

# RHEOMETRY FOR BLOOD COAGULATION STUDIES

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## ABSTRACT

This review considers some of the various rheometrical approaches that have been adopted to study blood coagulation, with special reference to the rheological assessment of clotting time and studies of the evolution of viscoelasticity during the course of fibrin polymerization and cross-linking. A common feature of many of these studies is that they attempt to detect a liquid-to-solid transition during coagulation and the significance of the Gel Point in blood coagulation studies is discussed. Coagulation studies based on various forms of shear viscosity measurements and complex shear modulus measurements are considered, the latter being based principally on instruments such as the various controlled stress and controlled strain rheometers. Also considered is the long established technique of thromboelastography, while several emerging techniques are described. The latter include damped oscillation rheometry, various forms of wave propagation measurements and other, less widely used techniques such as free oscillation rheometry, quartz crystal microbalance measurements and surface plasmon resonance.

**KEYWORDS:** Blood coagulation; Clot viscoelasticity; Incipient clot; Gel point; Clot formation time.

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## 1. BACKGROUND

### 1.1 Introduction

Venous and arterial thrombo-embolic disease and associated blood coagulation abnormalities cause significant morbidity and mortality in Western society. Pathological processes such as inflammation, sepsis, stasis and vascular wall damage are recognized to be implicated in activation of the blood coagulation cascade (Grignani and Maiolo, [1]; Slofstra *et al*, [2]) and the array of blood coagulation abnormalities facing the clinician in disease states is vast, occurring in many acute illness and chronic disease states. These coagulation abnormalities may be triggered

by the effects of surgery, trauma and even environmental factors, such as sedentary long-distance travel.

Blood coagulation involves the formation of haemostatic plugs called blood clots at lesions of the circulatory system. The process of blood clotting takes place by fibrinogen-fibrin transformation due to the catalytic action of thrombin and the subsequent polymerisation and network formation of fibrin fibres (Bailey *et al* [3]; Shulman *et al* [4]; Ferry *et al* [5]). The diagnosis and treatment of patients with thrombo-embolic conditions involves diagnostic tools, which rely upon conventional markers of coagulation abnormalities such as plasma thromboplastin (PT), partial thromboplastin time (PTT), platelet count and fibrinogen concentrations. Commonly, tests such as PT and PTT are used to measure the pathophysiological effects of disease states on the coagulation cascade and treatment regimes. These tests provide only an isolated measure of specific pathways of the coagulation system, rather than a global and continuous measurement of a coagulation problem.

The rheological technique of thromboelastography, which provides a measure of blood clot viscoelasticity, has been used as the basis of global coagulation profiling (Hartert [6]). Clot formation involves significant changes in viscoelasticity, some of which have been associated with pathologies such as myocardial infarction, peripheral vascular disease, cancer and diabetes. In clinical settings, some therapeutic procedures such as the intravenous introduction of dextran solutions, cardiological haemodilation, and vasodilation, significantly influence blood rheology, with hyperviscosity leading to lower blood flow rates, RBC aggregation and increases in clot formation (Bandey *et al* [7]). Associations between disease states and blood coagulation phenomena have stimulated increasing interest in blood viscoelasticity among researchers in clinical medicine and physiology (Isogai *et al* [8]; Chmiel *et al* [9]; Weisel [10]).

Viscoelastic properties are among the most sensitive measures of fibrin polymerization and clot structure and there are numerous examples of their physiological importance (Weisel [10]). The connection between disease and clot properties has been reported in terms of an increased incidence of myocardial infarction in patients with elevated levels of plasma fibrinogen, which results in less deformable clots (Scrutton *et al* [11]). In a partially occluded blood vessel the viscoelastic properties of a clot will determine whether the flowing blood causes it to deform reversibly or irreversibly, rupture, or embolize. The viscoelasticity of a blood clot also determines how it responds to treatments, such as coronary artery angioplasty.

## **1.2 Basic aspects of blood coagulation and clot formation**

Fibrinogen is a protein, consisting of two identical halves, each comprised of three peptide chains ( $A\alpha$ ,  $B\beta$ ,  $\gamma$ ), which are held together by a network of disulfide bonds. Fibrinogen is a trinodular structure of about 45nm in length and 4.5 nm in diameter. Upon activation, thrombin proteolytically removes fibrinopeptides A and B from the central node of fibrinogen thus converting it into fibrin and exposing binding sites (knobs) A and B on the central domain. These knobs interact with binding sites

(hole a and b) on the end domains. Fibrinopeptide A is cleaved first and upon the exposure of knob A, fibrin monomers assemble in a half-staggered fashion into two-stranded protofibrils. Upon growing to sufficient length, typically about 15 monomers, the protofibrils aggregate laterally to form fibres that branch into a three-dimensional network (Guthold *et al* [12]). (Release of fibrinopeptide B (exposure of knob B) aids lateral aggregation, but is not absolutely required (Blomback *et al* [13]). Platelets interact directly with the coagulation cascade (Kaibara, [14]) and with polymerised fibrin and form pseudo-crosslinks with fibres, which causes clot retraction (Fukada *et al* [15]). The intrinsic coagulation system is triggered by the activation of factor XII on negatively charged surfaces, causing a sequential activation of circulating zymogens in the blood, which leads to the conversion of prothrombin to thrombin. The extrinsic coagulation process is triggered by factor VII and tissue factor, where a complex is formed to activate factor X. The intrinsic and extrinsic coagulation systems subsequently unite. In vivo the network of fibrin fibres forms the primary microstructural basis of a clot whereas in vitro they form a fibrin gel. Fibrin gels are the main structural scaffolds of the haemostatic plug formed in vertebrate blood coagulation (Ferri *et al* [16]).

### **1.3 The significance of viscoelasticity in blood coagulation.**

The time required for conversion of the plasma protein fibrinogen into a clot network – the ‘Clotting Time’ – is an important parameter (e.g. Sagesaka [17]). It is important to realise that most current clinical coagulation tests consist of functional end point assays, in which samples of plasma or blood are incubated with exogenous reagents to activate the coagulation cascade. The time for clot formation is then assessed with the Clotting Time being compared to that of pooled normal plasma or whole blood samples, giving a standard measurement of a patient’s haemostatic status. The terms ‘Clotting Time’ and ‘Gel Point’ are used synonymously in studies of fibrin gels (Blomback and Bark [18]) which consist of three-dimensional networks of fibrin fibres whose physical properties, which include the extent of branching, determine the microstructural and viscoelastic properties of the entire network ( Blomback *et al* [19]; Blomback *et al* [20]; Ferri *et al* [16]).

In view of the clinical requirement to define and measure a blood clotting time (CT), it is natural to consider the significance of the Gel Point of coagulating blood insofar as it defines the transition between a pre-gel viscoelastic fluid and a post-gel viscoelastic solid. The key point is that the clot is required to perform a haemostatic function and the properties of a viscoelastic *solid* are necessary in order to perform this function. It follows that rheometrical detection of the Gel Point provides a basis for assessing the Clotting Time of blood in terms of the establishment of an *incipient* clot during coagulation. The Gel Point of coagulating blood has additional significance insofar as there is evidence that fibrin polymers formed after the Gel Point are incorporated into the general architecture of the existing network i.e. they do not form *de novo* networks within fluid spaces of the original network [Blomback and Bark [18]; Blomback and Okada [21]; Okada and Blomback [22]). Thus the detection of the

incipient clot, and the investigation of its microstructural characteristics, are of great scientific and clinical interest.

Studies of the conversion of fibrinogen to fibrin under varying conditions of ionic strength, fibrinogen and hydrogen ion concentrations have established that the Gel Point is the parameter of overriding significance in determining clot structure (Blomback and Bark [18]). By varying the physical-chemical conditions of the gelling solution, (such as pH, ionic species and concentration and the activating enzyme), fibrin gels with very different characteristics can be obtained (Ferry and Morrison [23]). Their structure has been studied by scanning electron microscopy (Kaibara *et al* [24]) and various light scattering techniques (Kita *et al* [25]). Takahashi *et al* [26] have reported a study of the three dimensional network structure of hydrated fibrin gels using laser scanning confocal microscopy, which ascertained that two length scales which characterize the gel network (the diameter of the polymer chain and the typical mesh size of the gel network) can be determined by a 3-dimensional box-counting analysis and a 3-dimensional Fourier transform (FT) analysis (to obtain the power spectra). Turbidity measurements were employed for the determination of average fibre diameter. The self-similar structure of the gel network was found in the range between those two scales, with the fibrin gels formed by larger amounts of thrombin showing a smaller fractal dimension, in good agreement with the result from 3-dimensional FT analysis and with the dynamic light scattering study of Kita *et al* [25]). Takahashi *et al* [26] note that the fibrinolytic and haemostatic kinetics of blood and various properties of clots (mechanical strength, syneresis, opacity, and clot retraction) are associated with the structural characteristics of fibrin networks. A key point is that the analysis of network structure, which is represented by fractal dimension, mesh size and orientational ordering, may be useful for understanding the physiological phenomena as well as the physical properties of clots formed under various physiological conditions.

Microscopic image analyses are generally limited to dilute systems and sample preparation for such analyses may damage gel structure. Rheological techniques have the advantage that they may be applied to concentrated systems. Kaibara *et al* [24] have attempted to relate rheological data to morphological studies of clots using electron microscopy and the results of absorbance spectrophotometry, while Kawakami *et al* [27] have used damped oscillation rheometry to study fibrinolysis induced by tissue plasminogen activator and have compared results with scanning electron microscopy (damped oscillation rheometry is considered later in this review). The goal of relating the viscoelastic properties of fibrin gels to their microstructure using complementary rheological and morphological studies has been an area of significant activity (e.g. see Thurston and Henderson [28]; Scott-Blair [29]; Ryan *et al* [30]).

