

A Review of Hemorheology: Measuring Techniques and Recent Advances

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Abstract

Significant progress has been made over the years on the topic of hemorheology, not only in terms of the development of more accurate and sophisticated techniques, but also in terms of understanding the phenomena associated with blood components, their interactions and impact upon blood properties. The rheological properties of blood are strongly dependent on the interactions and mechanical properties of red blood cells, and a variation of these properties can bring further insight into the human health state and can be an important parameter in clinical diagnosis.

In this article, we provide both a reference for hemorheological research and a resource regarding the fundamental concepts in hemorheology. This review is aimed at those starting in the field of hemodynamics, where blood rheology plays a significant role, but also at those in search of the most up-to-date findings (both qualitative and quantitative)

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in hemorheological measurements and novel techniques used in this context, including technical advances under more extreme conditions such as in large amplitude oscillatory shear flow or under extensional flow, which impose large deformations comparable to those found in the microcirculatory system and in diseased vessels. Given the impressive rate of increase in the available knowledge on blood flow, this review is also intended to identify areas where current knowledge is still incomplete, and which have the potential for new, exciting and useful research. We also discuss the most important parameters that can lead to an alteration of blood rheology, and which as a consequence can have a significant impact on the normal physiological behavior of blood.

KEYWORDS: hemorheology, blood viscosity, blood viscoelasticity, RBC deformability, RBC aggregation,

1. Introduction

Understanding the rheological properties and flow dynamics of whole human blood is of great importance for the early detection, diagnosis and therapy of circulatory disorders as well as for the development of cardiovascular devices and prosthesis, such as blood pumps, heart valves or stents. With clear evidence that the rheological properties of blood are related with diseases, such as cardiovascular disorders (Dintenfass 1979; Koenig *et al.* 1998), myocardial infarction (Jan *et al.* 1975; Marcinkowska-Gapinska *et al.* 2007), arterial hypertension (Lo Presti *et al.* 2014), diabetes mellitus (Le Devehat *et al.* 2004), cholesterol and triglyceride levels (Moreno *et al.* 2015) and sickle cell anaemia (Drasler *et al.* 1989), routine screening of hemorheological measurements could provide useful information for monitoring the development of such diseases.

Most investigations on rheological material functions concern steady shear measurements of whole blood. Other investigations have focused on transient and oscillatory flows, usually conducted under small amplitude deformation conditions (SAOS) (Vlastos *et al.* 1997; Picart *et al.* 1998; Yilmaz and Gundogdu 2008). The flow of blood in physiological conditions typically involves large deformations, large deformation rates and periodic forcing with large amplitude (Caro *et al.* 1974) and consequently valuable information can be obtained from the characterization of blood under more extreme conditions, such as in large amplitude oscillatory shear flow (LAOS) (Sousa *et al.* 2013) or under conditions of extensional flow (Sousa *et al.* 2011) since these can be observed in diseased vessels (e.g. stenosis) and in vessel bifurcations. Despite the many advances of rheological techniques, which have confirmed the non-Newtonian nature of blood, current viscometer/rheometer systems and measurement

methodologies are still complex and blood viscosity measurements are not yet routinely implemented to provide relevant clinical information. On the other hand, emerging microfluidic technologies have been investigated for various clinical purposes including the analysis of blood samples from patients. The potential of these systems lies on their small characteristic length and time scales, which besides enhanced portability, avoid the need for time consuming blood treatment prior to the measurement, and reduce the required sample volume.

Over the years, there have been a number of excellent reviews on different topics on the broad subject of hemorheology (Replogle *et al.* 1967; Thurston 1996; Thurston and Henderson 2007; Yilmaz and Gundogdu 2008). In the present review, we also focus on recent technical advances for the rheological characterization of blood using commercial instruments under less standard flow conditions (e.g. LAOS, extensional flows) and a diversity of novel micro-scale based techniques. The use of these technologies provided new data for the characterization of blood and new insights into its behavior under a broader range of flow conditions, which we compile and discuss in detail. We start by setting the background for those less experienced in the field with an introductory section, where the composition of blood is summarized, highlighting its constituents and functions, followed by a brief historical perspective of hemorheology, underlining the major developments in this area and finalizing with an up-to-date summary of the most important rheological properties of blood. Section 2 focuses on the experimental techniques used to measure blood and plasma rheological properties, followed by a detailed review on hemorheology on Section 3, including recent data on transient rheological characterization. In Section 3.1 we discuss whole blood viscosity, in Section 3.2 we focus on its thixotropy and yield stress, and in Section 3.3 we address

blood viscoelasticity. Finally, the concluding remarks and new directions for future investigation of blood rheology are outlined in Section 4.

1.1. Blood constituents

Blood is a concentrated suspension of corpuscles or formed elements, including red blood cells (RBC) or erythrocytes, white blood cells (WBC) or leukocytes, and platelets or thrombocytes in an intercellular liquid, the plasma (cf. Fig. 1) and accounts for 6–8% of body weight in healthy humans (Waite 2006). The density of blood under normal conditions is $1056 \pm 9 \text{ kg m}^{-3}$ (Eguchi and Karino 2008).

Figure 1 – near here

Plasma is mainly composed of water at a concentration of approximately 90 – 92 % by volume and several solutes, such as proteins (e.g. fibrinogen, albumin and cholesterol); lipids; glucids; and various electrolytes, essentially Na^+ , K^+ , Ca^{2+} and Cl^- (Thiriet 2008). Plasma accounts for approximately 55% of the blood volume (Thiriet 2008) and has a density of about 1021 kg m^{-3} (Shung 2006). The molecular weight of plasma proteins can vary by approximately two orders of magnitude (Cokelet and Meiselman 2007). Fibrinogen, which is essential in blood coagulation, occurs in different molecular weights, with the largest protein representing approximately 70% of the fibrinogen present in plasma (molecular weight of 340 kDa).

The erythrocytes (density of 1100 kg m^{-3} (Thiriet 2008)) are the most important cells in terms of their influence on blood properties and transport phenomena due to their overwhelming presence and multiple functions. The volume percentage of RBC in

blood is called hematocrit, *Hct*, and in healthy humans, typically varies between 38 – 46% in women and 42 – 53% in men (Thiriet 2008). The RBC are biconcave discs freely suspended in plasma, having typically 6-8 μm in diameter and 2 μm in thickness, as illustrated in Fig. 2. Inside the red blood cells there is a concentrated hemoglobin solution, which behaves as an incompressible viscous fluid. The cell membrane is made of a lipid bilayer and a cytoskeleton formed by protein molecules, and shows a complex rheological response with viscoelastic behavior (Puig-De-Morales-Marinkovic *et al.* 2007; Li *et al.* 2014). While at rest, RBC have a biconcave shape as shown in Fig. 2, aligning as ellipsoids with rotation of the membrane in arterial flow (Fischer *et al.* 1978) and deforming under the action of significant external forces, but returning to their original shape when the forces cease unless lysis of the cells occurs (Thiriet 2008). The ability to adjust their shape reversibly is crucial for flowing through capillaries with diameters smaller than their cellular dimensions at rest (Secomb 1987) and perform their main function of transporting nutrients to different parts of the body. It is well known that certain diseases, such as malaria and diabetes mellitus, are associated with reduced RBC deformation and may lead to perfusion of peripheral tissues (Simchon *et al.* 1987), blockage of the capillaries or cause ischemia (Lee *et al.* 2009).

Figure 2 – near here

1.2. Brief historical background of hemorrheology

Early hemorrheology investigations reported blood as a Newtonian fluid (Langstroth 1919; Pirofsky 1953). Interestingly, Poiseuille's law was derived by the French physician Jean Léonard Marie Poiseuille, when he aimed to determine the pressure drop

in capillary blood flow assuming a Newtonian fluid model (Sutera and Skalak 1993). Non-Newtonian characteristics were observed early in the 20th century, with Hess (1915) reporting that although blood viscosity at high shear rates could be described using Newton's law of viscosity, the apparent shear viscosity increased as the pressure drop decreased. He then linked such phenomenon to the ability of RBC to form aggregates at low shear rates. This was arguably the first observation of the non-Newtonian rheological behavior of blood linked with its structure. The dependence of blood viscosity on flow conditions has later been quantified by means of steady rheological measurements, as reported in the investigations of Pirofsky (1953), Chien *et al.* (1966), Chien (1970) and Thurston (1979). The aggregating properties of RBC were first described by John Hunter in 1786 (Bishop *et al.* 2001).

During the second half of the 20th century the viscoelastic characteristics of blood were recognized in the pioneering work of Thurston (1972), who reported results of unsteady pipe flow with blood. Thurston found that blood stores elastic energy with increasing strain deformation, corroborating the previous work of Schmid-Schonbein *et al.* (1968), who reported the ability of RBC to form aggregates and to deform elastically. Later, the viscoelastic nature of blood was investigated in more detail in several works (Thurston 1972; Thurston 1979; Thurston 1996). Most of the investigations on plasma rheology consider blood plasma to have a Newtonian behavior (Harkness 1971; Koenig *et al.* 1998), but a few argue that it cannot be considered a Newtonian fluid (Sharma and Bhat 1992; Brust *et al.* 2013).

In summary, the wealth of blood cells characteristics and their interactions result in a complex rheological behavior, such as viscoelasticity, shear-thinning and thixotropy (Thurston 1979). In addition, blood has also been reported to have a small yield stress (Morris *et al.* 1987; Picart *et al.* 1998). Besides the rheological properties of blood and

the physiological conditions (hematocrit, red blood cell deformability and aggregation), other parameters that affect blood dynamics are the blood pressure, the vessel shape and vessel wall properties. In addition, another important physiological property that also affects hemorheology is the Fåhræus effect. Fåhræus (1929) investigated the flow of RBC suspensions with different aggregation levels in glass capillaries and observed that increasing the aggregation led to a radial migration of the cells toward the center of the tube, and consequently to a cell-depleted layer near the vessel walls, as illustrated in Fig. 3.

