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● *Original Contribution*

ASSESSMENT BY TRANSIENT ELASTOGRAPHY OF THE VISCOELASTIC PROPERTIES OF BLOOD DURING CLOTTING

JEAN-LUC GENNISSON,[1]* SOPHIE LEROUGE,† and GUY CLOUTIER*

*Laboratory of Biorheology and Medical Ultrasonics, University of Montreal Hospital Research Center, Montreal, Quebec, Canada; and †Laboratory of Vascular Biomaterials, University of Montreal Hospital Research Center, Montreal, Quebec, Canada

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Abstract—Blood clotting is a natural process that can be both beneficial and life-threatening for the human body. It allows the maintenance of hemostasis after vascular injury, but it can also cause deep vein thrombosis and heart stroke. This study aimed better to understand the clotting process from a biomechanical point of view by using an acoustic method. The long-term objective is the staging of the age of clots in deep veins for therapy planning. The transient elastography method using a shear elasticity probe served to evaluate the shear wave velocity (V_S) and shear wave attenuation (α_S) of porcine whole blood during *in vitro* clot formation. By solving an inverse problem, it was then possible to provide images of the elasticity (μ_B) and of the viscosity (η_B) from clotting blood. The time-varying elasticity and viscosity were very similar to what has been observed for the sol-gel transition of polymers. The mechanical properties of blood clot, which were modified by varying the hematocrit and by adding heparin or fibrinogen, were clearly assessed by the transient elastography technique. It is concluded that the shear elasticity probe is an appropriate tool to quantify and follow the sol-gel transition of blood during clotting. (E-mail: guy.cloutier@umontreal.ca) **© 2006 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Transient elastography, Acoustic shear waves, Sol-gel transition, Blood clotting, Fibrinogen, Heparin, Hematocrit variations, Elasticity, Viscosity, Mechanical properties.**

INTRODUCTION

Several rheological approaches have been proposed to study liquid or coagulating blood, namely optical, mechanical and ultrasound characterization methods. In optics, blood coagulation was studied in a special beaker by analyzing the properties of the reflected waves. The blood samples were illuminated with a YAG (yttrium aluminum garnet) laser and coagulation was characterized from the refractive index and the absorption coefficient (Nilsson et al. 1997; Barton et al. 2001). To our knowledge, the viscoelastic properties of liquid blood were first reported in 1972 (Thurston 1972). Then, using conventional rotational rheometers, the viscoelasticity of blood was further studied and other properties such as the thixotropy were analyzed (Kiesewetter et al. 1982; Quemada and Droz 1983; Vlastos et al. 1997). The thromboelastography mechanical technique was employed to characterize blood coagulation from a qualitative point of view. With this technique, blood coagulation was characterized for different pathologies by analyzing the differences in the rotating amplitude of concentric cylinders (Wenker et al. 2000; Forestier et al. 2001).

By using ultrasound, the first rheological studies were performed on liquid blood (Sigelmann and Reid 1973; Shung et al. 1976) and almost simultaneously on coagulating blood (Jacobs et al. 1975; Shung et al. 1975). The common parameters assessed in these reports were the speed of sound, ultrasound attenuation and scattering, and the frequency dependence of these last two measures. Although this was not an objective of these studies, the speed of sound can be used as an indirect measure of the mechanical properties of a given medium, since it is related to the density and compressibility of the sample. It is to note also that the development of rheological methods based on ultrasound is mainly motivated

Address correspondence to: Dr. Guy Cloutier, Director of the Laboratory of Biorheology and Medical Ultrasonics, University of Montreal Hospital Research Center, Pavilion J.A de Sève (room Y-1619), 2099 Alexandre de Sève, Montreal, Quebec, H2L 2W5, Canada. E-mail: guy.cloutier@umontreal.ca and jl.gennisson@espci.fr

[1] Dr Jean-Luc Gennisson is now a CNRS Research Scientist at the Laboratoire Ondes et Acoustique, Paris, France.

by its ability to penetrate biologic tissues and thus by its potential to be used in non invasive *in vivo* imaging studies. Researches on ultrasound-based rheological methods are still a topical question (Machado et al. 1991; Cloutier and Shung 1993; Machado et al. 1997; Voleišis et al. 2002).

Since more than a decade, another growing field of research that allows to assess the mechanical properties of soft biologic tissues has been in development, namely ultrasound elastography, which relies on quasi static and dynamic methods. When applied to blood, quasi static elastography was used to assess the mechanical properties of clots, a major motivation being the staging of the age of clots in deep veins for therapy planning (Emilianov et al. 2002; Aglyamov et al. 2004). One limitation of these last studies was that the kinetics of blood clotting was not assessed and measurements were thus performed only on formed blood clots. Dynamic elastography based on a remote palpation technique was developed to assess the viscoelasticity of blood (Viola et al. 2004). With this technique, displacement fields are induced in blood during coagulation by a radiation force produced by a focused transducer. From the amplitude and relaxation time of the displacement fields, changes in elasticity and viscosity were estimated, but no absolute assessments were obtained.