In the following sections of this review we consider various rheological tests which have been used to study blood coagulation, with special reference to the rheological assessment of clotting time and studies of the evolution of viscoelasticity during the course of fibrin polymerization and cross-linking. A common feature of many of these studies is that they attempt to detect a liquid-to-solid transition during coagulation (e.g. Riha and Stoltz [31]; Williams *et al* [32]). These tests include various forms of thromboelastography (Hartert [33]), shear viscosity measurements (Fukada *et*

*al* [15]; Tran-Son-Tay *et al* [34]), and complex shear modulus measurements. The latter are based principally on instruments such as the various controlled stress and controlled strain rheometers (e.g. Copley *et al* [35]; Roberts *et al* [36]; Glover *et al* [37]); more specialised, custom-built instruments for coagulation studies, including the Variable Frequency Thromboviscometer, VFTV (Dintenfass [38]), and the Viscoelastorecorder (Fukada and Kaibara [39]); damped oscillation rheometry (Kawamoto and Kaibara [40]); various forms of wave propagation measurements (Bandey *et al* [7]); Alvaredo and Machado [41]; Viola *et al* [42]); and other, less widely used techniques such as free oscillation rheometry (Hansson *et al* [43]), and quartz crystal microbalance measurements (Cheng *et al* [44]). Various other tests, both optically and mechanically based, have been used to characterise blood coagulation. They include spectrophotometry and photometric techniques such as nephelometry and turbidimetry, which are commonly used to study coagulation of blood plasma in the bulk phase, while ellipsometry is used to study plasma/surface interactions. These techniques are not considered in detail herein as the focus of this review is mainly the direct assessment of viscoelasticity during blood clotting. References to other techniques may be found elsewhere (e.g. see Brown [45]; Sultan *et al* [46]).

## 2. TECHNIQUES FOR BLOOD COAGULATION MONITORING

### 2.1 Thromboelastography

The most widely used instrument in clinically based studies of blood coagulation is a simple coaxial cylinder device called a thromboelastograph<sup>1</sup> (TEG). Thromboelastography is a long established method (see the reviews by Mallett and Cox [47] and Thurston [48]), having been introduced in the late 1940's to provide a global coagulation profile (Hartert [6,33]). It has found particular use in assessing the consequences of major blood loss, such as dilutional coagulopathy and the effect of fibrinolysis during a variety of major surgical procedures, and has the added benefit of monitoring the effects of therapeutic interventions and blood replacement during surgery. As well as measuring the hypocoagulation defects associated with surgery it also has a role in determining the hypercoagulable states often seen with acute and chronic illness and surgery. The TEG is claimed to be capable of identifying numerous coagulopathies, and to be sensitive to platelet and fibrinogen levels in whole blood coagulation but the various parameters it provides have not been related in a rigorous way to those which are used to characterize the viscoelastic properties of materials, such as the complex shear modulus,  $G^*(\omega)$ .

In its most basic form the TEG consists of an inner cylinder (referred to as the 'pin') suspended on a torsion wire and an outer cylinder (referred to as the 'cup') in the form of a cuvette, which performs unsteady oscillation. A sample (typically 0.36

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<sup>1</sup> The term "thromboelastograph" may be inappropriate for such a device insofar as the term "thrombus" is usually reserved for *intravascular clotting during life*. The term "coaguloelastograph" or "blood clot elastograph" has been suggested as a more appropriate alternative.

ml) of blood is placed within the cup which moves back and forth, typically every 10 seconds, through an angle of 4 degrees 45 minutes. Chandler [49] provides details of TEG calibration. The eventual formation of a fibrin network (or clot) between the surfaces of the pin and cup is deemed to result in a coupling of the motion of the latter to the former. The resulting pin movement is recorded by an electromagnetic transducer and is plotted as a displacement (in *millimetres*) in both the clockwise and counterclockwise directions as separate curves on a chart-type record called a 'thromboelastogram'. TEG measurements will inevitably involve both elastic and viscous contributions from the evolving clot, and it is well established that liquids with a sufficiently high shear viscosity will generate substantial thromboelastograph readings, despite the absence of fluid elasticity (Scott-Blair and Burnett [50,51]).

The 'lag time' which elapses between the start of TEG data collection to a pin movement greater than 2 mm on the chart record is defined as the reaction time, which is referred to in thromboelastographic work as the 'R time', the interval necessary to initiate fibrin network formation (Traverso *et al* [52]). Note that in the TEG, clot 'rigidity' (loosely clot 'elasticity') is recorded in terms of a peak-to-peak amplitude of the pin displacement, which is measured in *millimeters*. Various other TEG parameters which are used to characterise the course of coagulation, clot formation and clot development include (i) the 'k value' - the time taken from the beginning of clot formation until the TEG amplitude of the TEG output trace reaches 20mm; (ii) the 'alpha ( $\alpha$ ) angle' between the line in the middle of the thromboelastogram and the line tangential to the developing "body" of the TEG trace; (iii) the 'maximal amplitude' (MA) - the greatest amplitude of the thromboelastogram; (iv) the amplitude of the thromboelastogram 60 minutes after MA and; (v) the 'clot lysis index' - the amplitude at 60 minutes, expressed as a percentage of MA. Some aspects of the relationships between thromboelastogram parameters and linear viscoelastic parameters derived from rheometrical studies of coagulation are discussed later in this review.

In addition to its original use in providing a global coagulation profile, other uses of the TEG have included measurements of the 'Shear Elastic Modulus Strength' (SEMS) of fibrin sealants - products which comprise separate preparations of thrombin and of fibrinogen/ factor XIII and which, when mixed at a site of injury, generate a clot similar to the natural haemostatic plug (Glidden *et al* [53c]). Rivard *et al* [54] have reported a possible method of linking TEG measurements with the generation of thrombin, which is usually assessed by thrombin/antithrombin complex (TAT). In this method, the total thrombus generation (TTG) parameter is calculated from the first derivative of the thromboelastogram and is compared with thrombin generation measured by TAT.

Despite its usefulness in some clinical settings, such as in coagulation monitoring during and following liver transplantation, little can be learned from TEG measurements in terms of fundamental aspects of blood coagulation and rheology. A principal drawback of the TEG is that the strain amplitude experienced by the sample is uncontrolled and changes significantly during the course of coagulation. This feature of the instrument's operation results in TEG measurements transgressing the non-linear viscoelastic regime during coagulation monitoring with the result that the TEG's operation can substantially modify clot structure during the measuring process.

It follows that the arbitrary, operational definition of clot formation time which it provides is inadequate as a basis for understanding the evolution of viscoelasticity in terms of the progressive conversion of fibrinogen into a clot network.

Burghardt *et al* [55] have confirmed that the TEG operates in the non-linear viscoelastic range and have studied the role of non-linear effects in thromboelastography. They report a well defined regime of linear viscoelastic behaviour of clots for strains less than approximately 2 %, while at larger strains clots show significant strain hardening (see also Riha *et al* [56]). An important consideration is that during a TEG experiment, the applied strain decreases progressively as the system clots. As a consequence the displacement recorded on the TEG chart trace cannot be considered to be directly proportional (or indeed solely attributable) to the growth of clot elasticity. In typical use, TEG measurements are associated with shear strain amplitudes in the range of 8-16 %, which is substantially greater than the strain required to maintain a linear viscoelastic response.

A modified TEG measurement procedure has been reported by Burghardt *et al* [55], this procedure being designed to study differences between clots formed during standard TEG measurements and those formed under quiescent conditions. In a standard clotting experiment, the evolution of clot elasticity (as measured by the TEG) was monitored continuously as a function of time. At the conclusion of the experiment, the cup displacement was fixed in a position corresponding to the center of the thromboelastogram, the cup being then in its "equilibrium" (null) position. The pin and cup were then replaced, and a fresh sample loaded. Coagulation then proceeded for one hour in the absence of cup oscillation, the clot thereby forming under quiescent conditions. Following this quiescent period, the cup was allowed to oscillate, and the maximum amplitude of the resulting TEG trace (MA) was measured. In the TEG, the cup (radius 4 mm, shearing gap 1mm) moves through an angular displacement of 4.75°, which corresponds to a total curvilinear displacement of 0.33 mm. Burghardt *et al* [55] have estimated the magnitude of the strain experienced by their samples during TEG experiments (noting that curvature effects will invariably lead to a non-uniform distribution of strain in the gap and that end effects associated with the gap between the bottom of the pin and the cup are difficult to characterize precisely). They estimate that prior to pin movement, shear strains of approximately 16% are experienced by the sample. The subsequent growth of clot elasticity leads to a progressive reduction in applied strain due to pin displacement, the final amplitude of the TEG trace was roughly half of the maximum possible value of 100 mm (Hartert and Schaefer [57]), which corresponds to a reduction of strain by a factor of two.

The representative thromboelastograph trace described by Burghardt *et al* [55] for the clotting of platelet-free bovine plasma reveals a detectable level of elasticity after approximately 10 minutes. This is followed by a period of rapid growth of the thromboelastogram amplitude, with clot elasticity growing more progressively thereafter and only gradual changes being registered after one hour. It is instructive to compare these TEG results with the corresponding results obtained by the authors using a controlled stress rheometer (Bohlin VOR), at two different levels of maximum strain amplitude. At the smaller strain amplitude involved in the experiments (0.9%), the first detectable levels of elasticity were recorded after approximately 10 minutes

(essentially the same elapsed period or lag time after initiation of clotting as in the corresponding TEG measurements). The value of  $G'$  at this time was approximately 60 Pa, the elasticity reportedly being outside the sensitivity range of the rheometer at earlier times. The second series of measurements, which were conducted at a larger strain amplitude (9%), also recorded the onset of detectable elasticity at approximately 10 minutes (again, the same as in the corresponding TEG measurements) but the value of  $G'$  in this instance was significantly lower, being only approximately 2 Pa. Following the rapid rise in clot elasticity, the rheological response is dominated by elasticity, with corresponding values of the phase angle,  $\delta$ , being (typically) less than  $5^\circ$  in this regime.

Burghardt *et al* [55] report that strains exceeding 2% lead to clots that are substantially weaker than their counterparts formed under strain amplitudes corresponding to linear viscoelastic conditions. The shear strain amplitudes associated with TEG measurements considerably exceed 2%, which suggests that exposure to shear during standard TEG test protocols could have a marked influence upon the recorded course of clotting i.e. resulting in the formation of clots with different properties than would be formed under quiescent conditions. The modified TEG protocol described by Burghardt *et al* [55] allowed this hypothesis to be directly tested, revealing that a clot formed quiescently exhibited a much larger TEG amplitude (MA) than achieved during the normal thromboelastographic testing protocol on identical plasma. Multiple experiments with bovine plasma were performed in the presence and absence of shear during clot formation. After one hour, normal TEG experiments yielded an average thromboelastograph amplitude of 46.6 mm, while clots formed under quiescent conditions yielded an average amplitude of 71.1 mm, an enhancement of over 50%. In summary, it may be concluded that the TEG operates in the linear viscoelastic range for only limited periods during the clotting process; and it is clear that, in the act of monitoring the evolution of viscoelasticity during clotting, the TEG has a direct influence on the clot formation process.