Figure 3 – near here

The near-wall plasma enriched zone is thin, but impacts strongly on the relation between flow rate and pressure drop. The reduction of the red blood cell concentration near the wall leads to a decrease of the apparent blood viscosity and shear stress, and consequently may lead to inaccurate measurements when using small diameter capillaries for blood rheology characterization. In particular, below a critical diameter of 0.3 mm, Fahraeus and Lindqvist (1931) found that the viscosity decreases markedly, as a consequence of a more effective radial migration of the red blood cells away from the vessel walls in relative terms, a behavior known as the Fåhræus-Lindqvist effect. In addition, the wall effect on blood viscosity was found to be negligible when the tube diameter is larger than about 0.4 mm (Fahraeus and Lindqvist 1931). It is interesting to note that a similar phenomenon was previously observed by Bingham and Green (1919), albeit in qualitative terms, when they investigated the rheological behavior of paints.

2. Experimental methods for rheological measurements

Different methods have been employed over the years to measure whole blood and plasma rheological properties, with most works focusing on the rheological characteristics under shear flow. We can divide the experimental methods for rheological measurements in two main groups, depending on their size: one using conventional devices, which we term “macro-scale” devices and the other using “micro-scale” devices. Conventional methods are the most common and most investigated, but the interest in micro-systems for rheological measurements has been growing considerably over the last decade because of the small blood samples required and also because such devices can be cheap, portable and easily disposable, features which are important also for implementation in routine clinical diagnosis. Whatever the technique used, the ideal procedure should: (1) use a small blood sample that can be easily collected from the donor; (2) ensure no blood coagulation during collection and measurement, but avoiding the use of anticoagulant if possible; (3) be able to perform the measurement for a range of relevant temperatures, including normal body temperature, $T \cong 37^{\circ}\text{C}$; (4) enforce simple and straightforward handling of blood sample; (5) provide known and controllable flow kinematics so that the principle of measurement is amenable to analytical mathematical solution (Muramoto and Nagasaka 2011).

2.1. Macro-scale rheological devices

2.1.1. Mechanical methods

The characterization of blood rheology (e.g. shear viscosity, yield stress and viscoelastic properties) has been mostly based on mechanical methods, in which the fluid experiences a shear flow and the resistance to the fluid motion is measured. In particular, rotational rheometers are extensively used, with emphasis on the parallel plates, cone and plate and the coaxial cylinders or Couette geometries. In this section we focus on the experimental techniques for the measurement of blood and plasma shear viscosity as well as yield stress and viscoelastic properties of blood.

- **Blood and plasma shear viscosity.** The foundations of blood rheology result from a multiplicity of experiments performed using a range of “classical” instruments: falling ball viscometer, capillary viscometer, tube viscometers and rotational rheometers (Bishop *et al.* 2001; Rosencranz and Bogen 2006). Techniques such as the falling ball viscometer, or the capillary tube, were widely employed in early investigations (Rosencranz and Bogen 2006). For example, early measurements of plasma viscosity were performed using a capillary viscometer, also known as Harkness viscometer (Harkness 1971), which was recommended as a standard measuring method for plasma viscosity (Haematology 1984) due to the simple and low-cost set-up, as well as the small amount of sample required, and the short duration of the test. However, this method is particularly suited for measurement of the viscosity of Newtonian fluids. Currently, there are several commercially available instruments based on different principles and techniques, which yield identical results if properly used (Baskurt *et al.* 2009). Commercially available rotational rheometers (Fig. 4a) are some of the most

versatile and commonly used devices for rheological characterization under shear. This type of rheometers has seen important improvements over the years, but also presents some disadvantages for measuring blood rheological properties, especially if to be used in a medical context. These systems are, in general, bulky, complex to use, and time-consuming. Issues related to the sedimentation of the cells and to the formation of a cell-depleted layer near the walls need to be carefully monitored to avoid erroneous measurements, particularly depending on the gap used for the measurement of blood rheology, which can be minimized using roughened surfaces (Merrill 1969; Sousa *et al.* 2013).

Most of the “*classic*” measurement systems require a relatively high volume of blood; usually more than 1 ml is necessary depending on the apparatus used. In order to overcome this limitation, and reduce the measurement time, oscillating torsional viscometers have been proposed, making blood rheological tests faster, more direct and feasible (Mark *et al.* 2006; Eugster *et al.* 2007; Travagli *et al.* 2008). As an example, Eugster *et al.* (2007) developed an oscillating viscometer based on a glass capillary which only requires 7 μl of blood for measuring blood viscosity.

Figure 4 – near here

Automatic tube-type viscometers have also been developed (Kim *et al.* 2000; Alexy *et al.* 2005) including a system able to measure the whole blood viscosity within 2 minutes at shear rates ranging from 1 to 375 s^{-1} (Kim *et al.* 2000). A falling-ball viscometer (Fig. 4c), used to measure the shear viscosity of whole blood and RBC suspensions, demonstrated high sensitivity to changes in blood viscosity, and can be useful clinically

for detecting abnormal blood viscosity values (Eguchi and Karino 2008) and thus can potentially be converted into screening platforms (Mark *et al.* 2006).

- **Yield stress.** Different factors can affect the measurement of blood yield stress, namely the torque accuracy and sensitivity of the instrument used at low shear rates, the cell sedimentation and the plasma layer formation, which is particularly relevant at low shear rates due to the formation of cell aggregates. Most of the yield stress values reported in the literature were determined using an indirect technique by extrapolating rheological data obtained using a Couette-type rheometer. In order to avoid the wall slip phenomenon, Picart *et al.* (1998) used a Couette rheometer in which the measuring system had roughened surfaces. Blood yield stress was also measured using a settling technique (Charm and Kurland 1967) and by recording stress relaxation curves (Benis and Lacoste 1968), both of which are direct measurement techniques. The values of the blood yield stress reported in the literature present a significant variability, which can be attributed to donor-to-donor variability, but also to deficiencies of the different measurements methods used (Picart *et al.* 1998).

- **Viscoelastic properties of blood.** Properties such as the storage (G') and loss (G'') moduli (or alternatively the viscous (η') and elastic (η'') components of the complex viscosity), are usually measured in small amplitude oscillatory shear flows (SAOS). In this type of experiment, a part of the measurement geometry oscillates to a preset maximum strain (an angle, typically small, in a rotational rheometer) and the time-dependent response is measured. The imposed strain is small in order to keep the sample microstructure unaffected, which allows probing the viscoelastic behavior in the linear regime. In addition to the geometries commonly used for measuring other

rheological properties of blood, porous media have also been used to measure G' and G'' . Porous media can exhibit tortuosity comparable to that of the vessels and were used to determine Maxwell relaxation times and to assess the importance of the blood elasticity relative to viscosity (Thurston and Henderson 2006). However, in this case, the cell aggregation phenomenon occurs to a small extent due to the restricted space in the pores, thus affecting the measurement of the complex viscosity. It is known that for a tube dimension smaller than about ten red-blood-cell diameters the formation of a cell depletion layer affects significantly the measurements (Thurston and Henderson 2006), which may well hinder rheological measurements with this type of device. It is important to note that the rheological measurements can be affected not only by the confinement size of the geometry used but also by the possible sedimentation of the RBC (Thurston and Henderson 2007), and proper care should be taken to minimize these effects. The use of roughened surfaces is common to reduce wall slip and the formation of a cell free layer at the wall and procedures that involve a homogenization step have been proposed to minimize RBC sedimentation (Marton *et al.* 2001; Lee *et al.* 2007; Sousa *et al.* 2013).

LAOS tests can be used in the measurement of the nonlinear viscoelastic response of blood, which can be associated with its internal structure. In LAOS (and SAOS), an oscillatory shear stress or strain is imposed to the sample and the other one is monitored, i.e. if the strain is imposed the shear stress is measured, and the viscous and elastic components of the viscoelastic sample are quantified. The imposed variable follows a sinusoidal evolution in time and for LAOS the corresponding response is no longer sinusoidal, containing higher harmonic contributions. The odd harmonics are related with the rheological response of the sample, while the even harmonics are due to experimental deficiencies (Ewoldt *et al.* 2008; Hyun *et al.* 2011). A technique

commonly used to analyze the material response is the Fourier transform, which is used to determine the storage and loss moduli of the viscoelastic sample (Hyun *et al.* 2011). The elastic properties of blood plasma have also been measured under uniaxial extensional flow (Brust *et al.* 2013). A capillary breakup extensional rheometer (CaBER), similar to the commercial apparatus illustrated in Fig. 4b, was employed to measure a characteristic relaxation time of blood in extensional flow. In this technique a fluid sample is placed between two circular plates, which are separated by a small distance and then the plates are separated and the liquid thread thins spontaneously until the occurrence of breakup, under the combined action of capillary and extensional resisting forces, which for viscoelastic fluids is essentially a viscoelastic force. The diameter of the filament ($D(t)$) is monitored as function of time and for a viscoelastic fluid, the filament diameter decreases exponentially and the relaxation time of the fluid is determined by fitting the experimental data in the linear region of $\log[D(t)]$ as a function of time. The drawback is that for fluids having a low viscosity and small elasticity, the filament thinning is very rapid, inertia may not be negligible and it is difficult to monitor filament time evolution with good resolution under these conditions. In addition, in the final stage of the thinning process, the filament diameter is of the order of a few microns, which is comparable to the RBC dimensions, and can lead to inaccurate measurements for whole blood extensional rheology measurements.

