Gel Point and Fractal Microstructure of Incipient Blood Clots are Significant New Markers of Haemostasis for Healthy and Anticoagulated blood

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ABSTRACT
This paper reports the first application of a fractal analysis of the viscoelastic properties of incipient blood clots. The study sought to ascertain whether the incipient clot’s fractal dimension, $D_f$, could be utilized as a functional biomarker of haemostasis. The incipient clot is formed at the Gel Point, GP, of coagulating blood, the GP demarcating a functional change from viscoelastic liquid to a viscoelastic solid. Incipient clots formed in whole healthy blood show a clearly defined value of $D_f$ within a narrow range which represents an index of clotting in health, where $D_f = 1.74 \pm 0.07$. A significant relationship is found between the incipient clot formation time, TGP, and the activated partial thromboplastin time (APTT), while the association of $D_f$ with the microstructural characteristics of the incipient clot is supported by its significant correlation with fibrinogen. The study reveals that unfractionated heparin not only prolongs the onset of clot formation but has a significant effect on its fractal microstructure. A progressive increase in unfractionated heparin concentration results in a linear decrease in $D_f$ and a corresponding prolongation in $T_{GP}$. The results represent a new, quantitative measure of clot quality derived from measurements on whole blood samples.
INTRODUCTION
Coagulation pathway changes play an important role in the outcome of both clot propagation and fibrinolysis. The structure-function relationship of the developing fibrin clot is known to be affected by many factors such as environmental, therapeutic and disease when compared to normal clot growth. A fibrin clot’s primary microstructure consists of a disordered network of entangled, branching fibrin fibers. Thinner fibers are associated with networks which display an increased number of branch points, creating denser, less permeable clots which have a known association with thromboembolic disease. More open/permeable networks are formed from thicker fibers, the latter displaying a reduced number of branch points for a given amount of fibrinogen and producing a more porous system. Clots with altered fibrin microstructure exhibit different susceptibility to fibrinolysis, clot permeability being the rate limiting factor for the activity of the fibrin network degradation enzyme plasmin. The permeability will aid or hamper the ability of tissue plasmin activator, tPA, and/or urokinase, uPA, to move through the three dimensional fibrin network and activate the zymogen plasminogen to fibrinolytic plasmin. The effect of anticoagulants such as heparin in the therapeutic manipulation of fibrin clot microstructure by thrombin inhibition increases clot permeability/porosity and produces clots with thicker fibres.

The evolution of clot microstructure is associated with significant changes in blood viscoelasticity (a measure of a material’s viscous and elastic properties). Viscoelastic properties are among the most sensitive measures of fibrin polymerization and blood clot structure. The present study focused on the formation of the incipient clot which provides the microstructural template which determines the future clot morphology. It involved measurements of the incipient clot’s viscoelastic properties by an oscillatory shear technique known as Fourier transform mechanical spectroscopy, FTMS. This technique provides an accurate determination of the Gel Point of coagulating blood and allows the microstructure of the incipient clot to be quantified by fractal analysis – a technique widely used in medicine and biology to characterize non linear growth in branching network structures. Previous studies of clot structure based on techniques such as scanning electron microscopy have reported qualitative descriptions of clot microstructure (involving terms such as “rigid clot structures”, “open / porous / dense / loose” etc) while studies of the fractal properties of fibrin gels based on light scattering techniques have been restricted to dilute solutions of fibrinogen, at concentrations below those of physiological relevance in whole blood.

A recent study of fibrin clot structure suggests a definitive diagnostic potential of characterizing clot structure and the modulation of clot architecture as a possible treatment for thrombosis. As a result there is a growing need to provide a functional biomarker in terms of a ‘Healthy Index’, for normal clotting, from which the effect of therapeutic manipulation and disease on clot quality and outcome can be monitored. This was one of the aims of the present study which sought to investigate the value of $D_f$ characterising incipient clots formed in samples of whole, unadulterated blood drawn from healthy subjects. The purpose of this investigation was to seek to establish a Healthy (i.e. normal) Index to represent an optimal value of incipient clot microstructure in terms of $D_f$. Further, by manipulation of the healthy blood using unfractionated
heparin, the investigation sought to ascertain whether altering the coagulation pathways and inhibiting thrombin production alters the value of $D_f$. Heparin significantly modifies fibrin assembly and clot structure by its effect on the coagulation pathways, with increasing levels of heparin causing the formation of thicker fibrin fibres,\textsuperscript{13} and this could be expected to correspond to a lower value of $D_f$ due to the concomitantly increased volume of the pore spaces. Another aim of this study involved comparing changes in $D_f$ against standard laboratory coagulation markers and thromboelastography.