## **2.2 Controlled Stress and Controlled Strain Rheometry**

Given the rheometrical inadequacies of the TEG, it is surprising that relatively few rheological studies of blood coagulation have exploited the availability of more sophisticated rheometers i.e. those which are able to independently control the amplitude and frequency of applied oscillatory shear or, alternatively, applied shear stress (e.g. see Henderson and Thurston [58]; Kirkpatrick *et al* [59]). Indeed, a feature of many coagulation studies is the employment of an *arbitrary* strain amplitude and a *single* frequency of oscillation throughout the coagulation process (e.g. Thurston [60]; Tran-Son-Tay [34]). Typically, an oscillatory test frequency of 0.1 Hz is employed (Ryan *et al* [30]; Yoon *et al* [61]) – in some instances this frequency is chosen to mimic the operation of the TEG (Burghardt *et al* [55]). Alternatively, a test frequency of 2 Hz is employed in an attempt to mimic aspects of circulatory blood pulsation.

Even where a range of frequencies has been applied (e.g. Fukada *et al* [15]), the results are not readily reconciled with established descriptions of blood viscoelasticity, but it may be argued that the latter, in terms of models such as the Maxwell fluid,

Kelvin-Voigt viscoelastic solid, or elastic solid are inappropriate, as are descriptions of clot rigidity based on a model of randomly interpenetrating stiff rods or the network theory for rubber elasticity (Tran-Son-Tay [34]; Copley *et al* [35]; Nelb *et al* [62]; Glover *et al* [37]). Some studies have involved more than one type of rheometer, supplemented by the results of thromboelastography. One such study of fibrin gels has been reported by Fukada and Kaibara [39]. This describes various oscillatory shear instruments that were used to monitor the temporal change of  $G'$  and  $G''$  during the clotting of fibrin. These instruments included the Multiple Viscoelastorecorder (MVER) instrument, a Viscoelastorecorder and a Low Shear Viscoelastometer (LSVE).

Isogai *et al* [8] have reported the use of the Viscoelastorecorder in their investigation of changes in viscoelasticity in the clot formed during the coagulation of blood samples taken from normal (healthy) subjects and from patients with a wide variety of disease, including connective tissue disease, neoplasm, hematological disease (polycythemia vera and chronic granulocytic leukemia) and diabetes mellitus. Their Viscoelastorecorder apparatus consisted of a stainless steel coaxial cylinder arrangement, which accommodated 1.5 ml of recalcified blood sample in the annular gap between the cylinders. The latter were attached to two strain gauges and were partially immersed in a water bath that maintained a constant test temperature of 25°C. The amplified signals from the strain gauges were fed to two phase-sensitive detectors as the outer cylinder was made to oscillate *vertically* at a constant frequency of 3 Hz by means of an electro-magnetic actuator. The oscillation involved a peak-to-peak amplitude of 60  $\mu\text{m}$  throughout the tests. The authors estimated that this corresponds to a maximum shear rate of 1.13  $\text{s}^{-1}$ . This suggests a rather large amplitude of the oscillatory shear strain in the light of subsequent findings, by other workers, concerning the limited range of the linear viscoelastic regime for such systems.

Isogai *et al* [8] considered that the viscoelastic properties of the coagulum formed under these rheometrical conditions would not be representative of those of an arterial thrombus (due to the low rate of shear produced in the apparatus) but a close relationship was assumed between them. Moreover they assumed that as coagulation proceeded, the eventual formation of a space-filling fibrin gel network between the cylinders resulted in a coupling of the stress generated by the motion of the outer cylinder to the inner cylinder (in this respect in a similar manner to that which is envisaged to occur in the TEG). Measurements of the phase relationship between the measured force and the imposed oscillatory strain provided by the phase-sensitive detectors provided estimates of the dynamic elastic modulus and dynamic loss modulus of the coagulum following its formation. Calibration of the instrument involved measurements with a standard viscous oil (1203 cP at 25 °C). The results were presented in the form of a 'dynamic viscoelastogram' of blood clotting, with the dynamic moduli being plotted as a function of elapsed experimental time, and were discussed in terms of four different phases of clot evolution. Some aspects of this dynamic viscoelastogram are, not surprisingly, reminiscent of the typical thromboelastogram, which is used to report the results of TEG measurements. Phase 1 of the viscoelastogram describes the period preceding that in which the base line of the 'clotting curve' begins to curve upward and is referred to as the *clotting latent phase*

(LP). Phase 2 refers to the period in which the viscoelasticity of the clotting blood increases rapidly, and is called the *accelerative clotting phase*. The part in which the increase in viscoelasticity is the most pronounced is called the *maximum clotting gradient* (MCG), the increase per minute of viscoelasticity being expressed in  $\text{dyn/cm}^2$ . Phase 3 of the viscoelastogram is the period of completion of clotting in which the curve reaches the peak points following a mild gradient and is called the *saturative clotting phase*. The peaks of the curves indicate that the formation of the fibrin network is complete and that the viscoelasticity has reached a maximum level termed saturated elastic modulus and saturated loss modulus. The loss tangent is reported in terms of a ratio of these saturated values. Phase 4 of the clotting curve is taken to refer to the clot lysis phase, the condition of the initial stage of lysis being expressed by the degree of decrease in viscoelasticity for 30 minutes following the period of maximum viscoelasticity.

The study reported by Isogai *et al* [8] is notable in that it is one of only a small number which have attempted to distinguish between healthy and abnormal clots on the basis of rheometrical techniques. They report that the results obtained using the Viscoelastorecorder clearly distinguish between healthy patients and those suffering from connective tissue disease or neoplasm, the values of  $E'$  of the latter patients being remarkably high relative to those found in healthy patients. The MCG of patients with connective tissue disease was also noted as being remarkably high – more than twice the value of normal subjects. However, Isogai *et al* [8] make the important point that values of parameters such as clot strength, which are obtained by using a variety of apparatus, cannot be readily compared with each other due to differences in experimental conditions. The latter include the frequency and amplitude of oscillation, test temperature and even the principle of measurement (i.e. whether it involves static or dynamic conditions). But a more subtle point is that even were the same apparatus to be used in different studies, differences in reported values of a parameter such as blood ‘clotting time’ would be likely to arise due to differences in operational definitions of clotting parameters and different criteria used in their assessment. As an example we may consider reference to the establishment of a clot in terms of the attainment of a particular value of dynamic rigidity, at a particular value of oscillatory frequency. This point is considered in more detail in a later section of the present review.

Another noteworthy rheometrical study involving samples taken from healthy and unhealthy donors has been reported by Scrutton *et al* [11]. Their study involved the characterisation of clot viscoelasticity using oscillatory shear in order to define a measure of clot *deformability*. Changes in clot deformability were then used as a possible explanation for the epidemiologic association between plasma-fibrinogen concentration and myocardial-infarction (plasma fibrinogen concentration has been related to the extent of atherosclerosis in the coronary arteries of patients who have had myocardial infarction). An important point is that the formation of ‘tight’ rigid fibrin gels may be associated with premature coronary disease and the storage modulus of the fibrin gel increases when the gel is formed from a solution containing a higher concentration of fibrinogen (Ferry [63, 64] Clark and Ross-Murphy [65]). The study involved viscoelastic measurements on fibrin gels formed by adding thrombin to

plasma samples from 99 subjects, with fibrinogen concentrations ranging from 1.45 to 4.14 g/l. In addition, measurements were reported for samples from 47 subjects in which fibrin cross-linking was blocked by addition of 0.1mM iodoacetamide (to inactivate factor XIIIa). Gel (or clot) deformability was defined as the inverse of  $G'$  (which is inversely proportional to fibrinogen concentration) at a specified measurement time during the course of fibrin gel formation. Clot deformability was estimated using measurements made at 25 °C in a controlled stress rheometer equipped with a 4cm 2° acrylic solvent trap cone. The measurements were performed at a nominal (controlled) maximum strain of 5%, at an oscillatory frequency of 0.5 Hz. The fibrin gel was formed *in situ* by placing a sample of citrated plasma containing CaCl<sub>2</sub> or purified fibrinogen solution containing CaCl<sub>2</sub> on the lower plate of the instrument, with thrombin being subsequently added to initiate fibrin formation.

The temporal evolution of  $G'$  in the experiments was as expected for a gelation process (Clark and Ross-Murphy [65]) and the results showed a highly significant correlation between plasma fibrinogen concentration and the storage modulus of the fibrin clot formed from this plasma after treatment with thrombin. In plasma samples obtained from a group of healthy subjects, the fibrin clots formed when plasma fibrinogen is at the upper end of its normal range were found to be less easily deformed than those formed when plasma fibrinogen concentration is at the lower end of this range. Scrutton *et al* [11] remark that such a relationship appears consistent with an influence of plasma fibrinogen concentration on the onset of myocardial infarction since formation of a less deformable clot in a small blood vessel appears more likely to restrict blood flow, and hence to cause arterial obstruction in the coronary circulation (with consequent myocardial infarction).

Glover *et al* [66] have reported a study of the effects of mechanical trauma on clot structure formation using a Weissenberg Rheogoniometer (R-18) fitted with a parallel plate geometry. Their tests were conducted on blood samples obtained by venipuncture from normal subjects and on platelet rich plasma (PRP) and platelet free plasma (PFP) in different types of experiments. These employed (i) different surface to volume ratios (gap width) at constant strain amplitude; and (ii) different gap/shear strain amplitudes. During coagulation of the PRP samples an initial lag period was recorded, followed by an 'explosive' increase in the moduli as the clot structure formed. In broad terms, these observations are similar to the general aspects of the thromboelastogram records produced by the TEG (and to the records produced by the Viscoelastorecorder apparatus). The recorded moduli were found to be nearly independent of frequency over the range 0.1 – 10 Hz. Interestingly, these tests indicated that a linear viscoelastic response could be obtained as long as the shear strain amplitude was less than 12%. The same rheometrical approach was used by the authors for a study of the rheological properties of fibrin clots in terms of the effects of fibrinogen concentration, Factor XIII deficiency and Factor XIII inhibition (Glover *et al* [37]).