2.1.2. Optical methods

An optical technique for measuring whole-blood viscosity based on laser-induced capillary waves was introduced by Muramoto and Nagasaka (2011). Pulsed YAG laser beams are used to heat the whole-blood sample and to generate a capillary wave, which is then detected by a diffracted probing laser beam at the heated region. The intensity of

the diffracted probing beam is converted into viscosity and surface tension measurements of the blood sample. Despite the short time and the small sample volume required, a wide range of viscosities can be measured using this method.

Another method that was investigated involves fluorescent viscosity-sensitive dyes, referred to as molecular rotors (Haidekker *et al.* 2002). Due to their high sensitivity, spatial and temporal resolution, these molecular probes are a good alternative to mechanical methods for measuring blood plasma viscosities. Upon photoexcitation, the fluorescent molecules form twisted intramolecular charge transfer states. Photoexcitation leads to an electron transfer and the quantum yield of a molecule rotor increases with decreasing free volume of the solvent, which is related with viscosity. The emission intensity of molecular rotors depends on the viscosity of the solvent. In the measurements of whole blood viscosity, the use of fluorescent molecular rotors shows some difficulties. In particular, blood protein binding may affect the measurements (Akers *et al.* 2005), which are also affected by the optical properties of the fluid, dye concentration and dye-solvent interactions. In addition, the use of fluorescent sensors also requires a calibration of the measurement system.

2.2. Micro-scale rheological devices

2.2.1. Microfluidics for rheological measurements

Microfluidics is an interesting alternative to commercial viscometers and rheometers and is gaining increased popularity. This technological field allows the miniaturization of macro-systems into micro-devices, which can integrate different processes generating point-of-care diagnostic devices that can be used in medicine and in life science

research besides applications in many other areas. The microchips typically used are cheap to fabricate, easy to handle and use, require very small volume samples (typically in the range between nano- and micro-liters) and consequently produce small amounts of waste and can be combined with optical systems for visualization (Fig. 5a).

Figure 5 – near here

Microfluidic devices able to operate as blood viscometers have been developed in order to overcome the drawbacks of the macro-scale devices and are a promising tool for use in clinical practice because of their fast response and minimum fluid requirement. Such micro-devices have been used to measure the shear viscosity (Srivastava *et al.* 2005; Zeng and Zhao 2009; Smith *et al.* 2010) and viscoelasticity (Brust *et al.* 2013; Kang and Lee 2013) of blood. Microfluidic viscometers are based on a range of different principles as reviewed by Ong *et al.* (2010), such as capillary pressure-driven flow (Srivastava *et al.* 2005), electrical impedance spectroscopy (Zeng and Zhao 2009), laser-induced capillary wave (Taguchi *et al.* 2009) and oscillating micro-structure induced by an electrostatic driving force (Smith *et al.* 2010). For instance, in the micro viscometer proposed by Srivastava *et al.* (2005), which is illustrated in Fig.5c, the pressure drop over the channel length is used to determine the plasma viscosity. The device requires a sample volume of 600 nL and is able to perform the measurement in less than 100 s. However, microfluidic devices often require a syringe pump or, in some cases, a pressurized reservoir and connecting tubes to be combined with the microchip in order to promote the blood flow, which typically implies larger sample volumes. An exception is the micro-optical capillary sensor based on laser-induced capillary wave developed by Taguchi *et al.* (2009), in which the sample is placed directly on the

sensor. Measurements using distilled water with a pigment have been performed for a proof-of-concept, but the device is yet to be tested with real blood. Two potential shortcomings of the method are the possible occurrence of sample evaporation and the micro-sensor being affected by surrounding vibrations. Due to the reduced measurement times required in these devices, it is expected that the addition of an anticoagulant to the blood sample is less critical, although it continues to be used in some works (Zeng and Zhao 2009). Interestingly, the shear rates reported in these investigations is in the approximate range between 1 and 10^3 s^{-1} , which is similar to the reliable range of shear rates used in macro-scale apparatus.

It is well known that an adequate temperature control in the measurement of rheological properties of blood is very important. Recently, a microfluidic viscometer that uses an array of microchannels equipped with a temperature controller was developed by Kang and Yang (2013). The temperature of the fluid within the channel was controlled using a Peltier system, micro-sized thermocouples and a feedback controller, which increases substantially the complexity of the microfabrication process. In addition, the micro-device requires the use of a reference fluid and the blood viscosity is measured by counting the number of indicating channels in the array which are filled with blood and with the reference fluid.

In terms of viscoelasticity, Kang and Lee (2013) proposed to measure a characteristic relaxation time of blood by controlling the transient blood flow in a microfluidic device analogue of a Wheatstone bridge (Fig. 5b). The microfluidic channel consists of one channel connecting two side channels and the characteristic time of blood, an important parameter for evaluating blood viscoelasticity, is determined examining the area of the connecting channel that is filled with blood for a certain pressure drop in both side channels. The determination of the area of the channel filled with blood is based on

image processing. This type of micro-device avoids the use of integrated sensors in the microchip and the need for calibration, although a reference fluid is required.

An interesting approach in microfluidics is taking advantage of the shape of the micro-channel to impose controllable deformations. Recently, the blood plasma viscoelasticity was assessed in a microchannel containing a contraction-expansion geometry (Fig. 5d) in order to promote a strong extensional flow (Brust *et al.* 2013). Measurements of the pressure drop in the channel revealed a nonlinear flow resistance with the increase of plasma flow rate, which is a typical behavior of a viscoelastic fluid.

In order to study the effects of extensional flow on RBC deformation, microchannels with a hyperbolic converging region (Fig5e) have been used due to the acceleration imposed by the converging section (Lee *et al.* 2009; Yaginuma *et al.* 2013). Such type of microfluidic devices that generate a strong extensional flow field are promising tools that can be applied in the detection and diagnosis of several diseases related to RBC deformability.

There are clear advantages of using microfluidics for measuring blood rheological properties as discussed above, but there are also some drawbacks that need to be considered, namely red blood cell clogging, measurement stability associated with weak signals and reproducibility. In addition, the formation of the cell-free layer is an added challenge in the rheological measurements at the micro-scale. Incidentally, Faivre *et al.* (2006) found that in a microchannel with an abrupt contraction followed by an abrupt expansion, the cellular content in the middle of the downstream channel increases 24% for a flow rate of 200 $\mu\text{L}/\text{h}$ and an initial hematocrit of 16 (v/v)%.

2.2.2. Microrheology and other micro-scale techniques

Microrheology techniques allow the measurements of the rheological properties of a complex fluid from the motion of colloidal probes directly embedded in the sample since the time-dependent trajectories of tracer particles dispersed in the fluid can be related to the linear viscoelastic properties of that fluid (Yao *et al.* 2009). These techniques, which can be divided between active and passive microrheology (cf. Table 1), are useful to perform *in situ* measurements and have also been used to assess the elastic character of blood. Contrarily to microfluidics, microrheology techniques typically do not require the use of accessories such as tubes and syringes for promoting the fluid flow and only requires the volume sample to fill the sample chamber, using volume samples of the order of 10 μL (Yao *et al.* 2009).

Active microrheology techniques involve the active manipulation of small probe particles using external applied forcings, whereas in passive microrheology techniques the tracer particles' motion is induced by thermal fluctuations (Squires and Mason 2010). Active microrheology techniques typically use optical tweezers, magnetic manipulation techniques or atomic force microscopy (Neuman and Nagy 2008).

Optical tweezers have been extensively used to investigate the mechanical (Dao *et al.* 2003; Lim *et al.* 2004; Li *et al.* 2009) and electrical properties of RBC (Fontes *et al.* 2011). In this non-invasive method, a highly focused beam of light focused through a microscope objective attracts and traps a high refractive index particle as sketched in Fig. 6a. Using optical tweezers it is possible to measure particle displacements of the order of nanometers with applied forces in the pN range (Yao *et al.* 2009). The technique requires a microscope and a high numerical aperture objective able to focus efficiently the laser beam. When a cell is attached to such a trapped particle, moving the focused laser beam forces the particle to move through the surrounding material,

Table 1. Microrheology and other micro-scale techniques used in the investigation of blood viscoelasticity.

	Technique	Principle	Measurement	Advantages	Disadvantages	References
Active microrheology	Optical tweezers			Non-invasive; Precise measure of pN forces;	Complex equipment required; Non-uniform deformation	Dao <i>et al.</i> (2003); Lim <i>et al.</i> (2004); Li <i>et al.</i> (2009); Fontes <i>et al.</i> (2011)
	Magnetic manipulation	Applied external forcing	Deformability/viscoelastic character of RBC	Non-invasive; Selectivity of the magnetic beads	Difficult measurement; Sensitivity	Puig-De-Morales-Marinkovic <i>et al.</i> (2007); Li <i>et al.</i> (2014)
	Atomic force microscopy			Simple and rapid sample preparation; pN resolution; Measurements under near-physiological conditions	Large size and high stiffness of the cantilevers; Possible interaction between AFM tip and molecule under study	Bremmell <i>et al.</i> (2006)
Passive microrheology		Thermal fluctuations	Viscoelastic moduli of whole blood	Linear viscoelastic regime since there is no external stress applied	Requirements in the probes selection; Temperature control	Campo-Deaño <i>et al.</i> (2013)
Other techniques	Micropipette aspiration	Applied negative pressure	Mechanical properties of RBC	Simple technique; For individual cells; Precise measure of pN forces	Time-consuming	Artmann <i>et al.</i> (1997)
	Filtration	Applied negative pressure	Deformability of RBC	Relatively simple instruments; High reproducibility	Selectivity: feasible only for reasonably deformable cells; Blockage of the pores	Koutsouris <i>et al.</i> (1988)
	Rheoscopy			Visualization; For individual cells	Complex design of the equipment	Dobbe <i>et al.</i> (2004)
	Ektacytometry	Shear flow	Deformability of RBC	Volume samples lower than 50 μ L; Measurement in less than 1 min	Complex equipment	Shin <i>et al.</i> (2007); Baskurt <i>et al.</i> (2009)

corresponding to the application of a local stress and the resultant displacement can be converted in terms of viscoelastic response using image analysis. The accuracy of the results depends on the precision of the image analysis technique which is typically of the order of 1 pixel but sub-pixel resolution can be achieved to measure the mechanical properties of RBC (Li *et al.* 2009).

