

The Effects of Temperature on Clot Microstructure and Strength in Healthy Volunteers

Matthew James Lawrence, MEng, PhD,*† Nick Marsden, BSc, MBBCh, MRCS,‡ Ranganwamy Mothukuri, MBBS, MRCS(Edin), FCEM, PG Cert,§ Roger H. K. Morris, BSc, PhD,|| Gareth Davies, BSc,*† Karl Hawkins, MEng, PhD,*† Daniel J. Curtis, MEng, PhD,¶|| Martin Rowan Brown, MPhys, PhD,¶|| Phylip Rhodri Williams, FICHEM, FInstP, FRSA, FLSW,¶|| and Phillip Adrian Evans, MD, MBBS, FRCS, FFAEM*†§

BACKGROUND: Anesthesia, critical illness, and trauma are known to alter thermoregulation, which can potentially affect coagulation and clinical outcome. This in vitro preclinical study explores the relationship between temperature change and hemostasis using a recently validated viscoelastic technique. We hypothesize that temperature change will cause significant alterations in the microstructural properties of clot.

METHODS: We used a novel viscoelastic technique to identify the gel point of the blood. The gel point identifies the transition of the blood from a viscoelastic liquid to a viscoelastic solid state. Furthermore, identification of the gel point provides 3 related biomarkers: the elastic modulus at the gel point, which is a measure of clot elasticity; the time to the gel point (T_{GP}), which is a measure of the time required to form the clot; and the fractal dimension of the clot at the gel point, d_f , which quantifies the microstructure of the clot. The gel point measurements were performed in vitro on whole blood samples from 136 healthy volunteers over a temperature range of 27°C to 43°C.

RESULTS: There was a significant negative correlation between increases in temperature, from 27°C to 43°C, and T_{GP} ($r = -0.641$, $P < 0.0005$). Conversely, significant positive correlations were observed for both the elastic modulus at the gel point ($r = 0.513$, $P = 0.0008$) and d_f ($r = 0.777$, $P < 0.0005$) across the range of 27°C to 43°C. When temperature was reduced below 37°C, significant reductions in d_f and T_{GP} occurred at $\leq 32^\circ\text{C}$ (Bonferroni-corrected $P = 0.0093$) and $\leq 29^\circ\text{C}$ (Bonferroni-corrected $P = 0.0317$), respectively. No significant changes were observed when temperature was increased to $>37^\circ\text{C}$.

CONCLUSIONS: This study demonstrates that the gel point technique can identify alterations in clot microstructure because of changes in temperature. This was demonstrated in slower-forming clots with less structural complexity as temperature is decreased. We also found that significant changes in clot microstructure occurred when the temperature was $\leq 32^\circ\text{C}$. (Anesth Analg 2016;122:21–6)

Extremes of body temperature occur in about half of patients in the operating room or in the critical care unit, contributing to morbidity and mortality.^{1–3} These alterations in temperature can result in fluctuations between hypothermia or hyperthermia, which may result from a wide range of clinical complications.⁴ Although

hypothermia is associated with hypocoagulable states, hyperthermia can result in a hypercoagulable state.^{5–10} These effects of temperature on coagulation can be demonstrated as abnormalities in the organization of the fibrin network that determines the microstructure of the clot.^{11–16} Clots formed under hypercoagulable conditions will have more tightly packed fibrin networks, which are more resistant to deformation and breakdown compared with clots formed under hypocoagulable conditions.^{14–16}

Previous studies have attempted to investigate the effects of temperature change on coagulation by using a variety of viscoelastic techniques, including thromboelastography (Haemoscope Inc., Niles, IL), rotational thromboelastometry (Tem International GmbH, Munich, Germany), and free oscillation rheometry.^{17–23} There is some evidence to suggest that hyperthermia causes a procoagulant effect.⁹ Although hypothermia is known to reduce the rate of clot formation, the effect of temperature on maximum clot strength is unclear, with some studies showing little or no change and others reporting significant changes from 33°C to 28°C.^{23–26}