To summarize, the abovementioned elastography reports had two main drawbacks. Namely, no absolute values of clots' elasticity and viscosity were given and the kinetics of blood coagulation was not assessed. The current paper reports a technique that allows to follow quantitatively and, potentially, *in vivo*, the clotting of blood as a function of time with a one-dimensional (1D) shear elasticity probe (Sandrin et al. 2002). This tool consists in detecting the velocity and attenuation of shear waves propagating in soft solids. The velocity is directly related to elasticity in a perfectly elastic medium (Royer and Dieulesaint 1996). Moreover, in viscoelastic solids, the shear wave velocity (SWV) and shear wave attenuation (SWA) are linked to the viscosity-elasticity couple.

The shear-wave elasticity method described here was previously used to assess SWV and SWA variations in agar-gelatin mixtures during percolation (Gennisson and Cloutier 2006). Results showed that very small variations in elasticity and viscosity could be estimated in real-time during solidification. Further, to assess the potential impact of this method, experiments were performed on porcine whole blood samples during clotting. SWV and SWA were measured to determine the time-varying elasticity and viscosity of each blood sample as a function of the hematocrit. Furthermore, to simulate pathologies and to test the sensitivity of the transient elastography technique to small changes in blood mechanical properties, fibrinogen or heparin were added to the samples to modulate the coagulation process.

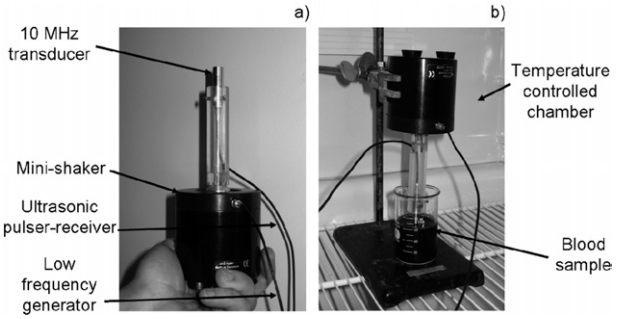

Fig. 1. (a) Experimental set-up. The shear elasticity probe was composed of a transducer mounted on a minishaker. The probe was placed in a temperature-controlled chamber at the surface of a 60-mL blood sample. (b) The shear elasticity probe.

MATERIALS AND METHODS

Preparation of the blood samples

Porcine whole blood samples were collected from a slaughterhouse, immediately anticoagulated with an EDTA (ethylenediamine tetraacetic acid, number E-9884, Sigma Chemical, St. Louis, MO, USA) saline solution (3 g/L) and stored at ambient temperature. Within 12 h to 72 h after blood collection, blood clot formation was studied *in vitro* in 60-mL samples in a beaker. The shear elasticity probe was placed at the surface of the sample in a temperature-controlled chamber at 23°C (Fig. 1b). In all experiments, coagulation was initiated by adding to blood 0.12 g of calcium ions ($CaCl_2$, calcium chloride dihydrate, Acros Organics, Geel, Belgium) diluted into 2.4 mL of saline solution.

Samples at different hematocrits (30%, 40%, 50% and 55%) were prepared by centrifugation. Hematocrits below 30% could not be tested, because red blood cell (RBC) sedimentation was too important and too fast to provide good homogeneous ultrasonic speckle during the 4-h experiment. Speckles are mainly produced by RBCs in whole blood.

In some experiments, heparin and fibrinogen were added to the blood samples before initiating coagulation with $CaCl_2$. The anticoagulant heparin (1000 units/mL of solution, number 0045380, LEO Pharma, Ballerup, Denmark) was added directly in blood at concentrations of 0.5, 1 and 1.5 units/mL of blood. Two hundred and 400 mg of the pro-coagulant fibrinogen plasma protein (immuno bovine fibrinogen, number 820212, MP Biomedicals, Irvine, CA, USA) were first diluted in 15-mL of saline solution and then mixed with the 60-mL blood sample to obtain final concentrations of 2.65 and 5.30 mg/mL of blood. In all cases, hematocrits were measured

before adding calcium ions or drugs. So, the hematocrits reported hereafter were corrected by considering the volume of solutions added.