Figure 6 – near here

Similarly to the optical tweezers, in magnetic twisting cytometry techniques spherical beads are attached to the cell cytoskeleton in order to generate stresses. A torque is applied to each bead, which causes the twist of the magnetic microparticle bounded to the membrane receptor and the resultant bead rotation is measured. Magnetic twisting cytometry generates larger range of stresses than in optical tweezers, in which a unidirectional force is applied to a single attached bead. The nature and magnitude of the applied stress in magnetic twisting torque cytometry and optical tweezers may lead to differences in the results obtained using both techniques (Laurent *et al.* 2002). The storage modulus of RBC was investigated using optical magnetic twisting cytometry and its numerical analog, the twisting torque cytometry (Puig-De-Morales-Marinkovic *et al.* 2007; Li *et al.* 2014).

Valuable information concerning physical properties, as well as alterations in RBC, can be obtained using atomic force microscopy (AFM). This technique provides nanoscale topographic resolution and has been used to investigate the elastic properties of RBC (Bremmell *et al.* 2006). Micro-spheres are attached to a cantilever (Fig. 6b), which is used to scan the sample surface. In a force profile mode, the probe approaches the cell surface, contacts and then retracts. The force profile becomes nonlinear and reflects the

mechanical properties of the cells (Neuman and Nagy 2008). AFM allows measurements of inter- and intra-molecular interaction forces with pN resolution. Recently, Steffen *et al.* (2013) measured the dextran-induced aggregation of RBC using atomic force microscopy-based single cell force spectroscopy. Different dextran molecules were used and the corresponding interaction energy between red blood cells was measured. The results obtained for the interaction energy can be described by the depletion theory, although a detailed investigation of the cause for stronger adhesion energies is still lacking.

Passive microrheology uses the Brownian motion of tracer particles to measure the rheology and structure of a material. The mean squared displacement (MSD) of the particles is measured and related to the viscosity of the material by the Stokes-Einstein equation, which is valid for Newtonian fluids. For a viscoelastic material, the generalized Stokes-Einstein relationship is used with the Newtonian viscosity replaced by the frequency-dependent complex viscosity of the material (Squires and Mason 2010). Hence, the time-evolution of the MSD of tracers can be used to obtain the linear viscoelastic moduli of the sample. Campo-Deaño *et al.* (2013) employed passive microrheology techniques in order to measure the viscoelastic properties of whole blood by measuring the storage and loss moduli. These techniques require the use of a microscope and corresponding optical accessories. In addition, care must be taken when choosing the colloidal probes in order to ensure its sphericity, polydispersity and the absence of interactions between the particles and the medium (Breedveld and Pine 2003). In this type of measurements the temperature may be difficult to control or it may be difficult to reach the physiological temperature, which is desirable for whole blood measurements. Campo-Deaño *et al.* (2013) performed the measurements at 27 °C and used the time-temperature superposition method, which is a common practice in

polymeric samples when the range of temperatures is modest, to infer the viscoelastic moduli of human blood at 37 °C.

Micropipette aspiration techniques have been extensively used to measure the mechanical properties of RBC membranes. Here, a glass micropipette is used to apply a negative pressure onto the RBC membrane leading to the aspiration of the cell membrane into the pipette and progressive membrane deformation is measured as a function of time using optical microscopy. The internal diameter of the micropipette and the applied pressure can be selected to control the extent of the deformation. The elastic modulus, viscosity and characteristic time for relaxation of the cell membrane can be determined by matching the experimentally observed changes of the cell, namely the aspiration length, for a given pressure and configuration with analytical models (Dao *et al.* 2003). Micropipette aspiration is an advantageous technique for mechanical characterization of cells since it is simple, relatively inexpensive and can provide forces as low as pN. Investigations of the human RBC membrane using this technique found an implied in-plane shear modulus of 4-10mN/m, a relaxation time of 0.10-0.13 s and a membrane viscosity of 0.6-2.7 $\mu\text{N}\cdot\text{s}\cdot\text{m}^{-1}$ (Lim *et al.* 2004). However, in micropipette aspiration, as well as in AFM and optical trap techniques, the deformation is nonuniform.

Other techniques have been used for measuring RBC deformability, such as filtration through pores of 3–5 μm in diameter with the transit time being determined during the measurement; rheoscopy; and ektacytometry (Shin *et al.* 2007). In rheoscopy, the cells are subject to simple shear flow in a rotating cone-plate system and a microscope is required for focusing the middle plane between the cone and the plate. In ektacytometry a laser beam is projected through a RBC suspension and the resulting diffraction pattern

analyzed in order to determine the RBC shape and consequently deformability. A comparison between instruments commercially available for ektacytometry was performed by Baskurt *et al.* (2009), using the LORCA (Laser assisted optical rotational cell analyzer, Mechatronics Instruments BV, Zwaag, The Netherlands), the Rheodyn SSD (Myrenne GmbH, Roetgen, Germany) and the RheoScan-D (RheoMeditech, Seoul, Korea) systems. The LORCA (Fig. 6c2) and the Rheodyn SSD systems cannot be strictly classified as micro-scale instruments given their characteristic dimensions; the RheoScan-D (Fig. 6c1) on the other hand combines microfluidics with laser-diffraction techniques. Shin *et al.* (2007) compared the deformability of RBC at various shear stresses measured using the RheoScan-D and the LORCA systems and the results obtained with both instrument were found to be in excellent agreement.

3. Hemorheology

Hemorheology deals with the flow and deformation behavior of blood and its formed elements (Popel and Johnson 2005). The complex RBC aggregation and deformation hysteretic processes have been extensively investigated and found to be relevant in the context of hemorheology as discussed in the following sections. A list summarizing relevant investigations on blood shear rheology performed in the last two decades is presented in Table 2.

3.1. Steady shear viscosity of blood

The combined effects of aggregation and deformation of RBC impart a shear-thinning rheological behavior to blood as illustrated in Fig. 7 and are known to be closely linked to the presence of plasma proteins. In healthy human whole blood, RBC form

Table 2. Review of blood shear-rheology studies using different measuring systems.

Reference	<i>T</i> (°C)	Measuring system/method	Type of measurement		<i>Hct</i> (%)	Blood sample	Blood preparation/ Anticoagulant	Remarks
			Steady	Oscillatory				
Vlastos <i>et al.</i> (1997)	25	Couette viscometer	$0.01 \leq \dot{\gamma} / \text{s}^{-1} \leq 100$	$0.05 \leq \gamma_0 \leq 13.11$ $f=0.5\text{Hz}$ $0.03 \leq f/\text{Hz} \leq 5$ $\gamma_0=0.2$	45	- Autologous plasma + RBC	- Heparin	- Steady and oscillatory shear tests combined in the same measurement - Blood viscoelasticity decreases when a steady shear rate is superimposed
Kim <i>et al.</i> (2000)	37	Scanning viscometer with capillary tube	$30 \leq \dot{\gamma} / \text{s}^{-1} \leq 375$	-	40.5 41 46.5	- Whole blood	- No anticoagulant	- Measurement performed in 2 min - Blood volume sample of 5 ml
Shin <i>et al.</i> (2004)	25	Pressure- scanning viscometer	$1 \leq \dot{\gamma} / \text{s}^{-1} \leq 1000$	-	n. m.	- Whole human blood	n. m.	- Measurement of the pressure variation with time in a collecting chamber - Comparison between new method and a rotating viscometer

Table 2. (continued)

Reference	<i>T</i> (°C)	Measuring system/method	Type of measurement		<i>Hct</i> (%)	Blood sample	Blood preparation/ Anticoagulant	Remarks
			Steady	Oscillatory				
Alexy <i>et al.</i> (2005)	37	Computerized scanning capillary viscometer	$1 \leq \dot{\gamma} / s^{-1} \leq 1500$	-	28-56	- Whole blood - RBC in plasma	- EDTA	- Comparison between: new method and a cone- plate; new method and Couette
Mark <i>et al.</i> (2006)	37	Oscillating resonator probe	-	✓	20-60	- RBC in Dextran 40 - RBC in Dextran 70	- EDTA	- Measurement system into the vacutainer tube for blood withdrawal - Comparison between new method and Couette system
Eugster <i>et al.</i> (2007)	37	Oscillating torsional resonator viscometer	-	✓	20-60	- Whole blood	- EDTA	- Couette viscometer for comparison purposes
Eguchi and Karino (2008)	37	Falling ball viscometer	✓	-	20-60	- Whole blood - RBC in serum - RBC in Tyrodes- albumin solution	- Sodium citrate	- Cone-plate viscometer for comparison purposes

Table 2. (continued)