Recent advances in studying viscoelastic changes in coagulation have led to the development of a novel rheological test for the analysis of clotting characteristics, including an assessment of clot microstructure.^{14–16,27} In contrast to standard coagulation assays, this technique uses whole blood, and it accurately measures coagulation based on the

From the *NISCHR Haemostasis Biomedical Research Unit, Morriston Hospital, Abertawe Bro Morgannwg University Health Board, Swansea, Wales, United Kingdom; †College of Medicine, Swansea University, Swansea, Wales, United Kingdom; ‡The Welsh Centre for Burns and Plastic Surgery, Morriston Hospital, Swansea, Wales, United Kingdom; §Emergency Department, Morriston Hospital, Abertawe Bro Morgannwg University Health Board, Swansea, Wales, United Kingdom; ||School of Health Science, Cardiff Metropolitan University, Cardiff, Wales, United Kingdom; and ¶College of Engineering, Swansea University, Swansea, Wales, United Kingdom.

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Address correspondence to Phillip Adrian Evans, MD, MBBS, FRCS, FFAEM, Emergency Department, Morriston Hospital, Abertawe Bro Morgannwg University Health Board, Swansea, Wales SA6 6NL, UK. Address e-mail to phillip.evans2@wales.nhs.uk.

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attainment of a gel point.^{14–16} The gel point identifies the transition of the blood from a viscoelastic liquid to a viscoelastic solid state, which signifies the first point at which the clot forms an unbroken structure. Taking rheological measurements at the gel point provides 3 important and related measurements: the elastic modulus at the gel point, G'_{GP} , which is a measure of clot elasticity; the time to the gel point, T_{GP} , which is a measure of how long it takes to form the clot; and the fractal dimension of the clot at the gel point, d_f , which quantifies the microstructure of the clot. In previous in vitro studies of hemodilution and anticoagulation, we have shown that the measurement of the gel point provides better prediction of coagulation abnormalities than conventional markers.^{14,16}

The aim of this preclinical in vitro study is to determine whether temperature change over a range of 27°C to 43°C will cause alterations in the microstructural properties of the clot. We hypothesize that because temperatures are reduced to the mild and severe hypothermic range, significant changes in the gel point parameters will occur, where the clot will form more slowly and produce reductions in d_f . We also hypothesize that increases of temperature into the hyperthermic range will also cause a significant change in the gel point parameters, where we will see quicker-forming clots with increases in d_f .

METHODS

The study was approved by the Wales Research Ethics Committee 6. Informed written consent was obtained from all subjects before enrolment. The exclusion criteria were as follows: (1) individuals with acute or chronic conditions known to effect coagulation (i.e., malignancy, hepatic, and/or renal dysfunction); (2) individuals undergoing antiplatelet or anticoagulation treatment; (3) individuals with a family history of bleeding or thromboembolic disorders; and (4) anyone younger than 18 years.

Blood Sampling

Before blood sampling, each healthy volunteer was assigned to 1 in vitro test temperature, ranging from 27°C to 43°C increasing in 1°C increments ($n = 8$ for each temperature). Blood samples were obtained from the antecubital vein through an 18-gauge needle. The first 2 mL of blood was discarded after which 20 mL of blood was collected in a plastic syringe. The blood sample was then immediately divided into 2 aliquots. The first whole-blood aliquot was transferred to the AR-G2 (TA Instruments, New Castle, DE) rheometer for testing. The second sample was used for subsequent standard coagulation screening and full blood counts to ensure that each volunteer had a normal coagulation profile.