**MATERIALS AND METHODS**

**Healthy Group**

The healthy group involved strict exclusion criteria to ensure all participants could be considered healthy. The exclusion criteria eliminated from the study any individuals who were taking anticoagulant or antiplatelet therapy and who had any personal or family history of a bleeding disorder or thromboembolic diseases as well as any acute illness, cancer, hepatic and/or renal dysfunction. The healthy group consisted of (n =) 52 healthy individuals (29 males, 23 females, mean age 33.6 years, range 20-69).

**Anticoagulant Group**

The anticoagulant group consisted of (n =) 38 healthy adults sampled from the healthy group (25 males, 13 females, mean age 35.2 years, range 25-55) subject to the same exclusion criterion as the control group. To study the effect of inhibiting thrombin production on the incipient clot, small volumes (<10μl) of unfractionated heparin (from stock concentration 1000 IU/ml Monoparin, CP Pharmaceuticals, UK) were added to 20ml of whole blood collected in vitro which produced an effective anti Xa concentration range 0.05–0.80 IU/ml. The comparatively small volume of heparin added to the larger bulk volume of blood (<0.05%vol) minimized any dilution effect. The heparin and blood were well mixed and transferred immediately to the test instruments. The range of heparin concentration was chosen with reference to the American College of Chest Physicians’ Conference on Antithrombotic Therapy Consensus recommendation regarding the monitoring of unfractionated heparin in the treatment of venous thromboembolism.\textsuperscript{24} The dose of unfractionated heparin was adjusted in order to prolong the activated partial thromboplastin time (APTT) to a range corresponding to a heparin level of 0.3 to 0.7 U/mL by heparin anti-factor Xa analysis. In the present study this range corresponded to values of the APTT > 60 seconds.

**Blood Sampling and Data Collection**

This study was undertaken with full ethical approval from the local regulatory ethics committee of South West Wales. The ethical approval was undertaken in two stages involving patient information and fully informed written consent. At all times the study followed the STARD guidelines\textsuperscript{25} on the validation of new diagnostic testing. In all cases blood was taken slowly andatraumatically from the antecubital vein via a 21-gauge butterfly line into a 20ml syringe.
**Laboratory Markers**

Four-ml aliquots of blood from the bulk sample were used for full blood count (FBC) analysis including a platelet count, samples being collected into plastic, full-draw dipotassium EDTA Vacuette (Greiner Bio-One, Stonehouse, UK Ref: 454286). FBC was analysed using a Sysmex XE 2100 (TOA Medical Electronics, Kobe, Japan) automated haematology analyser within 2 hrs of collection. The analyser was calibrated according to manufacturer’s instructions.

A further 4.5 ml of the venous blood sample was used for routine coagulation studies, being collected into siliconized glass Vacutainers (Becton-Dickinson, Plymouth, UK Ref: 367691). PT, APTT and total clotting time (TCT) were measured using a Sysmex CA1500 analyser within 2 h of collection by scattered light detection (percentage test end point method). TCT measurements involved adding 100µL thromboclotin to 100µL sample. Clauss Fibrinogen concentration was determined by addition of 10µL sample to 90µL Owren’s buffer followed by addition of 50µL thrombin. The time to clot was recorded and the concentration obtained from a standard calibration curve. Fibrinogen calibration was checked against the 2nd International Fibrinogen Standard Version 4 (NIBSC code 96/612). All reagents were obtained from Dade Behring, (Eschborn, Germany). Analysis of heparin concentration was performed on the Sysmex CA-1500 using a chromogenic anti-Xa assay by Biophen Heparin 3 (Hyphen Biomed) supplied by Quadrattech, UK.