Reference	T (°C)	Measuring system/method	Type of measurement		Hct (%)	Blood sample	Blood preparation/ Anticoagulant	Remarks
			Steady	Oscillatory				
Travagli <i>et al.</i> (2008)	37	Oscillating torsional viscometer	-	✓	0-100	- Whole blood - RBC in plasma	- EDTA	- Rheometer with cone- plate geometry for comparison purposes
Muramoto and Nagasaka (2011)	37	Laser-induced capillary wave viscometer	-	✓ (capillary wave)	39.2 44.6	- Whole blood	- Heparin	- Volume sample of 90 μ l - Blood considered as Newtonian fluid
Campo-Deaño <i>et al.</i> (2013)	27	Passive microrheology	-	✓ $3 < \omega / \text{rad s}^{-1} \leq 600$	44	- Whole blood	- EDTA	- Viscoelasticity quantified in terms of G' and G''
Sousa <i>et al.</i> (2013)	37	Rotational rheometer Plate-plate (rough surface)	$1 \leq \dot{\gamma} / \text{s}^{-1} \leq 350$	✓ (LAOS)	41.3 41.6	- Whole blood	- EDTA	- Anticoagulant has no influence on rheological results - Viscoelastic behavior found with the viscous character more marked than the elastic one

Legend: n.m. – not mentioned; $\dot{\gamma}$ (s^{-1}) – shear rate; f (Hz) – frequency; γ_0 (-) – strain amplitude; G' (Pa) – storage modulus; G'' (Pa) – loss modulus; ω (rad s^{-1}) – angular frequency; LAOS – large amplitude oscillatory shear.

aggregates at low shear rates and as a consequence blood resistance to flow is increased with whole blood shear viscosity being about one hundred times higher than that of water. Increasing the shear rate disrupts the aggregate structures above a critical shear rate, leading to shear-thinning behavior, as shown in Fig. 7. An initial lower degree of aggregation of RBC involves weaker attractive forces between the cells, causing a lower critical shear rate at which disaggregation of the RBC occurs (Thurston 1979). As RBC are dispersed, deformed and aligned with flow direction due to higher flow velocities (shear rates), the shear viscosity decreases substantially by approximately two orders of magnitude, as shown in Fig. 7 and its value is about three times the shear viscosity of water.

The aggregation of normal RBC taking place at low shear rates leads to a higher apparent viscosity than when RBC are suspended in a non-aggregating medium such as 11% (v/v) albumin-Ringer solution in plasma (Chien 1970). The differences between the flow curves for normal blood and for a suspension of RBC in the albumin-Ringer solution shown in Fig. 7 demonstrate the impact of cell aggregation on the shear-thinning properties at low shear rates. Fig. 7 also shows that when erythrocytes harden, as occurs in some diseases, the shear-thinning behavior is weakened or even lost due to the lower capability of the hardened RBC to deform under shear and to form aggregates.

Figure 7 – near here

During a cardiac cycle the flow is pulsatile, with pressure and flow velocity varying in time and space in the vascular network. Consequently, as a shear rate dependent property, whole blood viscosity also changes in time and location. At low velocities, the apparent viscosity of blood increases in bulk shear flow due to the reduced deformation

of RBC with decreasing levels of shear stress/rate and the tendency of RBC to aggregate (Pozrikidis 2003).

Figure 8 compares blood viscosity measurements by different authors under steady shear. Figure 8(a) includes data at different hematocrit levels, ranging from 41.2% to 51.7%, measured at the physiological temperature (37°C) using different methodologies, whereas Fig. 8(b) shows an overview of different flow curves measured at a range of lower temperatures ($T = 4^{\circ}\text{C}$ and 37°C) but over a narrower range of hematocrit values, from 41.3% to 46%. Due to the diversity of conditions used in different investigations, namely the hematocrit levels, temperature of measurement, range of shear rates, or the blood sample preparation method, it is difficult to make a direct comparison between them. Nevertheless, the shear-thinning behavior of blood viscosity is clear and in spite of the differences in the values of the *Hct*, the viscosity data obtained by different authors agrees well, particularly at high shear rates, as shown in Fig. 8(a). It is important to note that the data presented in Fig. 8 was obtained with human blood samples. Care must be taken when comparing data obtained using animal blood due to the large variation in size, heart rate, life style between animals as reviewed by Rampling (2007). For instance, Johnn *et al.* (1992) found that the blood viscosity of 31 mammals investigated may vary by some 2-fold at high shear rates and 18-fold at low shear rates due to the variation in *Hct* and plasma viscosity.

Figure 8 – near here

Blood viscosity is affected by the variability of the characteristics inherent to the blood donor as well as by the following important parameters:

- **Hematocrit** - It is well documented that blood steady shear viscosity increases exponentially with the hematocrit, as illustrated in Fig. 9, and that the variation of the viscosity is more pronounced at lower than at higher shear rates attesting the effect of the hematocrit on the values of the viscosity and on the shear-thinning behavior (Cokelet and Meiselman 2007).

Figure 9 – near here

- **Temperature** - The normal body temperature, also known as eutheria or normothermia, is approximately 37 °C, but lower temperatures are also important in various medical procedures such as surgery and it is thus of significant importance to understand the influence of temperature on the rheological properties of blood.

Many rheological measurements using human blood were conducted at low temperatures and therefore significant differences in steady shear rheology are observed (cf. Fig. 8(a) and 8(b)). The dependence of the viscosity on temperature in a narrow range can be described mathematically using an Arrhenius type equation, known as Andrade's equation (Barnes 2000). Thurston (1996) measured the plasma viscosity as well as the viscous and elastic components of the complex viscosity under oscillatory flow conditions by applying a constant frequency of 2 Hz and a strain of 1 for temperatures ranging from 9°C to 42°C. The equations found by Thurston, valid only for the conditions described above, are summarized in Table 3.

The effect of temperature on RBC aggregation and deformability and consequently on rheological properties of blood is also important. In fact, temperature plays a significant role in terms of cell function and dynamics. As discussed previously, blood viscosity increases with a decrease of temperature, while the aggregation index of RBC decreases

when temperature is decreased (Lim *et al.* 2010). This can be explained by the fact that plasma viscosity increases when the temperature decreases and the rate of formation of RBC aggregates is delayed, with plasma being considered as an attenuating medium for RBC suspensions.

Table 3. Summary of the equations describing the variation of the plasma viscosity and of the viscous and elastic components of the complex viscosity with temperature obtained by Thurston (1996) in the range $9 < T [^{\circ}\text{C}] < 42$.

Plasma	Blood (<i>Hct</i> = 47 %)
$\eta_{\text{plasma}} [\text{mPa s}] = 3.362 \times 10^{-0.01017T [^{\circ}\text{C}]}$	$\eta' [\text{mPa s}] = 21.47 \times 10^{-0.01264T [^{\circ}\text{C}]}$
	$\eta'' [\text{mPa s}] = 4.213 \times 10^{-0.01022T [^{\circ}\text{C}]}$

In addition, temperature also plays a ubiquitous role in the deformability of intact (Artmann *et al.* 1998) or pathologic (Mills *et al.* 2007) red blood cells. Artmann *et al.* (1998) found that intact human RBC experience the passage through a 1.3 μm micropipette only in the narrow range of temperatures of $36.4 \pm 0.3^{\circ}\text{C}$ due to the elastomeric fluid/gel-like transition of hemoglobin. Furthermore, the deformability of pathologic RBC harboring *Plasmodium falciparum* is significantly reduced at febrile temperatures (41°C) compared with normal physiological temperature (Mills *et al.* 2007).

- **Anticoagulant** - The addition of anticoagulants for carrying out blood measurements, which delays the blood coagulation and consequently allows for longer measurement times, has been argued to change blood rheological properties when inadequate concentrations are used. Sousa *et al.* (2013) showed recently that the use of

ethylenediaminetetraacetic acid (EDTA) at the maximum concentration (1.8 mg/ml) recommended by the International Society for Clinical Hemorheology (Baskurt *et al.* 2009) has no significant influence upon the whole blood shear viscosity but care must be taken regarding the type and amount of anticoagulant added to the blood sample.

The shear-thinning behavior is frequently modelled using a Generalized Newtonian fluid (GNF) model with the blood shear-thinning viscosity described by simple equations, such as: Ostwald-de Waele power law model, Carreau model, Carreau-Yasuda model, Casson model, and Herschel-Bulkley model, see Table 4. These models can describe accurately the shear-thinning behavior but are not able to account for elasticity.

Table 4. Non-Newtonian fluid models commonly used to describe blood shear viscosity. Note that the shear stress and shear rate are related as $\tau = \eta\dot{\gamma}$.

Model	Equation
Power Law	$\eta = k\dot{\gamma}^{n-1}$
Carreau - Yasuda	$\eta = \eta_{\infty} + \frac{\eta_0 - \eta_{\infty}}{[1 + (\Lambda\dot{\gamma})^a]^{(1-n)/a}}$ (The Carreau model is obtained using $a = 2$,
Cross	$\frac{\eta - \eta_{\infty}}{\eta_0 - \eta_{\infty}} = \frac{1}{1 + (\Lambda\dot{\gamma})^m}$
Casson	$\sqrt{\tau} = \sqrt{\tau_0} + \sqrt{k\dot{\gamma}}$
Herschel-Bulkley	$\tau = \tau_0 + k\dot{\gamma}^n$

Legend: η – shear viscosity; η_0 – zero-shear rate viscosity; η_{∞} – viscosity at high shear rates; k – consistency index; n – power law index; Λ – time constant; m, a – dimensionless parameters; $\dot{\gamma}$ – shear rate; τ – shear stress; τ_0 – yield stress.