The parallel plate geometry employed in these latter studies is less susceptible to problems associated with the presence of platelets and cells within whole blood samples than systems such as the cone-and-plate geometry. The issue of gap size and the interpretation of results in terms of various phases of coagulation must be carefully

considered in respect to the relevant length scales of clot features. Sakharov *et al* [67] have reported the results of a confocal microscopy study of the decomposition of the fibrin network and binding of plasminogen and plasminogen activators to fibrin during lysis. The important point which emerges is the finding that the zone of final lysis is located within a superficial layer whose extent is approximately 5-8  $\mu\text{m}$ . Under conditions of diffusional transport of fibrinolytic enzymes from outside a plasma clot, it appears that extensive lysis is spatially highly restricted - to a zone not exceeding 5-8  $\mu\text{m}$  from the clot surface. It is within this zone that the structure of the fibrin network undergoes significant changes. Shankaran and Neelamegham [68] have reported the effect of secondary flow on biological experiments in the cone-plate viscometer and describe methods for estimating collision frequency, wall shear stress and interparticle interactions in non-linear flow.

Kirkpatrick *et al* [59] reported the use of a wide gap parallel-plate arrangement in their study of coagulation and fibrinolysis in terms of the development of the dynamic shear moduli in coagulating PFP and PRP. Oscillatory shear measurements of  $G'$  and  $G''$  were performed using a Weissenberg R18 Rheogoniometer. Flat parallel stainless steel platens, 5 cm in diameter, were employed as the test geometry. In all cases, a 578  $\mu\text{m}$  gap between the platens was used, the frequency and amplitude of oscillation being maintained at 6 Hz and 0.0028 rad, respectively. The experimental conditions, which involved a maximum shear strain of 12%, were chosen to reproduce those reported by Glover *et al* [66]. To prevent drying and protein denaturation at the air-plasma interface, a thin coating of silicone oil was applied at the boundary, and a humidified mixture of  $\text{O}_2$ ,  $\text{CO}_2$ , and  $\text{N}_2$  was admitted to the test chamber. The flow rate of the gases was adjusted such that the partial pressure at  $\text{O}_2$  and  $\text{CO}_2$  was the same as that of venous blood ( $p\text{CO}_2 = p\text{O}_2 = 45 \text{ mm Hg}$ ).

After an initial lag period (typically 10-13 minutes, with the time of plasma recalcification being taken as time zero) during which there was no measurable change in  $G'$  and  $G''$ , both moduli increased rapidly in PFP. As the polymerization and crosslinking processes were completed,  $G'$  and  $G''$  approached asymptotic values, with  $G'$  being an order of magnitude greater than  $G''$ . The time between the addition of calcium to PRP and the onset of the sudden increase in  $G'$  was found to be less than in PFP (6-8 minutes). Also, the maximum value of  $G'$  ( $G'_{\text{max}}$ ) of the PRP clot was an order of magnitude greater than that of the platelet-free clot, and  $G'$  declined after  $G'_{\text{max}}$  was attained. These effects have been attributed by Kirkpatrick *et al* [59] to the interaction of platelets with polymerizing fibrin. In the Rheogoniometer, the clot is firmly attached to the platen surface, and thus, the isometric tightening of the fibrin network gives rise to the increased dynamic rigidity of the material. This is an energy-requiring process, and as the metabolic stores of the platelet are depleted, relaxation of the clot occurs, and  $G'$  therefore declines.

### **2.3 Linear and non-linear viscoelastic behaviour during coagulation**

Several studies acknowledge that the linear viscoelastic range varies considerably during the various stages of the blood clotting process. The effect of varying hematocrit on the critical ('fracture') strain of clots has been studied by Riha

*et al* [56], using controlled stress rheometry. Their results indicate a decrease of elasticity and an increase of the fracture strain of the clot with increasing hematocrit. Moreover, the elastic modulus of the clot is not constant but is found to increase with deformation. Dintenfass [38] summarizes extensive work demonstrating how steady shearing and large amplitude oscillatory flow influence the course of blood coagulation. Other studies of clot viscoelasticity using dynamic oscillatory measurements, with relatively small amplitudes, have demonstrated the nonlinear elasticity of clots and the ability of shear applied during clotting to substantially weaken clots in blood and plasma (Kaibara and Fukada [69]; Henderson and Thurston [58]) and solutions of fibrinogen (Fukada and Kaibara [39]; Roberts *et al* [36]). Fukada *et al* [15] have studied the ratio of the 3<sup>rd</sup> harmonic to the fundamental harmonic of a waveform response to a constant strain input of 10 % at a frequency of 1 Hz and showed that this ratio is negligible during the early stages of coagulation, indicating a linear viscoelastic response. As the system gels the ratio rapidly grows, indicating an increasingly non-linear viscoelastic response. But during the course of the experiment (conducted over 1 hour) this ratio begins to decrease at a time of approximately 10 minutes and eventually disappears. Fukada *et al* [15] explain the disappearance of non-linearity after approximately 10 minutes as being due to the presence of platelets. They consider that the retraction of thrombosthenin in platelets incorporated within the network stretches the fibrin fibres, with the resulting increase in clot rigidity giving rise to the linear stress-strain relation.

Two aspects of the non-linear viscoelasticity of clots have been studied by Burghardt *et al* [55] using controlled stress rheometry. Their studies involving platelet free bovine plasma are particularly noteworthy in that they clearly establish that clot elasticity becomes highly non-linear beyond a small critical shear strain amplitude (approximately 2%), and that when plasma is subjected to oscillatory shear of sufficiently large amplitude during coagulation the resulting clot may be considerably weakened. A salient point which emerges is that the critical strain amplitude necessary to maintain a linear viscoelastic response is notably less than that arising in numerous rheometrical studies by other workers (in which strain amplitudes of 10%, or more, are reported). The instrument used by Burghardt *et al* [55] was a Bohlin VOR model controlled stress rheometer fitted with a stainless steel, cone and plate geometry (2.5° cone angle, 30 mm diameter). Prior to each clotting experiment, the fixture was thoroughly washed with a non-phosphate soap, rinsed with deionized water, followed by methanol and acetone, and dried in an oven at 65°C. Appropriate amounts of plasma, CaCl<sub>2</sub> and buffer solutions were pipetted onto the lower plate and mixed, and the cone was then quickly lowered into position prior to the clotting experiment. When the (complex) modulus had grown to a measurable level, a thin layer of mineral oil was applied to the outer surface of the clot to reduce evaporation of water. All measurements were carried out at 37° C. Oscillatory shear was applied during the clotting process,  $G'$  and  $G''$  being measured as a function of time at a frequency of 0.1 Hz. Experiments were performed in which the strain amplitude applied during clotting was varied over a wide range, from 0.04% to 40%. After monitoring the evolution of the dynamic moduli for a period of one hour, a strain sweep experiment was performed at a frequency of 0.1 Hz in order to study the linear and nonlinear elastic properties of the formed clot.

Of particular note are the results of their strain sweep experiments on three fully-formed clots that had been subjected to oscillatory shear at strains of 0.09, 0.9 and 9% for 60 minutes during clotting. These clots exhibited a regime of linear elasticity for strains below approximately 2% but at larger strains all the clots exhibited pronounced strain hardening, the modulus being a strong function of the magnitude of the applied strain. In the nonlinear regime, the recorded stress response to the imposed deformation was non-sinusoidal, exhibiting higher-order Fourier components. Under these conditions,  $G'$  was extracted from the fundamental harmonic of the stress signal (being taken therefore as a measure of an *effective* storage modulus). For a clot formed under the lowest deformation amplitude (0.09%), an increase in strain amplitude from 8 to 16% (i.e. the approximate strain range spanned by the TEG in normal operation) resulted in an increase in modulus of 45%, the non-linearity being even more pronounced for the clot formed while experiencing a relatively large strain amplitude.

For plasma clots the amplitude of non-linear deformation required to substantially perturb clot microstructure is around 2%. Burghardt *et al* [55] comment that this is relatively low, in comparison to permanent or temporary networks of flexible polymers, but not surprising given that a fibrin network is comprised of relatively rigid building blocks. Since the structure of a previously formed clot is strongly perturbed by deformations exceeding 2%, then the application of shear strains larger than 2% *during* clot formation might inhibit the clotting process. Burghardt *et al* [55] report results which clearly support this supposition, for two clots formed while subjected to relatively mild shearing (at strains of 0.09% and 0.9%) under which conditions both exhibit nearly identical properties. But a clot formed while subjected to shearing with 9% strain was substantially weaker, the value of its (linear) elastic modulus being only approximately 25% of that measured in clots formed under lower strain amplitudes. An important aspect of this study is that it clearly establishes the strain regime in which oscillatory shear during clotting does not affect the resulting clot properties, and connects this to the transition between linear and nonlinear clot elasticity. Despite the fact that the study was restricted to PFP, its conclusions relating to the possible consequences of non-linearity in the TEG are likely to be pertinent to the coagulation of whole blood in view of the available evidence concerning the reduction of clot rigidity due to applied deformation during whole blood coagulation (e.g. see Kaibara and Fukada [69]; Dintenfass [38]).

At the conclusion of coagulation, it has been reported that clots of whole blood (Kaibara and Fukada [69]) or platelet-rich plasma (Fukada *et al* [15]) give less indication of nonlinear viscoelasticity (in terms of strain hardening), which has been attributed to inclusion of platelets and/or red blood cells into the clot network. But the period *during* clot formation is characterized by nonlinear behaviour which is similar to that seen in PFP and the important point is that applied deformation would be expected to have its most significant disruptive effect on the incipient clot (i.e. that formed during the *initial* formation of the fibrin network) due to its strain-sensitivity.

#### **2.4 Detection of incipient clot formation by oscillatory shear rheometry**

Many operational definitions of a blood ‘Gel Point’, ‘Clotting Time’ (CT) or ‘clot formation time’ criterion are based on the attainment of an *arbitrary* level of dynamic rigidity during coagulation, at a *particular* frequency of oscillation. An example may be found in the studies of fibrin-thrombin gels reported by Ryan *et al* [30], in which clot formation is defined operationally as the time at which  $G'$  attains a value of 0.73 Pa, at a frequency of 0.1 Hz. While useful in a comparative study involving a particular gel system, it is unlikely that an arbitrary operational definition of clot formation could be successfully applied more widely, e.g. as the basis of studying differences in coagulation rates for samples of blood drawn from different donors. It follows therefore that it is important to draw a distinction between operational definitions of CT and a more widely applicable definition of the Gel Point of coagulating blood.