**Rheometry**

The ‘Gel Point’, GP,\(^{26}\) defines the rheological transition between an elasticoviscous fluid and a viscoelastic solid. The clot’s haemostatic function requires the properties of a viscoelastic solid thus the GP identifies the establishment of the incipient clot. At the GP, the elastic and viscous components of the complex shear modulus \(G^*\) (the dynamic rigidity, \(G'\), and loss modulus, \(G''\), respectively) scale as power-laws in frequency, \(\omega\), as \(G'(\omega) \sim G''(\omega) \sim \omega^\alpha\). This feature enables the GP to be identified unambiguously by the corresponding frequency independence of the loss tangent, \(\tan \delta = (G''/G')\)\(^{18-20}\) where \(\delta\) represents the phase angle between stress and strain waveforms in small amplitude oscillatory shear measurements and is related to the exponent \(\alpha\) as \(\delta = \alpha \pi /2\).

The fractal characteristics of the 3-dimensional network cluster formed at the GP have been extensively studied and are described in various theoretical treatments of polymerisation and gelation in a wide range of systems. One such treatment, known as the percolation theory, describes the GP in terms of a connectivity transition. Below the GP, isolated clusters formed from polymerised monomers represent the sol phase whereas at the GP a polymeric cluster establishes sufficient connectivity to become ‘sample-spanning’, thereby conferring elastic solid-like properties upon the system. The percolation theory defines the polymerising system as macroscopically homogeneous at a length-scale \(L >> \varepsilon\) whereas for \(L << \varepsilon\) the sample-spanning network cluster is a ‘fractal’ object whose mass \(M\) scales with \(\varepsilon\) as \(M \sim \varepsilon^D\) where \(D_f\) is the fractal dimension. The value of \(D_f\) is calculated from analysis of the viscoelastic data at the GP using the established relationship\(^{27}\) \(D_f = (D + 2)(2\alpha - D)/2(\alpha - D)\) where \(D\) is the space dimension.
(D = 3 herein). The higher the value of $D_f$ the more compact is the network structure, whereas low values of $D_f$ correspond to more open/permeable networks.

Aliquots (10ml) of blood were transferred directly and immediately after sampling (<60 seconds) to the custom acrylic coaxial cylinder geometry (1mm shearing gap) of a TA Instruments ARES rheometer. All work was conducted at 37°C, all measurements being made on aliquots of the same sample, using similar measuring geometries, with identical measuring surfaces and surface preparation procedures. Sequential frequency and FTMS measurements of $G'$ and $G''$ were made in the linear viscoelastic regime immediately after sample loading, and the GP was identified by attainment of frequency independence of $\tan \delta (= G'' / G')$. The corresponding value of $\alpha$ was used to calculate $D_f$. The value of $D_f$ and the time, $T_{GP}$, taken to reach the GP were recorded and the results were correlated with other markers of haemostasis.

**Thromboelastography**

Alongside the rheometric measurements, aliquots from the same bulk blood samples were analysed using a thromboelastograph, TEG (Haemoscope 3000 Clot Analyzer). All work was conducted at 37 °C using plain cuvettes. The TEG data recorded included (i) the ‘R-time’ the time elapsed between the start of data collection to a pin movement greater than 2mm on the thromboelastogram; and (ii) the TMA – the time which elapses between the start of data collection to the maximum amplitude of the thromboelastogram; and (iii) the MA value or maximum amplitude of the thromboelastogram; and (iv) the ‘clot lysis index’ LY30—the amplitude at 30 minutes, expressed as a percentage of the MA value.

**Statistical Analysis**

Statistical analysis was performed using Minitab version 15 software (Havertown, PA). Pearson correlation coefficients were calculated on data assumed to be normally distributed. Two sample differences were calculated using the two-sample t-test. Standard and multiple regression analysis were performed to identify significant relationships between variables, with multicollinearity identified by calculating variance inflation. Data was assumed to be significant when p<0.05 throughout. The rheological analysis is performed on freshly drawn samples of whole blood. To determine its reproducibility we calculated the Coefficient of Variation (CV) for both $T_{GP}$ and $D_f$ for three normal samples and for samples with high and low levels of heparinisation. Both parameters were consistent in their measurability with CV’s of less than 5% for all the conditions tested.