There are other more complex models that consider the viscoelastic characteristics of blood. For example, Campo-Deaño *et al.* (2013) reported the fit to the shear viscosity as well as the viscoelastic moduli of whole blood using two commonly used viscoelastic multi-mode models, namely the simplified Phan-Thien-Tanner (sPTT) and the Giesekus models, while Owens (2006) proposed a new and more complex model that describes the viscoelastic behavior of blood. However, these more complex models that account for viscoelasticity are not used as often precisely due to the increased complexity. For a more complete description of blood rheology models the reader is referred to the reviews by Yilmaz and Gundogdu (2008) and Campo-Deaño *et al.* (2015), who report on more complex constitutive equations as well as on the parameters of the models that better fit experimental data.

3.2. Blood thixotropy and yield stress

Thixotropy is the reduction of viscosity with time in a fluid sample subjected to a constant shear rate (Barnes 2000), an effect which occurs due to shear-induced changes in the fluid microstructure. Another manifestation of thixotropy is the hysteresis found in fluid properties, such as the viscosity and shear stress, in a cycle of increase and subsequent decrease in the imposed shear rate, as shown in Fig. 10. In the context of blood rheology, thixotropy has been reported to occur (Dintenfass 1985) and has been linked to the aggregation and disruption dynamics of the RBC structures, in particular with the behavior and evolution of the rouleaux depending on the intensity and duration of the applied stresses. An increase and subsequent decrease of shear rate results in lower apparent viscosities than initially at the same shear rates, since RBC structures are only formed at low shear rates and require some time to form, leading to hysteretic behavior in the apparent shear viscosity. It is also important to notice that cell

sedimentation is faster with aggregates than with isolated RBC, and undue consideration of their effect may lead to a misrepresentation of the thixotropic rheology of blood.

Time-dependent changes of rheological properties of blood are of great importance to describe the physiological conditions in the circulation, where blood experiences rapid and cyclic changes from low to high shear flow conditions during the cardiac cycle. It is important to note that larger vessels are those closer to the heart, where the time-dependent effects are stronger on account of the proximity to the time-dependent stimulus.

Figure 10 – near here

Some materials are characterized by a critical yield stress, which must be exceeded for the material to flow. In a flow curve measured under steady shear flow, the yield stress can be defined as the limiting shear stress when the shear rate approaches zero (cf. Fig. 10(b)). The measurement of the blood yield stress is useful for the diagnostic of pathological conditions such as polycythemia (Merrill 1969), systemic sclerosis (Picart *et al.* 1999) or diabetes (Radtke *et al.* 1984) since the values of the blood yield stress when such diseases occur are higher than in healthy conditions. When blood is at rest, attractive forces between cells arise, such as van der Waals forces, Coulombic forces (Cheng and Evans 1965) and ion bridges or hydrogen bonds (Cho and Cho 2011). Consequently, a critical pressure gradient is required to overcome the yield stress and promote blood flow. Early studies established that the yield stress of blood is independent of temperature in the range between 10 °C and 37 °C (Barbee 1973). Subsequent investigations determined the blood yield stress indirectly by fitting the

Table 5. Summary of studies on whole blood yield stress (τ_0): proposed correlations as function of hematocrit level (Hct) and temperature of measurement (T_{meas}) (note: 1 dyne cm^{-2} = 100 mPa).

Reference	Correlations for yield stress	Parameters
Merrill <i>et al.</i> (1963)	$\tau_0[\text{dyne cm}^{-2}] = \tau_{y0}(Hct - Hct_c)^3$	$30 \leq Hct / \% \leq 50$ $\tau_{y0} \approx 0.008 \text{ dyne cm}^{-2}$ $1.3 \leq Hct_c(\text{critical hematocrit}) / \% \leq 6.5$
Chien <i>et al.</i> (1966)	$\tau_0[\text{dyne cm}^{-2}] = ae^{bHct}$	$Hct > 50\%$ a, b - empirical constants $10 \leq T_{\text{meas}} / ^\circ\text{C} \leq 37$
Zydney <i>et al.</i> (1991)	$\tau_0[\text{dyne cm}^{-2}] = \tau_{y0}(Hct - Hct_c)^3$	$Hct < 80\%$ $a = 0.007 \text{ dyne}^{1/3}\text{cm}^{-2/3}$ $b = 3.2 \%$
Picart <i>et al.</i> (1998)	$\tau_0[\text{dyne cm}^{-2}] = e^{(aHct+b)}$	$Hct > 80\%$ $a = 0.058$ $b = 6.36 \%$ $T_{\text{meas}} = 37 \text{ }^\circ\text{C}$
	$\tau_0[\text{dyne cm}^{-2}] = \tau_{y0}(Hct - 0.05)^3$	$\tau_{y0} = 0.71 \text{ dyne cm}^{-2}$ $T_{\text{meas}} = 37 \text{ }^\circ\text{C}$
	$\tau_0[\text{mPa}] = \tau_{y0}Hct^3$	$0.53 \leq Hct \leq 0.95$ $\tau_{y0} = 26.87 \text{ mPa}$ $T_{\text{meas}} = 25 \text{ }^\circ\text{C}$

experimental shear stress-shear rate data to generalized Newtonian models (such as the Casson model represented in Table 4) that incorporate a yield stress. Correlations reported by different authors for fitting blood yield stress as a function of hematocrit level are presented in Table 5. In spite of the efforts to determine the yield stress of blood, the results show considerable scatter since it is highly influenced by the hematocrit level, fibrinogen and RBC deformability. Moreover, the different experimental methods used and the complexity of blood contribute to the inconsistency between the values measured by different authors. In any case, it is clear that the blood yield stress is very low, of the order of 10^{-3} Pa for *Hct* in the range of 40% - 50% (Picart *et al.* 1998; Lee *et al.* 2011). Recently, it was found that yield stress is an onset phenomenon, which only occurs when the hematocrit exceeds a critical value, which in turn depends on the fibrinogen concentration (Apostolidis and Beris 2014).

3.3. Blood viscoelasticity

The viscoelasticity of blood is associated with its two-phase nature as well as with the morphological and elastic properties of the discrete phases, namely the RBC. Complex viscoelastic properties of RBC were reported by Puig-De-Morales-Marinkovic *et al.* (2007) and Li *et al.* (2014) when membrane rheology was investigated experimental and numerically using optical magnetic twisting torque cytometry or twisting torque cytometry, respectively. The interactions between the lipid bilayer and the cytoskeleton play an important role in the viscoelastic properties of RBC membrane and allow the cells to flow through capillaries of small diameter (Li *et al.* 2014). As a consequence, it comes as no surprise that the elastic behavior of blood generally increases with hematocrit level due to increasing cell interactions. For shear rates ranging from 1 s^{-1} to 10 s^{-1} the viscous component of the complex viscosity increases exponentially with

hematocrit whereas its elastic component increases nearly proportionally to the cube of the hematocrit. For shear rates above 10 s^{-1} , both viscous and elastic components of the complex viscosity increase exponentially with hematocrit (Thurston 1996).

Measurements of blood viscoelasticity have focused mostly on shear flow experiments including SAOS (Thurston 1972; Thurston 1996; Thurston and Henderson 2007) and, only more recently, LAOS (Sousa *et al.* 2013) and passive microrheology (Campo-Deaño *et al.* 2013). Recent studies have also recognized the importance of the rheological behavior of plasma and blood under extensional flow (Brust *et al.* 2013).

Small amplitude oscillatory shear (SAOS)

SAOS tests are commonly used to characterize the frequency dependence of the rheological behavior of a material. Considering a fixed oscillatory maximum strain, the viscous and elastic components of the blood complex viscosity decrease moderately with an increase of the frequency of oscillation (Thurston 1996) as shown in the inset of Fig. 11. Varying parameters such as hematocrit level or the RBC characteristics, namely deformability and aggregation, lead to modifications of the viscoelastic properties of blood as reviewed by Thurston and Henderson (2007).

Since blood viscoelasticity has a significant impact on blood flow, in particular on the propagation of the pressure pulse generated by the heart in the arterial system (Thurston and Henderson 2007), most of the works do not focus only on these fundamental measurements alone, but combine them with other rheological tests to create more realistic kinematic flow conditions as discussed in the following sections.

Figure 11 – near here

Most of the measurements, and in particular those discussed in relation to Fig. 11, were carried out at flow conditions mimicking real pulsatile flow, with oscillatory flow superimposed onto a steady flow (Thurston 1979; Thurston 1996; Thurston and Henderson 2007). Thurston (1979) measured the viscous and elastic components of the complex viscosity under oscillatory flow conditions by applying a constant frequency ($f = 2$ Hz) and varying the deformation applied to the sample. Additionally, a frequency sweep at constant strain amplitude was also performed. At low shear rates, viscoelasticity is dominated by the aggregation characteristics of RBC, but when the shear rate increases, RBC orient in the flow direction and aggregation is reduced. Although accompanied by the disruption of the aggregates of RBC at high shear rates, it is the deformability of RBC that becomes the main cause of the viscoelastic properties of blood.

Vlastos *et al.* (1997) investigated the blood response in steady and in SAOS using a strain amplitude sweep at a constant frequency of 0.5 Hz, as well as under a frequency sweep at constant strain amplitude. Constant steady shear rates were superimposed during oscillatory shear measurements in order to analyze their combined effect on the viscoelastic characteristics. The authors observed that increasing the steady shear rate component leads to a decrease of both viscous and elastic components due to the reduction of RBC aggregation by a non-zero average shear rate with the elastic component experiencing the highest decrease. Thurston and Henderson (2007) determined the Maxwell relaxation times of blood samples ($Hct = 44\%$) from oscillatory measurements at 2 Hz and 22 °C and showed how the blood characteristic time scale is associated with the structure and breakup of RBC aggregates (Thurston 1979). As depicted in Fig. 12, the Maxwell relaxation time reaches a plateau at low shear rates, which is explained by the formation of RBC aggregates. When the shear

rate is increased, the cell aggregates become progressively smaller and relax faster. For high shear rates, the aggregates no longer exist and the RBC deform and align with the flow leading to a further decrease of the relaxation time (Thurston and Henderson 2007).