Gel Point Measurements

The gel point technique has been validated previously.¹⁴ In this study, 6.6 mL of whole nonanticoagulated venous blood was placed into a double-gap concentric cylinder geometry testing surface mounted on a AR-G2–controlled stress rheometer. Immediately after sample loading, small amplitude oscillatory shear measurements were performed at test frequencies 2, 0.93, 0.43, and 0.2 Hz to obtain measurements

of the viscoelastic properties of coagulating blood, and the formation of the gel point was recorded. Identification of the gel point was then used to find (1) the time taken to reach the gel point, T_{GP} ; (2) the shear elastic modulus at the gel point, G'_{GP} ; and (3) the fractal dimension of the clot, d_f . A more detailed description of the gel point technique can be found in the Supplemental Digital Content (<http://links.lww.com/AA/B244>).

Close temperature control was achieved through an integrated Peltier concentric cylinder system (valid between –10°C and 150°C and is accurate within 0.1°C). The temperatures were checked using a thermocouple (P.I.8013, Portec type K thermometer, Wrestlingworth, United Kingdom). The process was repeated for all temperatures studied. The time taken for whole blood to reach the test temperatures after being loaded onto the testing surface (within 0.2°C) never exceeded 10 seconds.

Computational Simulation

In conjunction with the gel point measurements, we also provide a previously published computational simulation.²⁸ This simulation was used alongside the experimental data collected from the gel point measurements to help illustrate how any changes in incipient clot microstructure (d_f) will relate to changes in the mass of the clot. This computational simulation will use the established relationship between incipient fibrin clots and their fractal properties, where the mass, M , is related to d_f by the following power law equation: $M \approx \varepsilon^{d_f}$, where ε is a length scale value in the range 100 nm to 10 μm .²⁹

Standard Coagulation Markers

The second aliquot of blood (4.5 mL of the remaining blood) was immediately transferred to a siliconized glass Vacutainer (Becton-Dickinson, Plymouth, United Kingdom; Ref: 367691) for routine coagulation studies, including prothrombin time, activated partial thromboplastin time, and fibrinogen concentration measured using the method of Clauss.³⁰ These were measured with a Sysmex CA1500 analyzer (Sysmex Co., Milton Keynes, UK) within 2 hours of collection. Fibrinogen concentration was verified against the second International Fibrinogen Standard version 4 (NIBSC code: 96–612; NIBSC, Hertfordshire, UK). All reagents were obtained from Siemens (Frimley, UK). The analyzer was calibrated according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using Minitab® version 16 software (Minitab Inc., Havertown, PA) and GraphPad Prism® version 6.0 (GraphPad Software Inc., La Jolla, CA). To establish at what temperature significant changes in d_f would occur compared with the value at 37°C, the number of participants per temperature was calculated by a power calculation. The sample size of this study was based on the previous studies, where the d_f at 37°C was 1.73 ± 0.035 ,¹³ and expecting a decrease in d_f of approximately 0.06 as temperature is decreased into the moderate or severe hypothermic range (below 33°C). Assuming an α of 0.05 and a power of 0.90, we calculated that 8 subjects would be required for each studied temperature, with a total of 17

Table 1. The Normal Coagulation Profile of the Study Participants with Normal Ranges for Reference

Coagulation profile	Result	Normal range
PT (s)	10.4 ± 0.4	9–12.5
aPTT (s)	25.7 ± 1.9	22.1–30.9
Fibrinogen Clauss (g/L)	2.9 ± 0.5	1.5–4.2
Hemoglobin (g/L)	149 ± 15	130–180
Platelets (× 10 ⁹ /L)	263 ± 58	150–400
Hematocrit (vol/vol)	0.441 ± 0.039	0.40–0.52

PT = prothrombin time; aPTT = activated partial thromboplastin time.

different temperature graduations (ranging from 27°C to 43°C in 1°C intervals) giving a total of 136 participants. We used the Anderson-Darling method and inspection of the distribution of the residuals to ascertain that T_{GP} , G'_{GP} , and d_f were normally distributed. One-way analysis of variance was performed to allow for a Bonferroni-corrected multiple comparisons test to be performed post hoc, which was used (for all 17 different groups) to determine the exact temperatures when true differences arose from the values at 37°C. Pearson correlation was used to explore associations among temperature, d_f , T_{GP} , and G'_{GP} .

