco, "Growth kinetics and structure of fibrin gels," Phys. Rev. **63**, 31401 (2001).

[29] D. J. Curtis, K. Hawkins, M. Lawrence, T. M. Davies, M. R. Brown, and P. R. Williams, "Incipient fibrin gel microstructure as an indication of maturing clot growth processes," Ser. Biomech. **25**, 184 (2010).

[30] C. Baravian, G. Benbelkacem, and F. Caton, "Unsteady rheometry: can we characterize weak gels with a controlled stress rheometer?," Rheol. Acta **46**, 577 (2007).

[31] K. Atalik and R. Keunings, "On the occurrence of even harmonics in the shear stress response of viscoelastic fluids in large amplitude oscillatory shear," J. Non-Newtonian Fluid Mech. **122**, 107 (2004).