What is required is a rheometrical criterion which may be defined within the framework of the theory of linear viscoelasticity and which is (i) independent of the absolute value of dynamic rigidity attained by a particular blood, plasma or fibrin gel sample during coagulation, and (ii) independent of the oscillatory frequency of measurement. Such a criterion is provided by the Chambon-Winter Gel Equation (Chambon and Winter [70]; Winter [71] Yoon *et al* [61]), which accurately describes the Gel Point of samples of whole human blood (Williams *et al* [32]), and other biogel systems, such as gelatin (Jones *et al* [72], Michon *et al* [73]) and collagen (Forgacs *et al* [74]).

It is useful to recall that the formation of branched polymers and gels is similar to a percolation process. In the percolation model of polymerization and gelation the parameter  $p_c$  denotes a *connectivity transition* with  $p$  being the probability of two nearest neighbour monomers on the sites of a periodic lattice forming a bond between them. As  $p$  increases, larger and larger polymeric clusters of connected monomers are formed. In the vicinity of the Gel Point, GP, the elastic modulus of the network obeys a scaling law  $G \sim (p - p_c)^f$  where  $p > p_c$ , the shear viscosity of the sol phase diverging as  $\eta \sim (p_c - p)^{-k}$  where  $p < p_c$ . Kita *et al* [25] have reported a study of the formation of fibrin gels in fibrinogen-thrombin systems in which the dynamics of thrombin-induced fibrin gel formation were investigated by means of static and dynamic light scattering (DLS). The decay time distribution function obtained by DLS clearly revealed a stepwise gelation process involving the formation of fibrin and protofibril from fibrinogen, followed by the lateral aggregation of protofibrils to form fibrin fibres and the formation of a three-dimensional network consisting of fibres. The DLS correlation function was analyzed in terms of a sol-gel transition and gel structure and showed a stretched-exponential type behavior prior to the sol-to-gel transition point, and a power-law behavior at the Gel Point (see also Kubota *et al* [75]).

The significance of the GP in blood coagulation, and its specific relation to the incipient clot, is readily understood in terms of the clot’s requisite haemostatic function. At the GP, short-range connectivity is transformed into a three-dimensional sample-spanning network structure, with a corresponding transition from pre-GP viscoelastic fluid to post-GP viscoelastic solid behaviour. Only after the GP can the

haemostatic function be performed. In this way the GP defines the formation of the *incipient* clot. The complex shear modulus  $G^*$  is written as  $G^* = G' + i\omega\eta$  for which  $G^*(\omega, \varepsilon) \sim \varepsilon^f h_1(i\omega\varepsilon^{f+k})$ , where  $\varepsilon = |p - p_c|$  and  $h_1(x)$  is a scaling function. In the low frequency regime,  $G^*$  depends only on  $\omega$  with  $G^* \sim \omega^{-\alpha}$  and measurements of  $G^*(\omega)$  providing a rigorous basis for GP detection. At the GP, the elastic and viscous components of  $G^*$  (the storage and loss moduli,  $G'(\omega)$  and  $G''(\omega)$ , respectively) scale as power-laws in frequency, i.e.  $G'(\omega) \sim G''(\omega) \sim \omega^\alpha$ , enabling the GP to be identified by the frequency independence of the loss tangent,  $\tan\delta (= G''/G')$ . This self-similar behaviour at a critical point corresponds to the inverse power-law characteristic of the stress relaxation modulus  $G(t)$ , where  $G(t) \sim t^{-\alpha}$  at the GP (Goldenfeld and Goldbart [76]). The value of  $\alpha$  is a sensitive measure of the degree of branching in a gel. All values of  $\alpha$  in the range  $0 < \alpha < 1$  are possible for a fractal dimension  $d_f$  in the physically acceptable range  $1 \leq d_f \leq 3$ . Such behaviour is widely observed in chemical, physical and colloidal gels. Theoretical treatments of the GP, based on self-similar connectivity and a scaling theory of fractal correlations, predict values of  $\alpha$  in the range  $0.66 < \alpha < 1$  (Sahimi [77]).

Williams *et al* [32] have used the Chambon-Winter Gel Equation to characterise the rheology of incipient clots formed in samples of whole blood obtained by venipuncture from healthy human subjects. Aliquots of blood were transferred directly to the titanium parallel plate (6 cm diameter, 100-400  $\mu\text{m}$  gaps) geometry of a controlled-stress rheometer (TA Instruments, G2 model), and the stainless steel coaxial cylinder geometry (1 mm gap) of a controlled strain rheometer (Rheometrics ARES). An acrylic coaxial cylinder geometry (1 mm gap) was also used in work involving the ARES instrument alongside simultaneous TEG measurements on aliquots drawn from the same bulk blood samples. Five simultaneous test frequencies were employed in Fourier Transform Mechanical Spectroscopy (FTMS) to obtain time-resolved data throughout blood coagulation (Holly *et al* [78]). The lower and upper limits of the FTMS waveform (0.2 Hz and 3.2 Hz, respectively) were determined by consideration of sample mutation and fluid inertia effects, respectively. A Fourier analysis of recorded waveforms verified that tests were conducted within the linear viscoelastic regime, the maximum strain amplitude,  $\gamma$ , of the test waveforms being  $\gamma < 2\%$  throughout. In addition to measurements of  $G^*(\omega)$ , the normal force exerted on the upper plate by the maturing clot in the post-GP phase was recorded. A thin layer of low viscosity mineral oil at the outer rim of the sample prevented evaporation of the sample and all work was conducted at 37 °C.

Williams *et al* [32] report the evolution of viscoelasticity, which typifies blood coagulation, under controlled stress FTMS. The initial response is characteristic of a viscoelastic fluid, with increasing frequency of oscillation causing  $G'$  to increase and the loss tangent ( $\tan\delta$ ) to decrease. In this pre-clot viscoelastic fluid stage of coagulation (prior to the establishment of the incipient clot),  $G'$  varies from ca.  $5 \times 10^{-3}$  Pa to  $7 \times 10^{-2}$  Pa over the FTMS frequency range, with the phase angle  $\delta < 80^\circ$  throughout. The frequency dependence of  $\tan\delta$  decreases progressively as coagulation proceeds, becoming frequency *independent* as the incipient clot is established as a sample-spanning cluster at the GP, where  $\tan\delta = 2.35$  (corresponding to  $\delta = 67^\circ$ ,  $\alpha = 0.74$ ). A rapid increase in elasticity follows the establishment of the

incipient clot and in this post GP regime the loss tangent is found to increase with increasing frequency of oscillation, behaviour which is characteristic of a viscoelastic solid. The corresponding values of  $\delta$  decrease dramatically in this post-GP regime, rapidly achieving a value  $\delta < 5^\circ$ , with a concomitant decrease in the frequency dependence of  $G'$  (as expected for a viscoelastic solid).  $G'$  continues to increase, reaching maximum (and near frequency-independent) values of approximately 300 Pa after 17 minutes.

In broad terms the controlled-stress FTMS results described above for the post-GP stage are similar to those reported in several other rheometrical studies of blood coagulation. Notable features are the very low values of  $\delta (< 5^\circ)$  recorded as  $G'$  reaches its maximum value and the markedly low values of dynamic rigidity associated with incipient clot formation ( $G' < 0.1$  Pa at the GP). This incipient clot formation feature has not previously been reported, with most rheometrical studies (and those involving the TEG) registering the onset of a rapid increase in elasticity only after the attainment of approximately 1 Pa (or a substantially greater clot formation time threshold value in some instances). Typically this onset occurs after a period of approximately 10 minutes in the TEG, and in other measurements which involve large strain amplitudes. It is notable, however, that in their study of platelet free plasma coagulation, Burghardt *et al* [55] found a coincidence of the TEG response time with the onset of resolvable  $G'$  values in both linear and non-linear controlled-stress oscillatory shear tests ( $G'$  being approximately 60 Pa at a strain of 0.9% strain but only 1-2 Pa at a strain of 9%). It is instructive, therefore, to consider the relative assessments of clotting time which are provided by the GP analysis based on the Chambon-Winter Gel Equation and that provided by TEG measurements on aliquots of the same sample of blood, using similar measuring geometries, with identical measuring surfaces and surface preparation procedures.

The controlled strain rheometry experiments reported by Williams *et al* [32] involved the evolution of  $G'$  at four frequencies during the coagulation of samples of healthy human blood. The measurements were conducted in the linear viscoelastic regime ( $\gamma < 2\%$  throughout) on the Rheometrics ARES rheometer at 37 °C. The experiments utilised a custom acrylic coaxial cylinder geometry (1 millimetre shearing gap) corresponding to that used in simultaneous TEG measurements conducted on Haemoscope Model 3000 instrument. The incipient clot formation time associated with the attainment of a frequency independent loss tangent ( $\tan \delta = 1.8$ ,  $\delta = 67^\circ$ ,  $\alpha = 0.7$ ) was approximately  $t = 240$  seconds, the values of  $G'$  at the GP increasing with increasing frequency of oscillation and ranging from approximately  $10^{-2}$  Pa to  $10^{-1}$  Pa. The initial TEG response was recorded at  $t = 600$  seconds, with the time at which the TEG amplitude reached 2mm being approximately  $t = 630$  seconds. At this latter time (termed the 'clot formation time' or 'k-value' in thromboelastography) the corresponding values of dynamic rigidity recorded simultaneously in the ARES rheometer were approximately  $G' = 1$  Pa (being virtually independent of frequency as the corresponding values of  $\delta$  at that time were less than  $6^\circ$ ).

Broadly, the course of the thromboelastogram trace corresponds qualitatively to the temporal evolution of  $G'$  in the post-GP phase, but a significant error may be

involved in the association of the initial TEG response with the time required for the onset of the establishment of the fibrin network. The ARES results show that the incipient clot has formed some six minutes prior to the initial TEG response, by which time the rigidity of the clot has increased by (at least) an order of magnitude. Moreover, the TEG-based assessment of the MA time may be inaccurate. The maximum value of  $G'$  associated with the clot retraction phase (confirmed by simultaneous measurements of the corresponding maximum value of normal force due to clot retractile stress) occurs significantly before the MA time recorded in the TEG measurement (see also Jen and McIntire [79]). In respect of this latter point, it is necessary to recall that in TEG measurements the strain amplitude decreases progressively during the coagulation process. The apparent strain-hardening of formed clots reported by Burghardt *et al* [55], at large amplitudes, may be followed in TEG work by a decrease in measured elasticity at subsequent smaller strains and thereby lead to an incorrect association of a maximum value in the thromboelastogram trace with the onset of clot retraction and lysis.