RESULTS

The standard coagulation measurements for all 136 volunteers are reported in Table 1. These results confirmed that all subjects had normal coagulation. At 37°C, the values of d_f (mean ± SD, 1.73 ± 0.039) and T_{GP} (mean ± SD, 231 ± 55) were similar to the values of those reported for healthy volunteers measured in 2 previous studies.^{11,14,16} Figure 1 shows that increasing or decreasing temperature > or < 37°C results in incremental changes in the gel point measurements. Progressive increases in temperature from 27°C to 43°C resulted in a progressive increase in both d_f and G'_{GP} , showing significant positive correlations with temperature (d_f : $r = 0.777$, $P < 0.0005$; G'_{GP} : $r = 0.513$, $P = 0.0008$). T_{GP} decreased as temperature increased ($r = -0.641$, $P < 0.0005$). Moreover, d_f decreased from (mean ± SD) 1.73 ± 0.039 at 27°C with the change becoming significant at 32°C ($d_f = 1.67 ± 0.037$; Bonferroni-corrected $P = 0.0093$; 95% confidence interval of the difference, 0.010–0.120). A significant decrease in T_{GP} occurs at < 29°C (Bonferroni-corrected $P = 0.0317$; 95% confidence interval of the difference, -285 to -7). By comparison no significant change was observed for G'_{GP} as temperature decreased from 37°C (Bonferroni-corrected $P > 0.75$ for all the different temperature groups in this range). Correlations were found between T_{GP} and d_f ($r = -0.562$, $P < 0.0005$) and between d_f and G'_{GP} ($r = 0.719$, $P < 0.0005$).

The computational simulation results (Fig. 2A) showed that as d_f decreases with corresponding decreases in temperature (specifically ≤ 32°C), large decreases in clot mass are observed. Figure 2B shows that a d_f of 1.65 (corresponding to a temperature of 28°C) would produce a clot that contains approximately 40% the mass from a clot formed with a d_f of 1.73 (37°C; see Fig. 2C). Figure 2A shows that the small incremental increases in d_f would require exponential increases in mass. Figure 2D shows that a d_f of 1.80 (some of the highest single values observed) would require

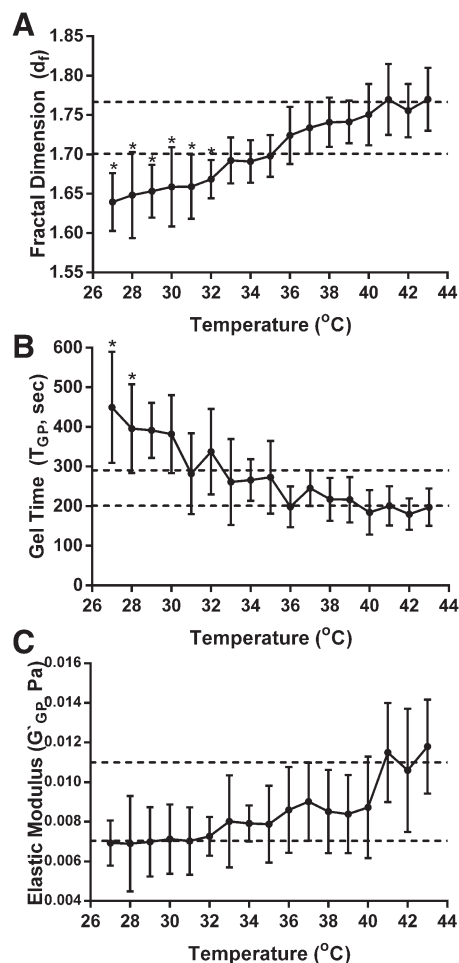


Figure 1. Graphs of the changes in (A) fractal dimension (d_f), (B) time to the gel point (T_{GP}), and (C) elastic modulus (G'_{GP}) with progressive alterations in temperature. A, At lower temperatures, a reduced value of d_f is observed, which represents a poorly formed, weak, porous clot. The reverse is seen at high temperatures, with a stronger, more densely formed clot produced. B, As temperature is decreased a prolongation in T_{GP} is observed indicating a hypocoagulable effect. C, A progressive increase in G'_{GP} is observed as temperature is increased, indicating a general increase in clot strength at higher temperatures ($P < 0.05$, 1-way analysis of variance Bonferroni multiple comparisons test). Dotted lines represent the mean and SD at 37°C.

a structure with 120% additional mass compared with one formed at a d_f of 1.73.