Data acquisition and analysis

The shear elasticity probe (Fig. 1a) was composed of a single-element 10-MHz ultrasonic transducer (model V312, Panametrics, Waltham, MA, USA) mounted on a minishaker (type 4810, Brüel&Kjær, Nærum, Denmark). The probe was placed at the surface of the blood sample and did not move during the experiment. Shear waves were generated by a low frequency pulse given with the front face of the transducer while it was working in pulse-echo mode. Ultrasound was generated by a pulse-echo system (5900PR, Panametrics) and low frequency pulses (25 Hz) were produced with a function generator (model 33250A, Agilent, Palo Alto, CA, USA), amplified (low frequency amplifier, type 2706, Brüel&Kjær) and sent to the minishaker. The amplitude of the low frequency pulse was not changed during the experiments. Measurements were performed at 25 Hz to reduce biases from shear wave diffraction effects (Gennisson and Cloutier 2006). At 25 Hz, the shear wavelength was large enough and attenuation effects were not too high to allow measurements of SWV and SWA. If a frequency below 25 Hz had been selected, one or less than one shear wavelengths would have propagated in the blood sample, and measurements would have been biased by the shear waves near field. A higher frequency would have increased attenuation. During a typical experiment, 300 echographic lines (A-scans) with a recurrence frequency of 1 kHz were recorded every 30 s for 4 h with an 8-bit digitizing acquisition card (Compuscope 8500, Gage, Lachine, QC, Canada) at a sampling frequency of 100 MHz.

During data analysis, the movement of the single-element transducer attached to the minishaker was compensated with a cross-correlation technique that used the strong echo from the bottom of the beaker, as performed earlier (Sandrin et al. 2002). Then, the longitudinal components of displacement along the ultrasonic beam were computed with a cross-correlation algorithm between successive ultrasonic signals stored in memory (Dickinson and Hill 1982; Ophir et al. 1991; Sandrin et al. 2002). From the displacement fields, an inverse problem approach based on the 1D Helmholtz equation was used to recover the shear velocity (V_S) and attenuation (α_S) of blood samples (Catheline et al. 2004; Gennisson and Cloutier 2006). Assuming that the mechanical behavior of blood samples is described by the Voigt's model, the Helmholtz equation can then be expressed as follow:

$$\frac{\partial^2 FT\left(u_z(z)\right)}{\partial z^2} + \frac{\rho\omega^2}{\left(\mu + i\omega\eta\right)} FT\left(u_z(z)\right) = 0, \qquad (1)$$

where FT is the Fourier transform, $u_z(z)$ is the longitudinal component of the shear wave displacement field, z is depth, ρ is the density of the medium (here ρ was considered constant at 1055 kg/m^3), μ is its elasticity and η its viscosity and ω is the pulsation frequency ($\omega = 2\pi f$, where f was fixed at 25 Hz in this study).

As detailed in Catheline et al. (2004), the local wave vector k is expressed as:

$$k = \frac{\rho\omega^2}{\left(\mu + i\omega\eta\right)} \qquad (2)$$

and the local SWV (V_S) and SWA (α_S) are given by:

$$V_S = \frac{\omega}{\mathrm{Re}\left[k\right]},\ \alpha_S = \mathrm{Im}\left[k\right]. \qquad (3)$$

Thus, from the complex wave vector, SWV and SWA can be expressed as a function of the elasticity and viscosity by:

$$V_S = \sqrt{\frac{2\left(\mu^2 + \omega^2\eta^2\right)}{\rho\left(\mu + \sqrt{\mu^2 + \omega^2\eta^2}\right)}}, \qquad (4)$$

$$\alpha_S = \sqrt{\frac{\rho\omega^2\left(\sqrt{\mu^2 + \omega^2\eta^2} - \mu\right)}{2\left(\mu^2 + \omega^2\eta^2\right)}}. \qquad (5)$$

Finally, by inversing this set of eqns (4) and (5), the elasticity μ and viscosity η of blood clots were retrieved as a function of time:

$$\mu = \frac{\rho V_S^2}{\left|\left(1 - \left(\frac{\alpha_S V_S}{\omega}\right)^2\right)\right|\left(\frac{2}{1 - \left(\frac{\alpha_S V_S}{\omega}\right)^2} - 1\right)}, \qquad (6)$$

$$\eta = \frac{\rho V_S^2}{\omega} \sqrt{\frac{\left(\frac{1}{1 - \left(\frac{\alpha_S V_S}{\omega}\right)^2} - 1\right)}{\left|\left(1 - \left(\frac{\alpha_S V_S}{\omega}\right)^2\right)\right|\left(\frac{2}{1 - \left(\frac{\alpha_S V_S}{\omega}\right)^2} - 1\right)^2}}. \qquad (7)$$

RESULTS

Typical displacement fields

Figure 2 presents a typical evolution of displacement fields during blood clotting. A low frequency pulse was transmitted to the blood sample at time t = 30 ms (abscissa on Fig. 2) for each observational time, going from 0 to 150 min in this example (the whole observation period was 4 h and recordings were performed every 30 s; only 10 displacement fields are presented on this figure). At the initial observation time t = 0 min, blood