## **2.5 Influence of the surface of the measuring geometry.**

Glover *et al* [66] have reported that surface effects were unimportant in work involving silicon-coated and silicon-free stainless steel platens. Other studies have shown that stainless steel surfaces prove to be the least reactive with blood, whereas silicone coated surfaces increase clotting time significantly (Fukada *et al* [15]). This suggests that rheological studies may monitor differences in enzymatic reactions at different surfaces, but few studies of blood coagulation have made reference to interactions among blood components and the vascular vessel wall. At a lesion, blood comes into contact with fibroblasts which are located in the adventitia and which play an important role in activating the intrinsic coagulation sequence and the accumulation of platelets. Suitably adapted rheometer geometries may facilitate measurements of the properties of surface layers, such as the effects of  $\gamma$ -globulin and  $\beta$ -lipoprotein in the rheology of fibrinogen or the procoagulant activities of different collagen types and structures.

What is generally modified by the *in vitro* conditions (i.e. in comparison with those within the circulatory system) is the *rate* of coagulation. This is due principally to the different initiation mechanisms of the coagulation process and, in particular, is related to adhesion of the clot to the walls of the instrument. The maximum possible stresses at the critical deformation of the clot (the onset of clot rupture) are considerably higher than the maximal physiological stresses (approximately 6 Pa) and much higher than the maximal pathological stresses (approximately 40 Pa). *In vitro* measurement shows that the fibrin fibres, which are the principal microstructural basis of the clot, are highly elastic and deformable. Although inhomogeneity in clot microstructure influences clot rupture in the circulation, another possibility is that clot retraction-induced residual stresses, as well as fibrinolysis or weaker links of the fibrin fibres to the vessel wall than to the metallic one, also play an important role.

Kaibara and Kawamoto [80] have reported rheological measurements of blood coagulation in vascular vessel model tube consisting of endothelial cells monolayer,

while Karino and Goldsmith [81,82] have studied the adhesion of human platelets to collagen on the wall, and the role of cell-wall interactions in thrombogenesis and atherogenesis. Kawakami *et al* [27] have studied blood coagulation tubes coated with cultured endothelial cells, which play an important role in the balance of anticoagulant and procoagulant mechanisms in hemostasis and thrombosis. The coagulation process of a blood sample in a BAEC (bovine aortic endothelial cells) coated tube was monitored in terms of changes in the logarithmic damping factor. The BAEC-coated tube underwent a damped rotational oscillation which produced an induced electromotive force in a coil which was in turn detected as a voltage and provided a measurement of the logarithmic damping factor, changes in which were monitored during coagulation using BAEC-coated and bare glass tubes. The initial increase in logarithmic damping factor is ascribed to the increase in viscosity of plasma from 1.1 mPas to about 6 mPas during fibrin polymerization and subsequent network formation (Murata *et al* [83]). In the glass tube an increase in logarithmic damping factor was recorded (after reaching a minimum value), this being taken to be due to clot retraction, where the clot detached from the glass surface. For whole blood in a BAEC-coated tube, the logarithmic damping factor began to decrease at about 30 minutes when coagulation started. (Conversely, a change of logarithmic damping factor in platelet-free plasma in a BAEC-coated tube did not occur within a time of more than 150 minutes).

## **2.6 Free Oscillation Rheometry in Blood Coagulation Studies**

A technique known as free oscillation rheometry has been used to study the contribution of platelets to the coagulation of plasma and to unanticoagulated fresh whole blood (native blood) (Ramstrom *et al* [84]; Ranby *et al* [85]). The technique has also found application in studies of whole blood coagulation on various surfaces, including protein adsorption-resistant PEG and peptide functionalised PEG-coated titanium (Hansson *et al* [43]). In the free oscillation rheometer, FOR, a cup containing the sample is attached to a perpendicular torsion wire, a near friction-free suspension being accomplished by air gap permanent magnet bearings (Bohlin [86]). Oscillation is initiated by a forced turn of the cup about its longitudinal axis followed by release of the cup. The ensuing rotational oscillation at a characteristic amplitude and frequency (ca. 10 Hz for an empty sample cup) is then repeated, typically every 2.5 seconds. The changing rheological properties of the sample during coagulation modify the characteristics of the oscillation, with the onset of clot formation (the “clotting time”) being associated with the time when the sum of change in damping and frequency reaches a preset value. This latter value has been deemed to correlate with a reference method involving the *visual* detection of clot formation (Ranby *et al* [85]).

The damping of the FOR oscillation is expressed as logarithmic damping ( $L_d$ ), which is the logarithm of the amplitude ratio of two consecutive oscillation cycles.  $L_d$  is determined as an average, typically over ten oscillations (Bohlin *et al* [87]; Whorlow [88]). The sample viscosity can be determined for a viscous sample by measuring the damping in the presence and absence of sample with a knowledge of the sample density, the angular velocity of the oscillation and a calibration constant

(Bohlin *et al* [87]). The FOR instrument used by Hansson *et al* [89] was a ReoRox model which was reported to be capable of resolving frequency changes of 0.0005 Hz and changes in  $Ld$  of 0.0001 (Global Hemostasis Institute MGR AB, Sweden). The instrument was thermostatted to 37°C and set a cylindrical cup into free oscillation around its vertical axis by means of an electromagnetic oscillation initiator and a torsion wire. After a hold time (1 second), the sample cup was released and an optical detector recorded the oscillation frequency and amplitude, the damping of the amplitude of an empty cup being around 1% per oscillation cycle. The damping and frequency of the oscillation were measured at intervals of 15 seconds.

The general aspects of FOR signals and their changes due to blood coagulation have been reported by Hansson *et al* [89]. When blood or blood plasma started to coagulate, the  $Ld$  increased due to increased sample viscosity. When the viscosity became sufficiently high during coagulation, several millimetres of the sample were effectively probed and participated in modifying the oscillation characteristics. Then, the  $Ld$  no longer increased but declined with increasing viscosity. As the viscosity increased further the entire sample was deemed to be in oscillation and the  $Ld$  was similar to that of an empty sample cup due to the low energy loss and lack of shear. Hansson *et al* [89] report that the oscillation frequency decreased when the blood sample started to coagulate and continued to decrease as the oscillating mass increased and ended up in a frequency minimum. The increase in the frequency after the minimum was due to the spring constant of the coagulum (i.e. its elasticity). Thus, the  $Ld$  monitored by the FOR at large penetration depths is not considered to reflect that experienced by the sample but the recording of frequency is considered to be reliable at high viscosities.

A key aspect of developing FOR procedures for clotting time assessment is the need for validation – a process which invariably involves comparison with suitable reference methods and, in some instances, intuitively and arbitrarily selected definitions of clotting time. Ranby *et al* [85] have reported clotting criteria based on corresponding changes in rheological properties derived from oscillatory shear measurements in a Bohlin VOR rheometer fitted with a Couette measuring geometry (at a frequency of 10Hz and a shear strain amplitude of 6.9% ). Note that this strain amplitude is relatively large in respect of the limits of linear viscoelastic response established by Burghardt *et al* [55] who also used a Bohlin VOR rheometer.

The association of clotting parameters with the detection of rheological changes clearly requires a suitable definition of a ‘reference’ rheological state. In the study reported by Ranby *et al* [85] the reference state refers to the recorded values from the instrument after 1 minute. Changes in  $G^*$  from the reference state (referred to as  $\Delta G^*$  values) were monitored as coagulation proceeded and, when a preset threshold value was reached, the clotting time was recorded. The clotting criterion  $\Delta G^* > 0.0017$  Pa resulted in the shortest recorded clotting time (5.17 minutes) whereas different clotting criteria,  $\Delta G^* > 0.01, 0.1$  or  $1$  Pa, resulted in increasingly longer estimates of clotting time (5.53, 6.17 and 7.21 minutes, respectively). Validation of the FOR procedure for clotting time determination involved *visual* inspection of the sample’s appearance, with the sample being inspected every 30 seconds. The following coagulation-related phenomena were noted: (i) the increased adhesion of the sample to the cup walls; (ii)

the loss of mobility of small air bubbles on surface; (iii) the appearance of a 'wrinkly' skin and other sample surface changes; (iv) a loss of sample surface orientation upon tilting; and (v) the ability to invert the sample cup without loss of sample. The first appearance of any of the foregoing (i)-(iii) was interpreted as the 'clotting time'. The first appearance of (iv) and (v) was interpreted as an auxiliary parameter termed the 'firm clot time'. Ranby *et al* [85] conclude that the FOR results were in agreement with those obtained with the conventional forced oscillation rheometer.

Note that FOR probes mainly bulk viscoelastic properties, e.g. during a change from a liquid state to a gel due to coagulation, or vice-versa, when a coagulum is lysed due to fibrinolytic actions. At 10Hz the sensing depth of FOR measurements is approximately 0.1 mm in biological fluids, under which conditions the FOR technique subjects a sample to shear rates equivalent to that experienced by blood in venous flow (approximately  $50 \text{ s}^{-1}$ ). FOR has been shown to perform well in monitoring blood plasma and whole blood coagulation and is considered particularly suitable for studies on coagulation of whole blood since the method is not restricted by sample turbidity and allows measurements in the presence of blood cells. Rheological techniques including those resembling the experimental arrangement reported by Ranby *et al* [85] have been used to analyse the performance of artificial materials in blood contacting systems (Kaibara and Date [90,91]; Gautam *et al* [92]).

## **2.7 Wave Propagation Techniques**

The marked shear strain sensitivity of blood clots and the rapidity of rheological change during coagulation are factors which suggest that high frequency wave propagation techniques might provide a suitable rheometrical basis for blood coagulation studies, as is the case for other gel systems (Jones *et al* [72]). Alvaredo and Machado [41] have reported experiments involving the transmission of shear waves (at 2 MHz) through a sample of plasma during the clotting process, and note that rheological changes in the medium impose modifications on the wave propagation characteristics, such as wave velocity. Clearly such techniques require careful consideration of the role of anomalous wave dispersion in viscoelastic media in general, and in gelling systems in particular (Davies *et al* [93]). Alvaredo and Machado [41] note that the transmitted wave amplitude and the phase fluctuate during the coagulation period, being registered by means of the in-phase and quadrature components of the transmitted wave. The fluctuations start at the beginning of the coagulation process and reportedly disappear once the fibrin network is completed. The authors refer to the corresponding coagulation period as the time interval when these fluctuations occur.