DISCUSSION

In this study, we observed significant changes in the gel point measurements when temperatures fell into the hypothermic range but not when they increased into the hyperthermic range. Furthermore, we found that the structural organization of the clot (d_f) is related to the kinetics of clot formation (T_{GP}) and its elastic properties (G'_{GP}) documented as significant correlations found between d_f and T_{GP} and d_f and G'_{GP} .

Hypothermia is common in critical illness, trauma, and during surgery, and it has been linked with excessive bleeding, blood transfusion, and poor outcomes.^{1,3,31} Previous in

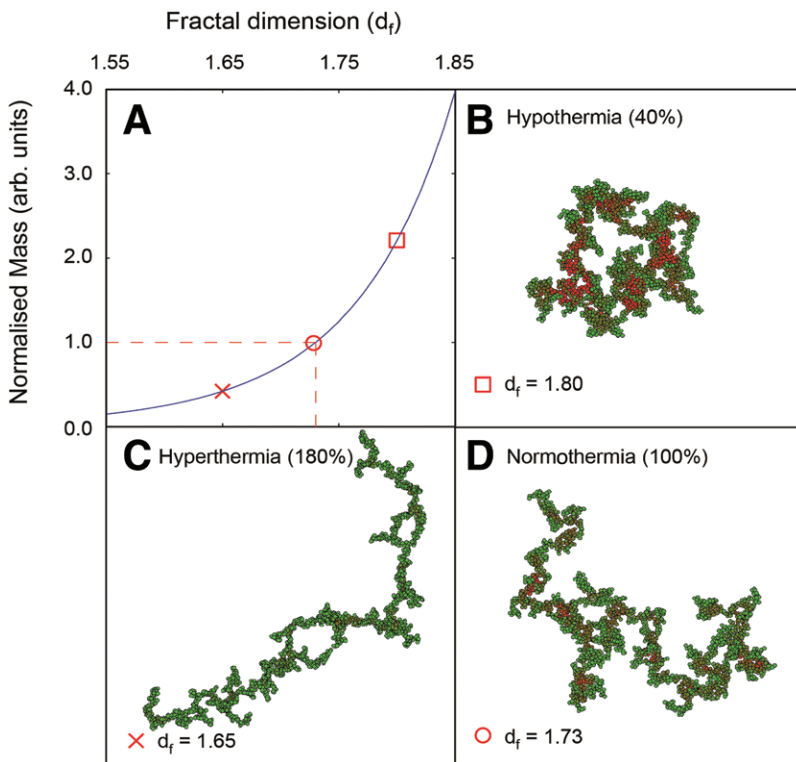


Figure 2. Computational simulation of fractal dimension d_f versus mass. A, Graph showing the relationship between the value of d_f of a particular structure and the mass incorporated into that structure. Substantial increases in mass are required to generate small increments of d_f . The mass value on the y-axis is normalized for the healthy index value of d_f (=1.73). B–D, Representations of different incipient microstructures that correspond to particular values of d_f are provided (cross = 1.65, circle = 1.73, and square = 1.80). The color of each node (unit sphere) within the fractal represents the local density of constituent nodes within a sphere of radii 5 units, and the color ranges from green (1 neighboring node) to red (20 neighboring nodes).