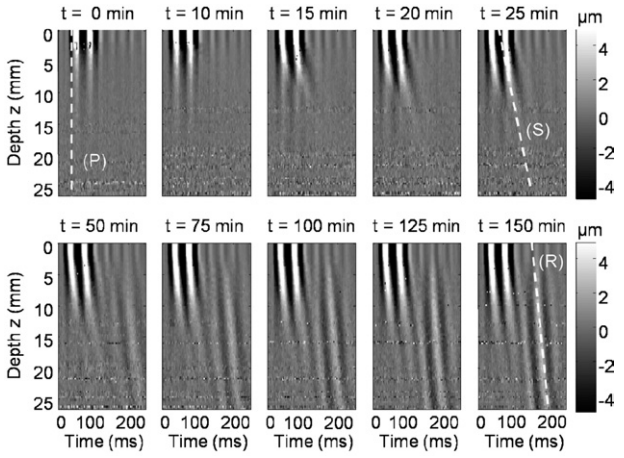

Fig. 2. Displacement field in blood during coagulation (52% hematocrit, sample no. 1) at different times (from 0 to 150 min). The displacement field was plotted in gray-color-scale, along the depth z as a function of time. The low frequency pulse was given at 25 Hz at the time 30 ms. Three different kinds of waves propagated. These were bulk waves (P), shear waves (S) and some secondary waves (R).

was liquid and only a very fast bulk wave with a velocity close to 1500 m/s was visible (longitudinal wave P requiring a few μs to travel 25 mm within the sample). Between t = 10 min and t = 15 min, the medium started becoming solid enough to allow the propagation of shear waves (quasitransverse wave S). Shear waves took a certain time to arrive at each depth, because they are much slower than the bulk wave, with a velocity of about 1 m/s. As soon as the medium became harder, the slope of the shear wave (depicted on the depth versus time plots) increased and could reach a constant value. At this point, the clot was mechanically stable. At time t = 50 min, some secondary waves (reflection wave R) appeared from reflections on the beaker side (Catheline et al. 1999). After t = 150 min, the displacement fields were similar and no big changes could be seen for all blood samples.

Shear wave velocity (SWV) and shear wave attenuation (SWA)

From the displacement fields (Fig. 2), SWV and SWA were computed according to eqn 3. In Figure 3, they are presented as a function of time for three samples drawn from the same animal (the experiment was repeated three times to appreciate the reproducibility of the method). If one defines the variable t_B as the time corresponding to reliable (valid) velocity and attenuation measurements, Fig. 3 shows, for example, with the sample no. 3 (line with triangles), that the velocity V_{StB} = 0.37 m/s and that the attenuation α_{StB} = 319 Np/m at t_B = 19 min. Before t_B, velocity and attenuation measurements were meaningless, either because no shear waves were propagating or because the signal-to-noise ratio was too low to estimate any parameter. At t_B, the state of coagulating blood changed from liquid to solid, thus allowing the propagation of shear waves (shear waves do not propagate in fluids) and permitting velocity and attenuation measurements (Gennisson and Cloutier 2006). This time can be defined as the sol-gel transition time in rheology (Djabourov et al. 1988).

From t_B, SWV increased (Fig. 3a), whereas SWA decreased as a function of time (Fig. 3b). Both reached a plateau at the end of the assessment of the coagulation process and mean values could be calculated over the last hour (V_{Sm} = 0.68 ± 0.03 m/s, α_{Sm} = 96 ± 9 Np/m, n = 3 samples). By using eqns 6 and 7, the clot's elasticity (Fig. 3c) and viscosity (Fig. 3d) were retrieved as a function of time. Elasticity and viscosity increased from t_B (μ_{BtB} = 20 ± 17 Pa, η_{BtB} = 0.6 ± 0.1 Pa.s, n = 3) and both measurements also tended to reach a plateau after several minutes (μ_{Bm} = 244 ± 28 Pa, η_{Bm} = 1.8 ± 0.1 Pa.s, n = 3, mean values computed over the last hour). According to these last measures repeated over three samples, the coefficients of variation of SWV, SWA, μ_B and η_B at the plateau were 4.4%, 9.4%, 11.5% and 5.6%, respectively. The coefficient of variation on t_B was 12.5%. This allows confirming the reproducibility of the technique. Table 1 presents a summary of all variables displayed in Fig. 3. As noted, the coefficients of variation on a single experiment were generally lower than the ones computed by considering all three measurements (see the last four columns of Table 1).