**RESULTS**

**Healthy Group**

**Laboratory Markers & Thromboelastography:** The results of the full blood count (platelets, Haematocrit etc), coagulation screen (Fibrinogen (Clauss), PT, APTT, TCT) and thromboelastography (SP, R-time, TMA & Lysis30) were within the expected
normal ranges for each of the parameters, excluding four (n = 4) individuals who presented with abnormal values in one or more of the tests leading to these individuals being removed from the study.

**Gel Point (T_{GP}) and Fractal Dimension (D_f):** Analysis of the viscoelastic data obtained for samples of whole, unadulterated blood drawn from healthy subjects indicates a clearly defined value of $D_f$, within a narrow range, which represents an index of clotting in health, where $D_f = 1.74 \pm 0.07$. We refer herein to this clearly defined value of $D_f$ as a ‘Healthy Index’. Data analysis (Table 1) demonstrates that $T_{GP}$ correlates significantly with $D_f$, platelet count and the APTT. The association of $D_f$ with the microstructural characteristics of the fibrin network is supported by its significant correlation with fibrinogen. The APTT result is particularly interesting since it is regarded as the most important global measure of the classical intrinsic or common coagulation pathways (in this regard the PT is a relatively insensitive measure). These results suggest that the GP is significantly associated with global coagulation.

**Anticoagulant Group**

**Laboratory Markers & Thromboelastography:** Increased concentrations of unfractionated heparin resulted in a marked increase in all the parameters designed for monitoring ‘clotting time’ or ‘clot growth with respect to time’ (PT, APTT, TCT, SP, R-time and TMA). Concentrations of cells and proteins, remained within normal ranges (FBC and Fibrinogen (Clauss)).

**Gel Point (T_{GP}) and Fractal Dimension (D_f):** Gel Point analysis further confirmed the existence of significant correlations (p<0.05) between $T_{GP}$, $D_f$, APTT and also anti-FXa (Table 2 & Figure 1). Interestingly, as anti-FXa concentration increases a decrease in $D_f$ is observed, indicating that highly heparinised blood produces incipient clot microstructures that are more open/permeable than those produced at lower heparin concentrations. Figure 2 shows examples of the evolution of $\delta$ in samples for which the anti-FXa concentration is 0.52 U/ml (Figure 2(a)) and 0.76 U/ml (Figure 2(b)). In viscoelastic systems $\delta$ has values in the range $0^\circ < \delta < 90^\circ$, where $0^\circ$ and $90^\circ$ represent the values of $\delta$ which characterize an ideal elastic solid and a Newtonian viscous fluid, respectively. The results illustrate the pre-incipient clot viscoelastic fluid response, with increasing frequency of oscillation causing $\delta$ to decrease. The frequency dependence of $\delta$ decreases progressively as coagulation proceeds, becoming frequency independent as the incipient clot is established at the GP. Thereafter, the frequency dependence of $\delta$ is characteristic of a viscoelastic solid.

There was a significant prolongation (p<0.05; two-sample t-test) in the mean value of $T_{GP}$ in samples treated with heparin (mean 16.28 ± S.D.5.75) as compared to non-heparin treated samples (mean 4.01± S.D.1.63). Furthermore, there was a significant reduction (p<0.05; two-sample t-test) in the value of $D_f$ for incipient clots formed in the heparinised samples as compared to normal samples, with the lowest recorded value of $D_f$ (1.58) indicative of a highly tenuous and friable network cluster at the highest heparin concentration. It is noteworthy that the y-intercept for the best-fit line, ($D_f = 1.74$ for anti-
FXa = 0) is highly predictive of the ‘Healthy Index’ value found for normal unadulterated blood samples ($D_f = 1.74 \pm 0.07$). At concentrations of heparin within the therapeutic range, and up to a concentration of 0.8 U/ml, significant correlation was observed with $D_f$ (for which, $r = 0.733$, $p < 0.05$, see Fig. 2) compared to the TEG R-time ($r = 0.527$, $p < 0.05$, see Fig. 3), or the TEG TMA ($r = 0.500$, $p < 0.05$, see Fig. 3). It should be noted that the TEG data presented in Figure 3 suggests that the R-time can only be detected accurately by the TEG at low concentrations (<0.4U/ml) of anti-FXa where the reduction in the complexity of clot structure is arguably less affected. The results of a multi-regression analysis (which included TEG parameters) revealed $D_f$, together with APTT, to be the most significant predictor of anti-FXa.