vitro studies have shown that temperatures ranging from 33°C to 35.9°C (mild hypothermia) have a minimal effect on the enzymatic activity of coagulation factors; however, reduced platelet adhesion and aggregation are observed.^{7,8} Temperatures below a threshold of 36.4°C and 34°C decrease the activation of glycoprotein IIb/IIa and P-selectin, respectively, processes important for platelet adhesion.³¹ Because temperature is decreased below 33°C, into the moderate (32.9°C–28°) and severe (<27.9°C) hypothermic ranges, a further inhibitory effect on platelet activity is observed.^{7,8,32} The inhibitory effect on platelets is coupled with a progressive decrease in the enzymatic activity of coagulation factors, where at 25°C, enzyme activity ranges from 0% (FVIII and FIX) to 5% (FII and FVII).³³

In this study, we report a progressive decrease in d_f and G'_{GP} with a prolongation of T_{GP} as temperature is reduced below 37°C (see Fig. 1). The progressive changes observed in d_f and T_{GP} with hypothermia are indicative of a worsening hypocoagulable state, producing d_f and T_{GP} values more commonly associated with anticoagulated or diluted blood.^{14,16} However, the changes in d_f do not become significant until a temperature of 32°C is reached, where d_f decreases from 1.73 ± 0.040 at 37°C to 1.67 ± 0.037 at 32°C ($P = 0.0093$). In contrast to standard coagulation testing, the gel point technique includes the vital contribution of platelets and cellular components of coagulation. However, over the temperature range from 33°C to 35.9°C, the observed inhibitory effect of reduced temperature on platelet adhesion does not seem sufficient to produce significant changes in the gel point parameters. When temperatures reach those synonymous with moderate hypothermia (<33°C), the additional inhibition of platelet and coagulation enzyme activity is likely to be the cause of this significant

reductions in d_f . We observed a significant change in T_{GP} only when the temperature decreases to 29°C (245 ± 44 at 37°C to 391 ± 69 at 27°C, $P = 0.0317$), and no significant change was observed in G'_{GP} ($P > 0.75$).

Hyperthermia is associated with hypercoagulability, where increases in temperature result in an increase in thrombin generation.⁹ The results of this study, however, show that increases in temperature >37°C and up to 43°C (temperatures synonymous with hyperthermia) had no significant effect on d_f , G'_{GP} , or T_{GP} (Fig. 1). This finding suggests that, in hyperthermic conditions, temperature alone does not significantly affect coagulation.

To better understand the changes we observed with d_f , we performed computational analysis that illustrate the nonlinear relationship between d_f and clot mass at the incipient clot stage of development. When temperature is reduced, a progressive decrease in d_f is observed, indicating a decrease in the complexity of the clot microstructure. This is illustrated in Figure 2, where a clot with $d_f = 1.67$ (33°C) has approximately 60% of the mass of a clot for which $d_f = 1.73$ (37°C). This value is reduced further at the lowest temperature investigated in this study (27°C), with a mean d_f of 1.62, where the clot would contain approximately 40% of the mass of a clot formed at 37°C. Small increases in d_f will result in an exponential increase in the mass of the clot network. A change in d_f from 1.73 to 1.80, for example, indicates an increase of 120% in the clot mass. The consequences of increased clot mass are 2-fold. First, it will affect clot lysis, because there is an increase in polymerized fibrin that is more tightly packed. Second, more polymerized clot mass will result in increases in clot elasticity, which is supported by the significant positive correlation we observed between d_f and G'_{GP} . This may be understood in terms of

the viscoelasticity of cross-linked polymeric gel networks in which G' is a function of polymer chain length between cross links. For idealized polymer systems with fixed cross links, increases in both G' and d_f will result from an increased cross link density.³⁴