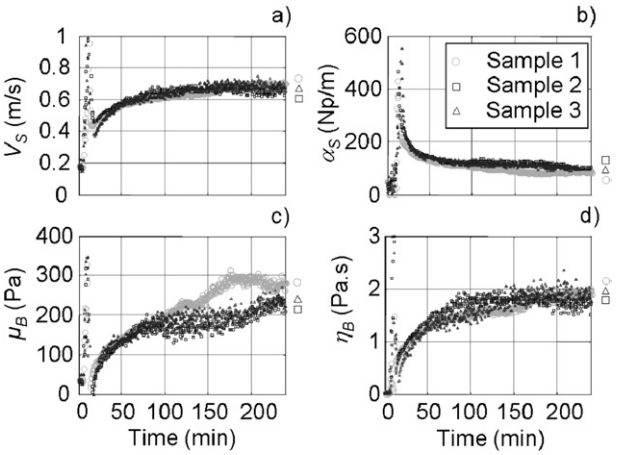

Fig. 3. Reproducibility test on three samples from the same whole blood (50% hematocrit). (a) Shear wave velocity SWV or V_S, (b) Shear wave attenuation SWA or α_S, (c) Elasticity μ_B and (d) Viscosity η_B of blood clot during coagulation as a function of time. The mean sol-gel transition time was found to be at t_B = 16 ± 2 min, when the blood clot became solid.

Table 1. Data from the reproducibility test: SWV, SWA, elasticity and viscosity at the transition time t_B and at the plateau as a function of the sample number from the same whole blood ($n = 3$). The hematocrit was 50%

Sample (#)	Time t_B (min)	Velocity at t_B: V_S (m/s)	Attenuation at t_B: α_S (Np/m)	Elasticity at t_B: μ_B (Pa)	Viscosity at t_B: η_B (Pa.s)	Velocity at the plateau: V_S (m/s)	Attenuation at the plateau: α_S (Np/m)	Elasticity at the plateau: μ_B (Pa)	Viscosity at the plateau: η_B (Pa.s)
1	14	0.46	319	17	0.6	0.69 ± 0.01	87 ± 3	277 ± 9	1.9 ± 0.1
2	16	0.50	318	5	0.6	0.64 ± 0.01	100 ± 4	224 ± 13	1.7 ± 0.1
3	19	0.37	319	39	0.5	0.69 ± 0.01	101 ± 4	232 ± 13	1.9 ± 0.1
Mean	16 ± 2	0.44 ± 0.07	319 ± 1	20 ± 17	0.6 ± 0.1	0.68 ± 0.03	96 ± 9	244 ± 28	1.8 ± 0.1

Effect of hematocrit variation

Blood clot formation was also studied as a function of the hematocrit (Fig. 4). Four samples with different hematocrits were prepared (from 28.8% to 52.8%) from the same animal blood. As the hematocrit was raised, SWV decreased (Fig. 4a) and SWA increased at the plateau (Fig. 4b). Likewise, μ_B (Fig. 4c) and η_B (Fig. 4d) decreased at the plateau as the hematocrit was raised. In other words, the lower the hematocrit, the harder and more viscous was the blood clot. As listed in Table 2, the transition time t_B decreased from 47 to 15 min when the hematocrit was increased. Likewise, SWV, μ_B and η_B at t_B declined while SWA increased.

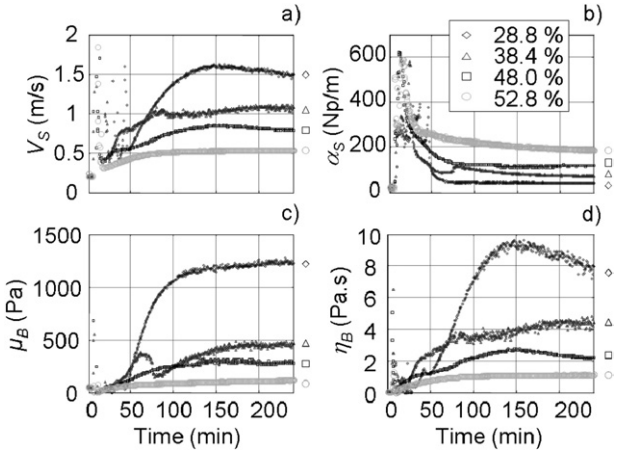

Fig. 4. (a) SWV, (b) SWA, (c) Elasticity and (d) Viscosity as a function of time for different hematocrit levels. The lower the hematocrit, the harder was the clot.

Effect of fibrinogen addition

Figure 5 and Table 3 summarize the effect of fibrinogen on clot formation of three samples from the same blood. Its addition (from 0 to 5.30 mg/mL of blood) increased the velocity (Fig. 5a) at the plateau. Likewise, there was no important change in attenuation at the plateau (Fig. 5b). The transition time t_B increased with the amount of fibrinogen added (from 12 to 22 min). One can also notice from Table 3 that, at t_B, SWA decreased and the viscosity slightly increased with the fibrinogen concentration. At t_B, the variation of SWV and of the elasticity did not follow a clear trend. In terms of viscoelasticity after more than 1 h of experiment, a large change in viscosity was noted for the highest concentration of fibrinogen tested (the viscosity reached a mean of 12.5 Pa s). The variability was important and results were less predictable for the elasticity (there was no progressive increase in elasticity as a function of the amount of fibrinogen added). Additional experiments on different blood samples would thus be required to confirm these last observations.