DISCUSSION
There have been previous attempts, by various methods, to investigate the relationship between abnormalities or alterations in coagulation pathways and clot quality. A definitive diagnostic potential of characterizing clot structure and the modulation of clot architecture has been suggested and there is an evident need to provide a functional biomarker in terms of a ‘Healthy Index’ for normal clotting. The present investigation involving samples of whole, unadulterated blood drawn from healthy subjects, suggests that such a Healthy Index representing an optimal value of incipient clot microstructure can be established in terms of $D_f$. Moreover, the present study shows how the functional relationship between changes in coagulation pathways and eventual clot outcome in anticoagulated blood can be explored by utilizing changes in $D_f$. Interestingly, the narrow range of values of $D_f$ obtained for samples of whole healthy blood (which could be considered the optimum structural template to maximize clot function) is in excellent agreement with predictions of the percolation theory of polymerization and gelation for a heterogeneous system at the sol-gel transition (GP), and similar to that characterizing fractal structures in other biological systems formed by non-linear growth processes (e.g. the bronchial branching system in the lung). In addition, this study demonstrates that by using this new rheological technique, the alteration of fibrin microstructure by manipulation of thrombin production using unfractionated heparin can be recorded over a much wider range, and with greater linearity of response, than by the long established and widely used technique of thromboelastography.

The finding that a progressive increase in heparin concentration within the therapeutic range results in a corresponding decrease in $D_f$ is particularly significant in the context of the incipient clot’s role as a microstructural template for ensuing clot development. The results reported herein demonstrate that the effect of heparin is not confined to prolongation of the onset of clot formation but that it has a significant effect on clot microstructure. In this respect our results confirm the findings of a previous study of the effect of heparin on fibrin assembly and clot structure. That study evaluated the sensitivity to t-PA-induced lysis of clots prepared from plasma preincubated in vitro with therapeutic concentrations of heparin. The extent of t-PA-induced lysis was significantly increased by preincubation with heparin and a turbidimetric assay revealed that heparin significantly modified fibrin assembly and clot structure. It was concluded that due to the formation of thicker fibrin fibres, the effect of heparin on clot sensitivity to lysis could be attributed to an increased permeability of the clots to fibrinolytic components. This would
correspond to a lower value of $D_f$ due to the concomitantly increased volume of the pore spaces. The present work is the first to report such an effect of heparin on the fibrin network structure of incipient clots formed in samples of whole blood. In this respect, and given the incipient clot’s templating role, it is interesting to note the significant correlation at low concentrations of heparin between $D_f$ and the TEG-derived clot lysis index (LY30) which is related to breakdown of the fully-formed clot.

The importance of $D_f$ in predicting the level of anticoagulation when heparin is added to whole blood in-vitro was confirmed by the results of a multi-regression analysis (which included TEG parameters) which revealed $D_f$, together with APTT, to be the most significant predictor of anti-FXa. Gel Point and fractal analysis can provide a reproducible measure of clotting status in whole blood in a near-patient setting. The present study suggests that $D_f$ and $T_{GP}$ provide significant global markers of haemostasis by determining accurately changes in anticoagulant status of whole blood and determining the functional relationship between pathway changes and eventual clot quality and outcome. The ability of the FTMS technique to provide time-resolved (hence more accurate) measurements of viscoelastic change during coagulation, and an unambiguous determination of the GP, are important rheometric features which could be exploited in future hemorheological studies to supplant present methods such as the TEG.