Figure 12 – near here

Thurston and Henderson (2006) showed experimentally that the Maxwell relaxation time increases when the cell deformability is decreased and the aggregation is increased, as in pathologic blood. Moreover, the influence of the flow geometry on blood viscoelasticity was also investigated by the same authors, who found that in small vessels, represented by microtubes, the elastic component of the complex viscosity increases for high shear rates, showing a shear-thickening behavior.

Recently, Campo-Deaño *et al.* (2013) used passive microrheology to measure the storage and loss moduli of whole blood at 27 °C and $Hct = 44\%$, confirming the non-negligible elastic behavior of whole blood (cf. Fig. 13).

Figure 13 – near here

Large amplitude oscillatory shear measurements (LAOS)

Sousa *et al.* (2013) recently investigated the response of whole human blood under LAOS. Strain amplitudes of 10, 50 and 100 were imposed and the measurements were performed at the physiological temperature, $T = 37$ °C. Lissajous-Bowditch plots were used to quantify the nonlinear viscoelastic properties and to determine the parameters that relate the shear stress response of the blood sample with strain or shear rate. These

parameters, presented in Table 6, are the minimum elastic modulus (G'_M), the large strain elastic shear modulus (G'_L), the minimum-rate dynamic (η'_M) and large-rate dynamic viscosities (η'_L), the strain-stiffening ratio (S) and the shear-thickening ratio (T). The elastic shear moduli (G'_M, G'_L) represent the local elastic response of a sample at small and large instantaneous strains and the minimum and large-rate dynamic viscosities (η'_M, η'_L) give information about the instantaneous viscosities at the smallest and largest shear rates of an oscillatory test, respectively. Further details of the physical meaning of these parameters can be found in Ewoldt *et al.* (2008) and Hyun *et al.* (2011). For the rheological conditions investigated, whole blood revealed intra-cycle strain-stiffening ($S > 0$) and intra-cycle shear-thinning behavior ($T > 0$). The Lissajous-Bowditch plots of $\tau(\gamma)$ and $\tau(\dot{\gamma})$ presented in Fig. 14 for an angular frequency of 0.1 rad s^{-1} illustrate the viscoelastic character of blood, since the internal area of $\tau(\gamma)$ and $\tau(\dot{\gamma})$ represent the loss and storage energies, respectively. For the higher strain amplitudes tested (50 and 100), the nearly circular shape of the $\tau(\gamma)$ plots reveal a dominant viscous behavior, which is confirmed by the small internal area of the $\tau(\dot{\gamma})$ plot. For the lower strain amplitude tested, $\gamma = 10$, both Lissajous-Bowditch plots demonstrate a viscous and elastic character, but the viscous effects are still dominant. These rheological data were recently used by Apostolidis *et al.* (2015) for validation purposes of a blood flow model in the transient shear regime. The viscoelastic model captured the main features observed experimentally by Sousa *et al.* (2013) for both elastic and viscous components of blood, when the amplitude or frequency of oscillation was varied. However, due to its simplicity and the use of a number of fitting parameters, which were not always obtained from experimental data, only a semi-quantitative agreement between the results of both investigations is observed. Additionally, the

viscoelastic model was able to capture the thixotropic behavior of blood, but has a limited region of applicability, working only at low to moderate shear rates ($\dot{\gamma} \sim 1 \text{ s}^{-1}$) due to the isotropic microstructure assumed for blood.

Table 6. Elastic moduli (G'_M and G'_L), dynamic viscosities (η'_M and η'_L) and dimensionless indices of nonlinearity (S and T) obtained by Sousa *et al.* (2013) for two different strain amplitudes and angular velocities.

γ	ω	G'_M	G'_L	S	η'_M	η'_L	T
[-]	[rad s ⁻¹]	[Pa]	[Pa]	[-]	[Pa s]	[Pa s]	[-]
10	0.158	2.50×10^{-3}	5.84×10^{-3}	0.572	1.77×10^{-2}	1.61×10^{-2}	-0.100
100	1.0	-6.50×10^{-4}	-7.50×10^{-4}	0.133	4.04×10^{-3}	2.85×10^{-3}	-0.417

Figure 14 – near here

Extensional flow measurements

It is well known that extensional properties should also be considered in the rheological characterization of complex fluids, as for example in DNA or polymer solutions (Larson 2005). In particular, it is of utmost interest to determine the relaxation time of whole blood in extensional flow to complement the existing rheological shear data. For example, Sousa *et al.* (2011) investigated the flow of two well-established blood analogue solutions through micro-contractions, and showed that despite their similar steady shear viscosity curves, their behavior is significantly different when subjected to extensional flow conditions comparable to those in the microcirculation. In this respect, it is important to note the scarcity of works investigating the flow behavior of blood under extensional flows. To the best of our knowledge, the only investigation of the

relaxation time of human whole blood in extensional flow is the recent work of Brust *et al.* (2013), where a characteristic CaBER relaxation time $\lambda_c = 7.8 \pm 0.6$ ms at 37°C is reported. However, no details of the measurements are provided and a more detailed investigation of this property, involving different physiological parameters, is still lacking. Interestingly, the authors found clear evidence of viscoelastic behavior when the blood plasma was subjected to elongational flow in a capillary breakup extensional rheometer. On the other hand, under steady shear flow, the human plasma showed a constant shear viscosity suggesting a Boger-like behavior, but evidence of viscoelastic behavior was also observed in microfluidic experiments using a contraction/expansion geometry. Brust *et al.* (2013) confirmed that viscoelastic behavior is not due to a protein surface layer but rather due to the elongational properties of the proteins, similarly to the phenomenon that occurs when adding a small amount of polymer to a solvent (Sousa *et al.* 2010) or even to a suspension (comparable to whole blood) (Barnes 2003) conferring a slight viscoelastic behavior. Investigating the whole blood behavior under strong elongational flow will certainly bring better insight about its elastic nature.

4. Concluding remarks and future directions

This review summarizes fundamental concepts and important investigations on hemorheology. The main factors that can lead to an alteration of blood rheology, and which have a significant impact on the normal physiological behavior of blood, are discussed. Quantitative comparisons of different studies are also provided in tabular form. A deviation of the whole blood viscosity from normal values can be linked with specific diseases, thus making blood viscosity a material function that can be used to identify various pathologies. Blood exhibits viscoelastic characteristics but these

properties are less investigated. The vast majority of viscoelastic measurements were performed under steady and small amplitude oscillatory shear flow conditions. Blood characterization under extensional flow and large amplitude oscillatory shear flow has been investigated only recently despite the recognized relevance of these nonlinear canonical flows in hemodynamics, e.g. LAOS measurements are relevant as pulsating blood flow under normal flow conditions is subject to large amplitude deformations.

Due to the complexity of blood flow, some simplifications are frequently assumed in hemodynamic studies. A significant number of investigations consider blood as a Newtonian fluid in spite of its non-Newtonian properties being well documented in the literature, while some works focus on cell behavior rather than on whole blood flow. Nevertheless, there has been a growing interest and increased efforts devoted to the investigation of whole blood rheological behavior under more realistic flow conditions.

Blood flow in small vessels shows complex flow features not observed at the macro-scale with enhanced non-Newtonian fluid flow characteristics, such as viscoelasticity. The increasing interest in microfluidics and the advances in the field in recent years, motivated among others by the development of micro-devices in the medical field, constitute an interesting framework for hemodynamic experiments. Furthermore, lab-on-a-chip technology combines portability, integration and automation in a single chip, and is thus a promising platform for point-of-care devices, which may be particularly useful for detection and diagnosis of circulatory disorders.

Future directions

Despite notorious advances there are still many important research challenges and opportunities to be explored in hemorheology, including better characterization and deeper understanding of the behavior of individual components of blood such as the

rheology of plasma and whole blood under different flow conditions, and in particular in diseased states to improve diagnostics, but also in terms of instrumentation and methodologies, in order to translate the measurements and simulations performed in research laboratories into a clinical context. In the near future, it will be possible to move toward the development and implementation of novel clinical micro-devices to be employed in medical diagnosis of hematological and cardiovascular diseases. However, there are still several fundamental topics that require further investigation for these applications to become routine:

- i. **Plasma rheology under extensional flow conditions.** Recent investigations showed a viscoelastic behavior in extensional flow, and constant viscosity in shear flow measurements (Brust *et al.* 2013). Further insight into plasma rheology in extensional flow and in combined shear and extensional flow is required in order to better characterize its rheological behavior.
- ii. **Whole blood rheology in extensional flow** or in combined shear and extensional flows should also be investigated in order to better assess its viscoelastic nature. It is known that blood only has a slight elastic character (Brust *et al.* 2013; Sousa *et al.* 2013), but a thorough quantitative characterization under extensional flow is still missing. So far, a characteristic CaBER relaxation time $\lambda_c = 7.8 \pm 0.6$ ms at 37°C was reported (Brust *et al.* 2013) but a more detailed investigation of this property, involving different physiological parameters, should be carried out. In addition, studying whole blood under extensional flow will allow analyzing RBC deformability in more detail.

- iii. **Blood yield stress** values reported so far present some inconsistencies due to the different methods used for measuring this property in addition to the inherent variability because of its dependency on physiological conditions. Further investigations of this property under well controlled conditions, in particular using direct measurements, are needed to obtain more accurate values and unveil the reasons for these inconsistencies.