Effect of heparin addition

In Fig. 6, SWV (Fig. 6a) and SWA (Fig. 6b) are plotted as a function of the heparin concentration added. The data were obtained with the same whole blood sample. SWV dropped and SWA increased at the plateau when heparin was added. Heparin had a large impact on the gelation time t_B, which increased from 45 min to 130 min. As summarized in Table 4, both the elasticity (Fig. 6c) and viscosity (Fig. 6d) were reduced at the plateau

Table 2. SWV, SWA, elasticity and viscosity at the transition time t_B and at the plateau as a function of the hematocrit

Hematocrit (%)	Time t_B (min)	Velocity at t_B: V_S (m/s)	Attenuation at t_B: α_S (Np/m)	Elasticity at t_B: μ_B (Pa)	Viscosity at t_B: η_B (Pa.s)	Velocity at the plateau: V_S (m/s)	Attenuation at the plateau: α_S (Np/m)	Elasticity at the plateau: μ_B (Pa)	Viscosity at the plateau: η_B (Pa.s)
28.8	47	0.54	179	91	1.1	1.50 ± 0.02	42 ± 1	1230 ± 14	8.1 ± 0.3
38.4	25	0.42	393	26	0.6	1.07 ± 0.01	73 ± 2	452 ± 15	4.3 ± 0.2
48.0	17	0.39	472	34	0.5	0.79 ± 0.01	120 ± 2	280 ± 7	2.2 ± 0.1
52.8	15	0.33	590	23	0.3	0.53 ± 0.01	191 ± 2	114 ± 1	1.1 ± 0.1

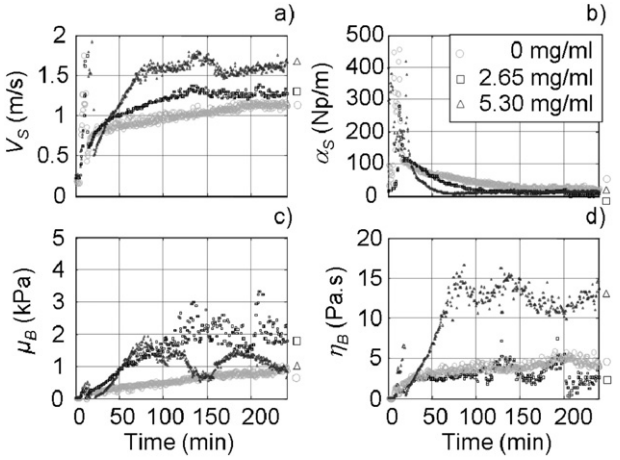

Fig. 5. (a) SWV, (b) SWA, (c) Elasticity and (d) Viscosity as a function of time for different fibrinogen quantities added in mg/mL of blood. Due to more noisy data, elasticity and viscosity are plotted with a median filter on three points.

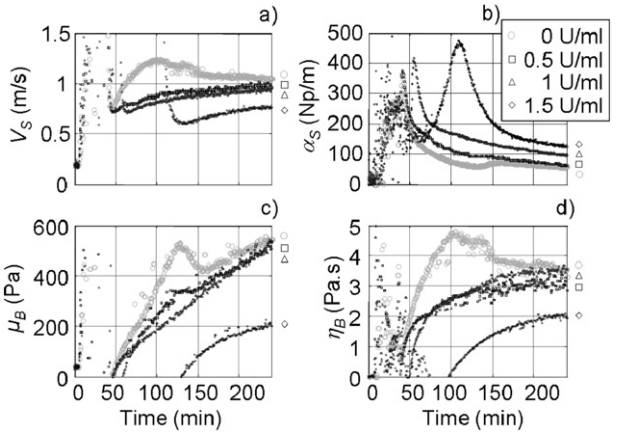

Fig. 6. (a) SWV, (b) SWA, (c) Elasticity and (d) Viscosity as a function of time for different concentrations of heparin added in units/mL of blood.

when the highest amount of heparin was added (*i.e.*, 1.5 units/mL of blood).

DISCUSSION

Coagulation transforms liquid blood into a gelatinous product consisting of a fibrin network with entrapped RBCs. The coagulation process involves two possible pathways with a "cascade of reactions" (Fig. 7), each leading to the formation of thrombin which catalyzes the transformation of fibrinogen, a plasmatic protein, into fibrin filaments. Thrombin also activates factor XIII (fibrin stabilizer factor), which interacts with fibrin, leading to cross-linking of fibrin networks into a hard insoluble polymer. *In vitro*, only the intrinsic pathway is activated. The formation of thrombin by this pathway is known to be the slowest step in coagulation; it is followed by fibrinogen polymerization and clot formation in a few seconds (Dintenfass 1971; Marieb 1997). This could correspond to the delay between $t = 0$ min and t_B.