The results from this study demonstrate a significant relationship between coagulation pathways and a new, quantitative assessment of the complex, highly disordered microstructure of incipient clots in healthy and anticoagulated blood. Previous attempts at measuring clot quality have led to only subjective functional outcomes when clot structure has been associated with the coagulation pathways. Consequently, our findings increase significantly the understanding of clot development in whole blood and introduce a new reproducible measure of clot structure in both normal blood and in blood treated with the anticoagulant heparin. Many disease states affect global haemostasis and clot structure. Our present work is now being extended to clinical studies of thrombotic patients to investigate whether Gel Point analysis and fractal dimension will provide functional biomarkers in such cases.

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Authorship Contributions and Disclosure of Conflicts of Interest
Contribution: K.H. and M.J.L. performed all rheometrical and thromboelastogram experiments. N.T. was responsible for recruiting, consenting and collecting blood samples from volunteers. L.W. and R.M. organized all coagulation and full blood count screens. R.H.K.M. performed the statistical analysis and interpreted the results. P.A.E.
and P.R.W. designed the research, analyzed and interpreted the results, and wrote the paper.
Conflict-of-interest disclosure: The authors declare no competing financial interests. The EPSRC grant (EP/C513037/1) and the Brian Mercer Feasibility Award were both obtained for translational research for developing techniques from different backgrounds for clinical research.
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References


Tables

Table 1: Pearson correlation coefficients for Gel Time ($T_{GP}$) and Fractal Dimension ($D_f$) vs haemostatic and haematological parameters: Correlation coefficients were calculated against Gel Time ($T_{GP}$) and fractal dimension in normal subjects (n=52). * denotes a significant result (p<0.05).

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<th>Gel Time ($T_{GP}$)</th>
<th>Fractal Dimension ($D_f$)</th>
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*APTT* = Activated Partial Thromboplastin Time; *PT* = Prothrombin Time
Table 2: Pearson correlation coefficient for Gel Time ($T_{GP}$) and Fractal Dimension ($D_f$) vs haemostatic parameters in heparin treated samples

Correlation coefficients were calculated against $T_{GP}$ and $D_f$ in samples treated with a range of unfractionated heparin concentrations (n=38).

| Fractal Dimension ($D_f$) Correlation Coefficient |
|------------------|------------------|
| $T_{GP}$         | -0.483*          |
| Fibrinogen       | 0.345*           |
| APTT             | -0.747*          |
| Anti FXa         | 0.733*           |
| R-time           | 0.527*           |
| MA               | 0.648*           |
| TMA              | -0.500*          |
| Ly30             | -0.548*          |
Figure Legends

**Figure 1:** Regression graph of fractal dimension ($D_f$) vs Anti FXa for blood samples treated with heparin: The best fit regression curve of $D_f$ against Factor Xa calculated for citrated blood samples to which a range of unfractionated heparin had been added (n=38). A significant relationship is clearly visible demonstrating the value of measuring fractal dimension during prolonged and abnormal clotting.

**Figure 2:** Examples of the evolution of the phase angle $\delta$ over a range of test frequencies identifying the Gel Point where frequency independence of $\delta$ is observed: Figure 3a has an anti-FXa concentration of 0.52 U/ml corresponding to $D_f = 1.69$ and Figure 3b has an anti-FXa concentration of 0.76 U/ml resulting in $D_f = 1.58$. Oscillatory shear frequencies are 3.2 Hz (light blue circles); 1.0 Hz (red circles); 0.5 Hz (brown circles); 0.2 Hz (dark blue circles).

**Figure 3:** Regression graph of TEG R-time and TMA vs Anti FXa for blood samples treated with heparin: The best fit regression curve of R-time and TMA against Anti FXa calculated for citrated blood samples to which a range of unfractionated heparin had been added (n=38). For the R-time a significant relationship is observed (particularly at low concentrations of anti FXa < 0.4U/ml) and a general increase in R-time is seen. For the TMA a significant relationship is demonstrated over the whole range of anti FXa. *Inset:* Typical TEG thromboelastogram showing the ‘R-time’, the TMA, the MA value and the ‘clot lysis index’, LY30.
Figures

Figure 1
Figure 2(a)
Figure 2(b)
Figure 3
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