The major limitation of the study was that gel point measurements at each of the different temperatures for each of the volunteers were not performed. The test is designed to be used in a near-patient setting, and it is performed with the use of whole nonanticoagulated blood. Hence, because of the limited numbers of AR-G2 instruments available and to avoid multiple blood samplings in each volunteer, not all 17 temperatures were studied in each subject. Thus, blood from different healthy volunteers was used at each temperature testing condition. To limit any possible variations between the testing conditions, all participants were determined to have normal coagulation. The gel point measurements are taken at the initial or incipient stage of clot development and are not measures of the mature clot. This has the advantage of providing a very rapid measure of the clotting properties. In addition, we have shown previously how the incipient clot acts as a template of mature clot growth²⁸ and that d_f is significantly correlated with other viscoelastic measurements of the mature clot.¹⁶ Regardless, this study highlights the potential of the gel point technique to measure the effects of temperature on coagulation. The gel point measurements have now been validated in the models of anticoagulation, dilution, and temperature.^{14,16} The results of this study suggest that preventing moderate-to-severe hypothermia (<32°C) and maintaining normothermia could be important for maintaining normal clot microstructure formation. However, the changes in temperature >37°C (hyperthermic conditions) do not seem to have such a pronounced effect on clot microstructure. Our results highlight the need for further clinical investigations, such as studying the role of temperature in critical illnesses such as sepsis and in patients with burn injury. ■■

DISCLOSURES

Name: Matthew James Lawrence, MEng, PhD.

Contribution: This author helped design the study, conduct the study, collect the data, analyze the data (rheology), and prepare the manuscript.

Attestation: Matthew James Lawrence approved the final manuscript and attests to having reviewed the original data reported in the manuscript.

Conflicts of Interest: The author declares no conflicts of interest.

Name: Nick Marsden, BSc, MBCh, MRCS.

Contribution: This author helped recruit the subjects, collect the data, design the study, and prepare the manuscript.

Attestation: Nick Marsden approved the final manuscript.

Conflicts of Interest: The author declares no conflicts of interest.

Name: Rangaswamy Mothukuri, MBBS, MRCS(Edin), FCEM, PG Cert.

Contribution: This author helped recruit the subjects, collect the data, design the study, and prepare the manuscript.

Attestation: Rangaswamy Mothukuri approved the final manuscript.

Conflicts of Interest: The author declares no conflicts of interest.

Name: Roger H. K. Morris, BSc, PhD.

Contribution: This author helped design the study, statistical design and analysis, and prepare the manuscript.

Attestation: Roger H. K. Morris approved the final manuscript and the statistical analysis reported in the manuscript.

Conflicts of Interest: The author declares no conflicts of interest.

Name: Gareth Davies, BSc.

Contribution: This author helped collect the data (rheology), revise the article for scientific and intellectual content, and prepare the manuscript.

Attestation: Gareth Davies approved the final manuscript.

Conflicts of Interest: The author declares no conflicts of interest.

Name: Karl Hawkins, MEng, PhD.

Contribution: This author helped revise the article for scientific and intellectual content, interpret the data, and prepare the manuscript.

Attestation: Karl Hawkins approved the final manuscript.

Conflicts of Interest: The author declares no conflicts of interest.

Name: Daniel J. Curtis, MEng, PhD.

Contribution: This author helped revise the article for scientific and intellectual content (computer simulation), interpret the data (computer simulation and rheology), and prepare manuscript.

Attestation: Daniel J. Curtis approved the final manuscript.

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Name: Martin Rowan Brown, MPhys, PhD.

Contribution: This author helped design the study, analyze the data, revise the article for scientific and intellectual content (computer simulation), perform the computer simulation, and prepare the manuscript.

Attestation: Martin Rowan Brown approved the final manuscript.

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Name: Phylip Rhodri Williams, FICHEM, FInstP, FRSA, FLSW

Contribution: This author helped design the study, analyze the data, revise the article for scientific and intellectual content (rheology), interpret the rheological findings, and prepare the manuscript.

Attestation: Phylip Rhodri Williams approved the final manuscript.

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Name: Phillip Adrian Evans, MD, MBBS, FRCS, FFAEM.

Contribution: This author helped design the study, analyze and interpret the data, and approved the final version to be submitted.

Attestation: Phillip Adrian Evans approved the final manuscript and attests to having reviewed the original data reported in the manuscript, and is the archival author and the corresponding author.

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