The kinetics of blood clot formation (SWV, SWA, elasticity, viscosity) (Fig. 3) have been observed and they can be shown to be very similar to agar-gelatin mixtures in the solidification or polymerization phase (Gennisson and Cloutier 2006). As blood is grossly constituted of biologic monomers (fibrinogen in plasma) and particles (RBCs), it is not surprising to find the same behavior as in agar-gelatin mixtures where gelatin is the monomer and agar, the particles.

Before the transition time (t_B) is reached, the absence of shear wave propagation in blood does not allow the determination of velocity and attenuation. The parameter t_B is defined as the transition time between the liquid and solid phases of blood. At this precise time, the medium becomes solid enough to allow the propagation of shear waves, as in agar-gelatin phantoms (Gennisson and Cloutier 2006). From this time, as noted in Fig. 3, velocities and attenuations evolved up and down, respectively, until they reached a plateau when the clot was mechanically stable. The elasticity and viscosity of the clot (Fig. 3) followed a power law and also reached a plateau, as in agar-gelatin phantoms (Joly Duhamel et al. 2002). Experimentally, we thus found similarities between nonbiological polymer solidification, known as percolation, and blood coagulation.

Table 3. SWV, SWA, elasticity and viscosity at the transition time t_B and at the plateau as a function of the fibrinogen quantity added. The hematocrit was 37.2%

Amount of fibrinogen added (mg/ml of blood)	Time t_B (min)	Velocity at t_B: V_S (m/s)	Attenuation at t_B: α_S (Np/m)	Elasticity at t_B: μ_B (Pa)	Viscosity at t_B: η_B (Pa.s)	Velocity at the plateau: V_S (m/s)	Attenuation at the plateau: α_S (Np/m)	Elasticity at the plateau: μ_B (Pa)	Viscosity at the plateau: η_B (Pa.s)
0	12	0.44	366	36	0.7	1.13 ± 0.02	23 ± 4	869 ± 74	4.6 ± 0.6
2.65	15	0.60	238	135	0.9	1.27 ± 0.05	5 ± 4	2132 ± 609	2.1 ± 1.6
5.30	22	0.53	201	20	1.1	1.61 ± 0.04	15 ± 2	1035 ± 136	12.5 ± 1.2

Table 4. SWV, SWA, elasticity and viscosity at the transition time t_B and at the plateau as a function of the heparin concentration added. The hematocrit was 40.3%

Heparin concentration (units/ml of blood)	Time t_B (min)	Velocity at t_B: V_S (m/s)	Attenuation at t_B: α_S (Np/m)	Elasticity at t_B: μ_B (Pa)	Viscosity at t_B: η_B (Pa.s)	Velocity at the plateau: V_S (m/s)	Attenuation at the plateau: α_S (Np/m)	Elasticity at the plateau: μ_B (Pa)	Viscosity at the plateau: η_B (Pa.s)
0	45	0.74	256	12	1.4	1.05 ± 0.01	59 ± 2	528 ± 15	3.6 ± 0.1
0.5	47	0.71	216	8	1.3	0.97 ± 0.02	67 ± 3	494 ± 24	3.1 ± 0.2
1.0	60	0.80	334	10	1.3	0.93 ± 0.01	101 ± 3	487 ± 19	3.5 ± 0.2
1.5	130	0.61	303	1	1.0	0.75 ± 0.01	131 ± 4	196 ± 10	2.0 ± 0.1

This shear wave elastography technique, applied for the first time to study blood clotting, showed good reproducibility. SWV and SWA behaviors were very similar for the three samples drawn from the same animal (see Fig. 3). Values at the plateaus were close. For instance, the coefficients of variation at the plateaus were constrained to 4.4% for the velocity and 9.4% for the attenuation. For the viscosity and elasticity, the coefficients of variation were slightly higher (5.6% and 11.5%, respectively). This can be explained by the fact that the variance is amplified by the quadratic operation on SWV (V_s) and SWA (α_s) that is required to estimate the elasticity and viscosity in eqns 6 and 7. Besides, the 12.5% variation of t_B (14, 16 and 19 min for the first, second and third experiment) can be explained by blood aging. For instance, three consecutive experiments were performed and blood hemolysis was observed during the duration of all measurements (the three experiments lasted close to 16 h). Nevertheless, this variation was very small compared with those obtained when drugs were added. This allows confirming the reproducibility of the transient elastography technique to assess the sol-gel transition of blood during clotting.