The experiments described by Alvaredo and Machado [41] demonstrate the relative insensitivity of ultrasonic *longitudinal* waves (at 2 MHz) to coagulation induced rheological changes. They conclude that only the shear waves which are transmitted through the sample are affected by such rheological changes but an increase in echogenicity associated with the formation of a thrombus due to an increase in ultrasonic backscatter has been reported by other workers, along with increases in attenuation coefficient and sound speed (Shung *et al* [94]).

Viola *et al* [42] have also attempted to exploit longitudinal wave propagation in studies of blood coagulation. Their technique, known as *sonorheometry*, uses acoustic radiation force to produce small, localized displacements within a sample (Walker *et al* [95]). The apparatus described by Viola *et al* [42] consists of a 1.0-cm diameter single-piston transducer which had a fixed focus at 4 cm and was held with the focus at the center of a 4.5 ml polystyrene cuvet sample holder fitted with an acoustically transparent window on the acoustic beam axis. The transmitted pulses were Gaussian enveloped sinusoids with a center frequency of 10 MHz and a full-width half maximum fractional bandwidth of 75%. A series of 4000 acoustic pulses was transmitted at a repetition frequency of 5 kHz to generate acoustic radiation force within samples of blood whose coagulation was initiated by addition of thrombin. The returning echoes of every tenth pulse were acquired in order to estimate displacements induced by radiation force. The results of these measurements were analysed in terms of a modified Kelvin-Voigt viscoelastic solid model, on the basis of a comparison with the results of creep measurements of the displacement of a concentric cylinder geometry in a conventional controlled stress rheometer. It is clear that this rheological model can only serve as a basis for interpreting data in the post-GP period of coagulation and thus the sonorheometrical technique is unlikely to provide a suitable basis for studying the rheological properties of incipient clots.

Several authors have reported blood rheological characterization using a wave based technique involving the thickness-shear mode (TSM) resonator. Muramatsu *et al* [96] have used multiple TSM sensors to detect blood coagulation factors VIII and IX—factors which are deficient in the blood of patients with hemophilia. Others have monitored only the resonant frequency of the sensor as whole blood coagulates (Cheng *et al* [44]; Si *et al* [97]). Tessier *et al* [98] have used the TSM resonator as an immunosensor to discriminate between A and B bloodgroups.

The TSM resonator has the advantage of using small sample volumes and of providing real-time, continuous measurements. In addition, equivalent circuit models may be used to obtain rheological parameters (Bandey *et al* [7]). The TSM resonator consists of a thin wafer of AT-cut quartz with metal electrodes deposited on both faces. When an alternating voltage is applied between these electrodes, a shear strain results in the crystal due to the piezoelectric effect, with the maximum amplitude of vibration occurring at the mechanical resonant frequency. When a sample of fluid is in contact with one of the resonator surfaces, the oscillating surface interacts mechanically (or acoustically) with it, changing both the resonant frequency and the magnitude of vibration. The latter parameters allow the extraction of information concerning the physical properties of the fluid (Martin *et al* [99]; Frye and Martin [100]; Ballantine *et al* [101]) and suitable mathematical models allow the extraction of appropriate material parameters from the measured responses (Bandey *et al* [102]).

Bandey *et al* [7] have used the TSM resonator technique to study blood rheology, including the temperature dependence of viscosity and coagulation. Their TSM resonators incorporated gold over chromium wrap-around electrodes with a single sensor encased in a sealed stainless steel fixture designed to expose one surface to the fluid environment. The fluid cavity was configured to dissipate compressional waves which otherwise interfere with shear mode resonator characteristics (Schneider

and Martin [103]; Tessier *et al* [98]). The volume of the cavity and the tubing accommodated approximately 3 ml of sample (heparinized bovine blood and plasma), the TSM resonator being cleaned in a 10% bleach solution prior to each experiment. Once cleaned and dried, a baseline resonator response was measured in air prior to fluid exposure.

The sensor response is recorded in one of two ways. In the first method the sensor fixture was attached to a 'lever'-type oscillator circuit (Wessendorf [104]) which drove the resonator, producing an RF signal related to the series resonant frequency and a voltage which was proportional to resonator damping. Sensor frequency, damping voltage, and temperature were recorded every 3 seconds, the sensor system being calibrated using viscosity standards. In the second method the sensor fixture was attached to a Hewlett Packard HP8751A network analyzer operating in reflectance mode. The network analyzer was used to excite the TSM resonator every 10 seconds with a controlled amplitude incident voltage and to measure the amplitude and phase of the reflected signal over a range of frequencies around crystal resonance. This signal was converted to an input impedance, the impedance spectra being modeled using equivalent circuit analysis to extract physical parameters of the surface material (Bandey *et al* [102]).

The initial experiments reported by Bandey *et al* [7] involved monitoring the viscosity-temperature dependence of heparinized bovine plasma and blood using a Physica rotating cup rheometer at a shear rate of  $1000 \text{ s}^{-1}$ , this being approximately the shear rate corresponding to the TSM resonator at 5 MHz in water. Viscosity measurement was carried out simultaneously with the TSM resonator sensor, the sealed cell being filled with either heparinized blood or plasma and the sensor voltage being measured once the system had equilibrated at a specific temperature. The TSM resonator sensor was employed in the vertical position to avoid red blood cell (RBC) sedimentation, its damping voltage, which is proportional to the square root of viscosity for a Newtonian fluid, decreasing with increasing temperature. Little difference was noted between the measured responses for heparinized bovine blood and plasma due to the fact that a liquid at the surface of a quartz resonator is viscously entrained, with the shear acoustic wave penetrating only a small distance into the fluid. This decay length,  $\zeta$ , is given by  $\zeta = (2\eta/\omega\rho)^{1/2}$  where  $\eta$  and  $\rho$  are the fluid viscosity and density, respectively, and  $\omega = 2\pi f$  is the angular excitation frequency of the resonator (Kanazawa and Gordon [105]). For a 5 MHz resonator in water at 20°C, the decay length is 0.25  $\mu\text{m}$ . Based on known plasma density and viscosity, the maximum decay length is less than 0.5  $\mu\text{m}$  - much smaller than the dimensions of RBC's (which are approximately 8  $\mu\text{m}$  in length). It follows that unless the cells are very close to the resonator surface, the mechanical movement that entrains the fluid does not perturb them and the resonator senses only the plasma surrounding the cells. Bandey *et al* [7] point out that this method would allow the extraction of information on plasma viscosity without the need to separate plasma from whole blood.

When whole blood is not heparinized, the clotting process can be studied using the TSM resonator. A range of phase characteristics and transitions then occur, from the Newtonian fluid behavior of the plasma to viscoelastic properties, which are associated with fibrin linkage of the RBCs to the solid mass of the dried clot. Bandey

*et al* [7] have studied the coagulation of whole blood using the TSM resonator and the network analyzer. A drop of fresh blood without anti-coagulating agent was distributed in a thin layer onto the surface of a 5 MHz resonator and the impedance response was recorded in terms of the real and imaginary parts of the impedance during coagulation. The real part of the impedance represents energy loss (through viscous dissipation), while the imaginary part represents energy storage. At the beginning of coagulation the real and imaginary impedance responses were both small and equal, with the subsequent early stage of polymerization being recorded by the TSM resonator as an increase in both the real and imaginary parts of the impedance. As polymerization proceeded further, Bandey *et al* [7] interpreted the TSM resonator response as corresponding to the sample passing through a “gel” phase, its properties being characteristic of a ‘lossy’ viscoelastic layer. The final stage of clot formation was indicated by a dramatic decrease in the real part of the impedance after 5 minutes, the sample layer then being deemed to behave as a rigid mass (indicated by a small value for the real impedance and an appreciable and constant value for the imaginary part of the impedance).

The large variation in the physical properties of the blood required the use of several TSM resonator equivalent circuit models to extract physically meaningful parameters throughout the duration of the clotting process. These included Newtonian fluid; semi-infinite viscoelastic layer; finite viscoelastic layer; and an ideal mass layer. Initially the blood was modelled as a Newtonian fluid, or a semi-infinite viscoelastic layer with zero storage modulus. However, as coagulation proceeded, the real and imaginary parts of the impedance deviated from one another and the blood could no longer be considered Newtonian. The low-frequency viscosity of this non-Newtonian medium was found to increase rapidly, with the shear acoustic wave penetrating the layer and being reflected at the blood-air interface. At this point, the blood was considered to be best modelled as a finite viscoelastic layer. Interestingly, the value of the loss tangent at this incipient clotting stage was found to be approximately 1.9 – close to that value reported by Williams *et al* [32] at the incipient clot formation point (GP) of samples of whole blood. After a further 30 seconds, the loss tangent of the modelled viscoelastic layer in the TSM resonator experiment had decreased (to approximately 1.2) while the modulus magnitude remained constant. Bandey *et al* [7] have attributed this behaviour to stiffening of the blood layer as a result of immobilized cells. In the final clot stage, the blood was found to be best represented as an ideal mass layer, the value of the loss tangent being approximately zero.

## **2.8 Surface Plasmon Resonance and Quartz Crystal Microbalance based studies of coagulation**

The FOR technique described earlier has been used in coagulation studies in conjunction with a complementary technique known as Surface Plasmon Resonance, SPR. SPR is an optical phenomenon of interference between light and matter which has been used to study plasma and whole blood coagulation, blood-surface interactions, and other aspects of biomaterials (Davies [106]; Hansson *et al* [107]; Nelson *et al* [108] Viking *et al* [109, 110]). SPR has a small sensing depth,

typically around 20-30% of the wavelength of the incident light beam (approximately 200 nm) making it a surface- or near-surface-sensitive method (Johansen *et al* [111]). When light is reflected under total internal reflection (e.g. at a glass/liquid interface) an electromagnetic field - the evanescent field - exists outside the glass. If a sufficiently thin metal film (such as gold) is applied between the glass and the liquid, the evanescent field can excite a charge-density wave known as a surface plasmon which propagates at the metal/liquid interface). The surface plasmon can only be excited for certain combinations of incident angles and wavelengths of the light but SPR can be performed in real-time during static and flow conditions and can be used for the analysis of non-transparent medias such as whole blood.