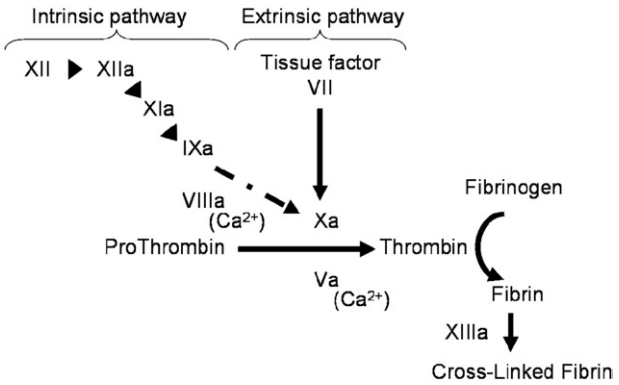

Fig. 7. The coagulation cascade. *In vitro*, only the intrinsic pathway is activated. Roman numerals represent the different factors involved. This cascade leads to the formation of thrombin, which induces the polymerization of fibrinogen in fibrin. Finally, factor XIII produces cross-linked fibrin. Figure adapted from Dee et al. (2002).

The transient elastography technique showed sensitivity to factors known to modify the composition of blood clots. As noted in Fig. 5 and Table 3, the addition of fibrinogen led to a small increase in plateau velocity, *i.e.*, to harder clots with a higher elasticity modulus (Royer and Dieulesaint 1996). This is in accordance with previous studies, where rigidity of clots was shown to be directly proportional to the fibrinogen concentration (Carr et al. 2002). Fibrinogen plays a fundamental role in clot formation, since it is the source of the fibrin network. As reported by Carr and Carr (1995), increasing fibrinogen concentration in plasma leads to thinner fiber formation and a linear increase in the gel elastic modulus. Normal fibrinogen concentration in porcine plasma is 1 to 5 mg/mL (Fries et al. 2005). In the present study, the addition of fibrinogen (up to 400 mg in 75 mL of blood, or 5.3 mg/mL) resulted in a significant increase of up to 100% of the original concentration, which increased substantially the elasticity and viscosity of the clot.

Decreasing the hematocrit led to large increases in shear wave velocity and also to small reductions of attenuation (Fig. 4 and Table 2). Therefore, harder clots with a higher elasticity modulus and a higher viscosity were noted for lower hematocrits. This can be partly explained by the effect of "apparent" increases in the fibrinogen concentration when the hematocrit is lower, since the blood volume was constant (elevation of plasma/RBC volume ratio). However, the variation of the fibrinogen concentration was much more limited than in the experiments reported in Fig. 5 and Table 3 (the initial fibrinogen concentration was not known; however, it varied from a maximum of 1.25 mg/mL in Fig. 4 compared with variations from 3 mg/mL or 6 mg/mL in Fig. 5). Consequently, further explanation is needed to elucidate the strong effect observed. RBCs are usually considered to be passive participants in the process of hemostasis and they are not directly involved in blood clotting (Hanson 2004). Modifying the volume concentration of RBCs and fibrinogen can, by analogy, be related to changes in the ratio of agar-gelatin and its effect on solidification. As noted recently (Gennisson and Cloutier 2006), an increase in gelatin concentration

induced a harder gel consistency. Although RBCs or agar may play a passive role in the gelation process, it is affecting the rigidity and viscosity of the medium by a mechanism that is unknown to us. Further research would be necessary to elucidate this process.

In Fig. 4 and Table 2, variations of the transition time t_B, velocity and attenuation at t_B may also be related to the "apparent" variation of the fibrinogen concentration. At a low hematocrit, there was a larger amount of fibrinogen for the same concentration of calcium ions added [the catalyzer (Ca^{2+}) that must be present in sufficient concentration to affect each step of the coagulation cascade (Fig. 7)]. It is to note that the growths of SWV at the plateau and of t_B are qualitatively in good accordance with the results for different concentrations of added fibrinogen (Fig. 5, Table 3).

In contrast, as noted in Fig. 6 and Table 4, heparin addition was shown to delay blood clot formation, as expressed by the increase of t_B. This was expected, since heparin is usually used in clinical practice to prevent thrombosis (Bussey and Francis 2004). More specifically, it prevents the formation of thrombin, which is responsible for fibrin formation and crosslinking (Carr et al. 2003). Afterwards, heparin inactivation allows blood coagulation to occur. In the current study, heparin addition was also shown to produce a softer clot and elasticity did not seem to reach a plateau after 4 h following the addition of $CaCl_2$. This may be related to the lower amount of thrombin generated in the presence of heparin, in a closed system such as our *in vitro* blood beaker.

CONCLUSION

In this paper, the transient elastography technique has demonstrated its efficiency to follow liquid to solid transition during blood clotting. SWV, SWA, elasticity and viscosity variations have been measured as a function of the hematocrit and following the addition of fibrinogen and heparin. These studies allowed better to understand the clotting process in terms of mechanical properties. However, further work would be needed theoretically to describe and better to comprehend these processes. In addition, future work should also be done on human blood on a large number of samples. The investigation of human samples would allow better to understand the etiology of some pathology, before hopefully applying transient elastography to study coagulation disorders such as deep venous thrombosis *in vivo*.

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