Viking *et al* [109] report representative SPR response curves for experiments involving blood plasma and different thromboplastin concentrations. The SPR response change is usually interpreted as a change in refractive index at the sensor/liquid interface. A response of 1000 resonance units (RU) corresponds to a refractive index change ( $\Delta n$ ) of approximately  $10^{-3}$ . In the experiments of Viking *et al* [109] the clotting process was initiated by addition of  $Ca^{2+}$  and corresponded to a steep response fall due to dilution of the sample with a solution of lower refractive index. The resulting 'sensorgram' (a plot of RU .v. time) was fitted using a sigmoid curve equation, with the extracted features being  $k$  (the derived time constant of the coagulation process),  $R_{eq}$  (the maximum recorded RU response) and  $t_{lag}$  (the time at which the response is half of  $R_{eq}$ ). The lag time of the response was found to be thromboplastin dependent and clotting was observed (by eye) to correspond closely to the development of the sensorgram slope. The plot of  $k$  value versus lag time overlapped for samples with and without heparin, which appeared, somewhat unexpectedly, to indicate that the clotting process follows the same kinetics whether it is accelerated by thromboplastin or delayed by heparin. Viking *et al* [109] acknowledge that this contradicts results reported by others (Heuck and Baumann [112]), who state that the  $k$  parameter is more sensitive to the clotting kinetics than the lag time, and conclude that the role of the detection method and the fitting equation requires further investigation.

The SPR instrument reported by Hansson *et al* [89] utilised a fixed set of incident angles and white light, the resonance being monitored by a spectrophotometer which detected a dark band at the resonance wavelength, which in turn depends on the refractive index close to the interface. Their SPR measurements were performed in combination with FOR to study haemostasis and interactions between whole blood and artificial surfaces. SFLLRN peptide-stimulated coagulation of native whole blood presented a higher SPR signal with different appearance than plasma coagulation, while the FOR signals corresponding to plasma and whole blood coagulation were similar. This indicated that the SPR technique was more sensitive to cell-surface interactions than to fibrin formation in whole blood during coagulation, while the FOR technique was equally sensitive to coagulation in whole blood and plasma. Spontaneous coagulation of native whole blood in contact with methyl- and hydroxyl-terminated self-assembled monolayers (SAM) on gold and gold surfaces regenerated after coagulation were also studied. The regenerated gold surfaces displayed the shortest coagulation times, although the contact-activation of blood coagulation for

these surfaces was low. The methylated and hydroxylated surfaces were comparable in terms of coagulation activation, while the hydroxylated surfaces presented FOR signals that indicated detachment of the coagulum from the surface.

An interesting finding reported in this work is that the regeneration of used gold-coated sensor probes and sample cups resulted in gold surfaces with a remanent proteinaceous layer. The thickness of the protein remnants on the surface were analysed with ellipsometry and found to be at the angstrom level, indicating less than a monolayer of plasma proteins. The regenerated and clean gold sample cups and sensor probes performed similarly in a coagulating system although the frequency declined immediately after start of the analysis for the regenerated gold surface (which was not the case for a clean sample cup). The early decrease in FOR frequency was interpreted as a higher tendency by the re-used surface to adsorb proteins, associate proteins, and/or adhere cells. Despite its force measuring capability and its recent application in studies of the tensile properties of thin fluid films (Barrow *et al* [113]), the AFM has rarely been exploited in blood coagulation studies (Blinc *et al* [114]) being mainly limited to imaging sensor surfaces involved in clotting experiments, in conjunction with SPR work (Vikinge *et al* [109]).

Hansson *et al* [89] report the general aspects of SPR and FOR signals registered during the coagulation of citrated blood plasma after initiation by thromboplastin and  $\text{Ca}^{2+}$ . The time of frequency minimum was interpreted as the coagulation time and the  $Ld$  increased prior to clotting, reaching a sharp peak and declining rapidly during clotting. The SPR response increased by approximately 2 kRU during coagulation and displayed a sigmoidal shape with an increase of the signal during the coagulation process. Interestingly, the coagulation signal feature in the FOR curves (i.e. the frequency minimum and the peak in the logarithmic damping) coincided with  $(dR/dt)_{\text{max}}$  of the coagulation phase in the SPR signal. In most respects the SPR and FOR signals obtained by Hansson *et al* [89] during plasma coagulation display typical blood plasma coagulation features, i.e. the  $Ld$  peak (FOR), frequency minimum (FOR) and an increase in the SPR signal appear simultaneously. The FOR and SPR features in this work are similar to those previously described (see Ranby *et al* [85], Vikinge *et al* [109]). When the coagulation of native whole blood was accelerated through the addition of SFLLRN peptide (Hui *et al* [115]), coagulation coincided for the SPR and FOR methods since the peak in  $Ld$ , the frequency minimum and the local SPR maximum were all recorded at around 13 minutes. In the coagulation of native whole blood, which was accelerated through activation of the thrombin receptor on platelets with the thrombin receptor activating peptide (SFLLRN), the total increase in the signal was five to six times larger than that for blood plasma. When the sample coagulated, a local SPR maximum appeared at 13 minutes, nearly coincident with the minimum in the FOR frequency. After the coagulation had occurred, a slow increase in the SPR signal due to the clot retraction was observed at 25 minutes, the FOR signals showing characteristics similar to those for plasma.

It is noteworthy that although their SPR sensing surface probe was placed vertically, the SPR signal features reported by Hansson *et al* [89] closely resemble previous curve shapes obtained during the coagulation of citrated whole blood when initiated with thromboplastin and  $\text{Ca}^{2+}$  on a horizontal flat sensing surface (Hansson *et*

*al* [107]). The increase in the signal in the early phase of the analysis was then thought to be due to deposition of cells but in a vertical arrangement no deposition due to gravity would be expected. The most probable cause of the initial SPR signal increase seemed to be protein adsorption, in concert with cell adhesion. The magnitude of the SPR response from the start of the analysis until a completed coagulation occurs (ca. 10 kRU) indicated that the mass density change was much higher than expected due to protein adsorption or plasma coagulation. The increase in both the SPR and FOR signals after 25 to 30 minutes was suggested to be due to retraction of the blood clot. The increase in SPR signal may then be a consequence of increased refractive index when the clot contracts and serum is squeezed out of the coagulum. The increase in *Ld* and frequency are then possibly due to a more liquid state near the sample cup wall when the clot retracts from the sample cup surface, the coagulum contracting around the SPR probe and retracting from the sample cup walls surrounding it.

In addition to its use in combination with FOR, the SPR technique has been combined with the quartz crystal microbalance (QCM) technique (Marx, [114]; Vikinge *et al* [110]). A variant of the piezo quartz crystal (PQC) technique, QCM measures changes in the amount of coupled mass via a frequency shift, and has been used to detect plasma and whole blood coagulation (Muramatsu *et al* [87]; Cheng *et al* [41]). It comprises a thin AT-cut quartz wafer sandwiched between two metal excitation electrodes at its fundamental resonance frequency – typically 10 MHz. The change in oscillation frequency is related to the mass accumulated on the crystal (Sauerbrey, [117]). In situations where the adsorbed film is not rigid the Sauerbrey relation becomes invalid. A ‘soft’ (i.e. viscoelastic) film will not fully couple to the oscillation of the crystal which dampens the crystal's oscillation and in these circumstances an appropriate viscoelastic model is required for the interpretation of results. Piezo-crystal sensors have found application in the determination of fibrinogen concentration over the range from 50 to 500 mg/dl based on gelation time measurement (Muramatsu *et al*, [118]).

A variation of QCM provides a measure of energy dissipation (*D*) of the sensor signal (Rodahl *et al*, [119]) which is related to the viscoelastic properties of the adsorbed layer. QCM with dissipation monitoring (QCM-D) has been used in studies of single protein adsorption (Hook *et al* [120]), blood coagulation density and immune complement activation on artificial surfaces, and contact activated blood coagulation (Sellborn *et al*, [121]; Andersson *et al*, [122]; Berglin *et al*, [123]; Andersson *et al*, [124]; Hook *et al* [120]). Assessing potential surface induced blood coagulation often involves measurement of the soluble thrombin–antithrombin complex (TAT) with enzyme immuno assay (EIA) as the standard method. QCM-D provides a possible alternative for determination of surface induced blood coagulation, since the fibrin formation can be detected *in situ* and in real time on the inductive surface. Andersson *et al*, [122] report that surface associated fibrin formation could be described with at least four parameters using the QCM-D method, these being; (1) time to onset of clot formation, (2) rate of clot assembly, (3) total frequency shift at stable plateau and (4) clot density. They conclude that few other methods (if any) can describe the surface induced clot formation with so many parameters, reasoning that the success of the QCM-D technique is that the clot analysis takes place directly on the modified sensor

surface. However, they note that the D-factor does not provide any new analytical information about the possible complement activation mechanisms. Nevertheless, QCM-D was found to be a reliable tool for the analysis of surface induced complement activation. Andersson *et al*, [122] have also compared the QCM-D technique with EIA measurements of soluble products from the surface activation of the complement and coagulation systems and find that the results from EIA and QCM-D measurements correspond well for the complement activation but not for the coagulation. The latter finding is deemed to be probably due to the biological complexity of the coagulation system.

Puckett *et al* [125] have described a new coagulation monitoring sensor technique based on magnetoelastic sensors in which the ribbon-like magnetoelastic element oscillates at a fundamental frequency which shifts linearly in response to applied mass loads, or a fixed mass load of changing elasticity (Loiselle and Grimes [126]; Grimes *et al* [127]). The magnetoelastic sensors emit magnetic flux which can be detected by a remote pick-up coil. Changes in viscosity during coagulation shift the characteristic resonance frequency of the sensor, providing a record called a 'blood clotting profile'. Puckett *et al* [125] describe experiments in which fresh and anticoagulated whole blood from different animal species were evaluated. They report that a distinct coagulation profile could be seen which was similar to the results reported with PQC experiments (Cheng *et al* [44]). The decrease in signal amplitude was explained by the fact that as blood coagulates, the resonance frequency shifts further away from the monitoring frequency, the eventual levelling-off of the signal versus time profile being indicative of the end point of coagulation where no further changes in viscosity occur. The technique may offer advantages over SPR and QCM/PQC in that it is simple, inexpensive and requires a minimal sample volume.

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