- iv. **Red blood cell aggregation**, which is of the utmost important phenomena in hemodynamics, is not yet fully understood. Unraveling the mechanism involved in cell adhesion and consequently in rouleaux formation remains an important challenge. A recent study shows that dextran-induced RBC aggregation is explained based on depletion effects (Steffen *et al.* 2013) but consideration of other macromolecules, in particular fibrinogen, an important plasma constituent, is still lacking. The same study indicates that bridging effects can describe the adhesion mechanisms for contact times longer than a few seconds, which needs to be confirmed.

- v. **RBC deformability**. Since it is now recognized that several pathologies lead to alteration of the mechanical properties of RBC and to changes of blood rheology, investigating RBC deformability and blood rheological properties of unhealthy human blood in shear and extensional flows, with control of the disease condition, is needed to develop diagnostic procedures. For example, microfluidic devices composed of hyperbolic shaped contractions (Lee *et al.* 2009; Yaginuma *et al.* 2013) have been used to investigate normal and altered

RBC. Other fluidic devices with customized shapes can be used to generate different (and relevant) flow conditions in which RBC are stretched. In particular, microfluidics devices that offer easy and inexpensive fabrication have great potential to be used in the assessment of RBC behavior.

- vi. **Micro-rheological studies.** The small quantities of sample employed in these techniques make them very interesting for blood characterization, but it should be investigated how far these can be used for the measurement of rheological properties, in spite of the occurrence of the Fåhræus effect. It is also important to understand how to extrapolate the rheological properties obtained to conditions in which the plasma layer is not formed. In addition, further work on blood dynamics at the micro-scale should be considered in order to capture possible flow phenomena not easily achieved at the macro-scale. Microfluidics can be a valuable platform to develop micro-total analysis systems, which combines portability and automation for a rapid and efficient analysis of a small blood sample drawn from the patient. Examples representative of its effective potential are the lab-on-a-CD systems for biochemical (Lee *et al.* 2011) or electrochemical bioanalysis or blood separation (Li *et al.* 2013) using only whole blood volume samples of the order of 10-100 μL or even a cytometry platform developed using a cell-phone (Zhu *et al.* 2013), to mention only a few.

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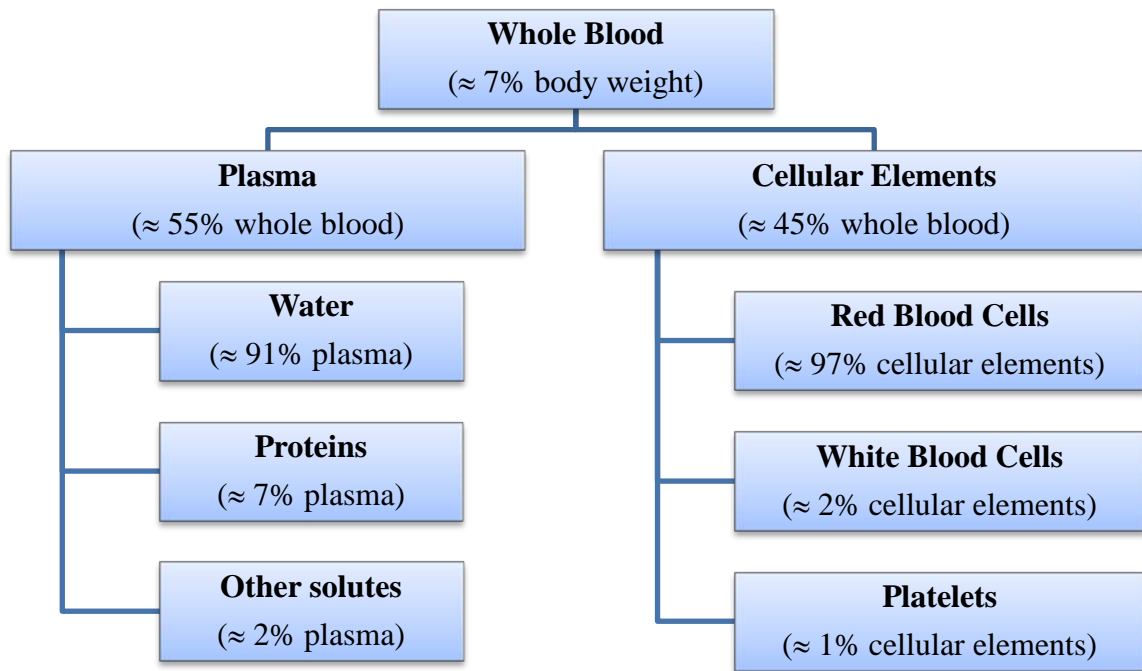


Figure 1. Main components of whole human blood and their typical volume concentrations.

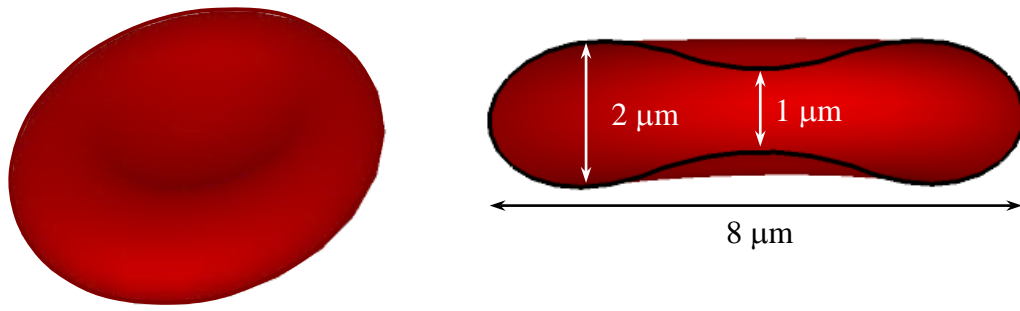


Figure 2. Schematic representation of a healthy red blood cell at rest and its typical dimensions.

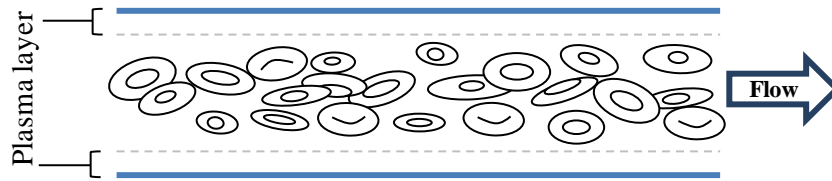


Figure 3. Sketch of the formation of a plasma enriched layer near the vessel walls and accumulation of RBC at the center of the vessel when blood is flowing (wall effect).

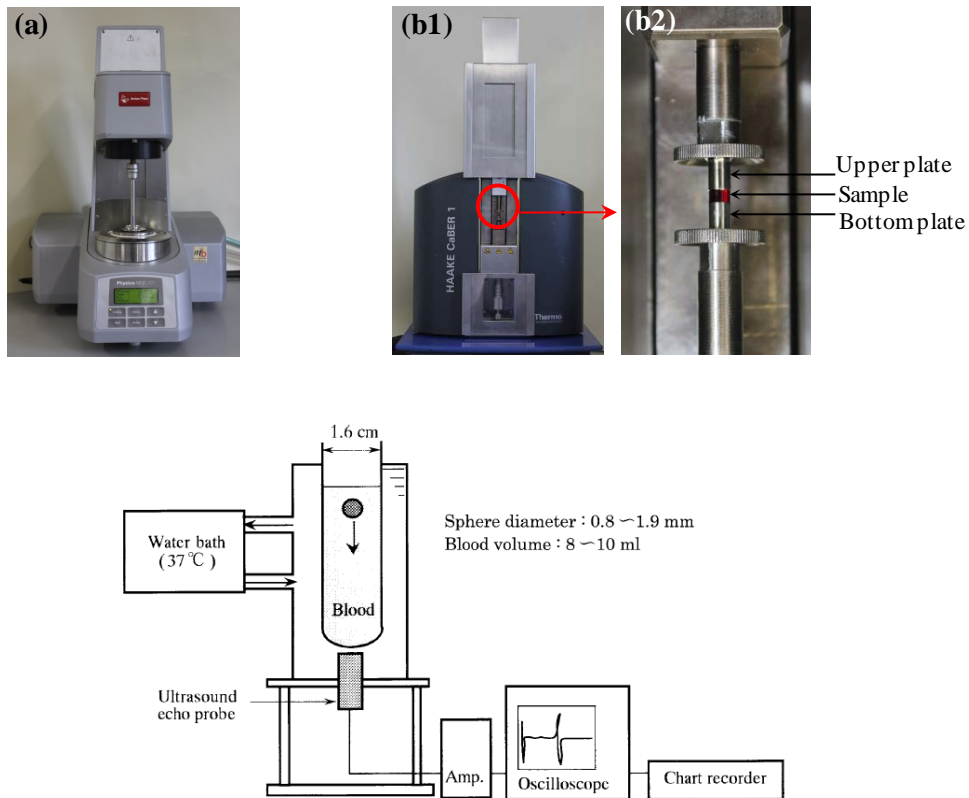


Figure 4. Examples of mechanical devices employed in rheological measurements of blood. (a1) Rotational rheometer (Physica MCR301, Anton Paar). (b1) Capillary break-up extensional rheometer Haake™ CaBER™ 1 (Thermo Scientific). (b2) Zoomed view of the test section. The sample is placed in the space between the upper and the bottom plates. (c) Schematics of the falling-ball viscometer used by Eguchi and Karino (2008). Reprinted with permission from Eguchi and Karino (2008).

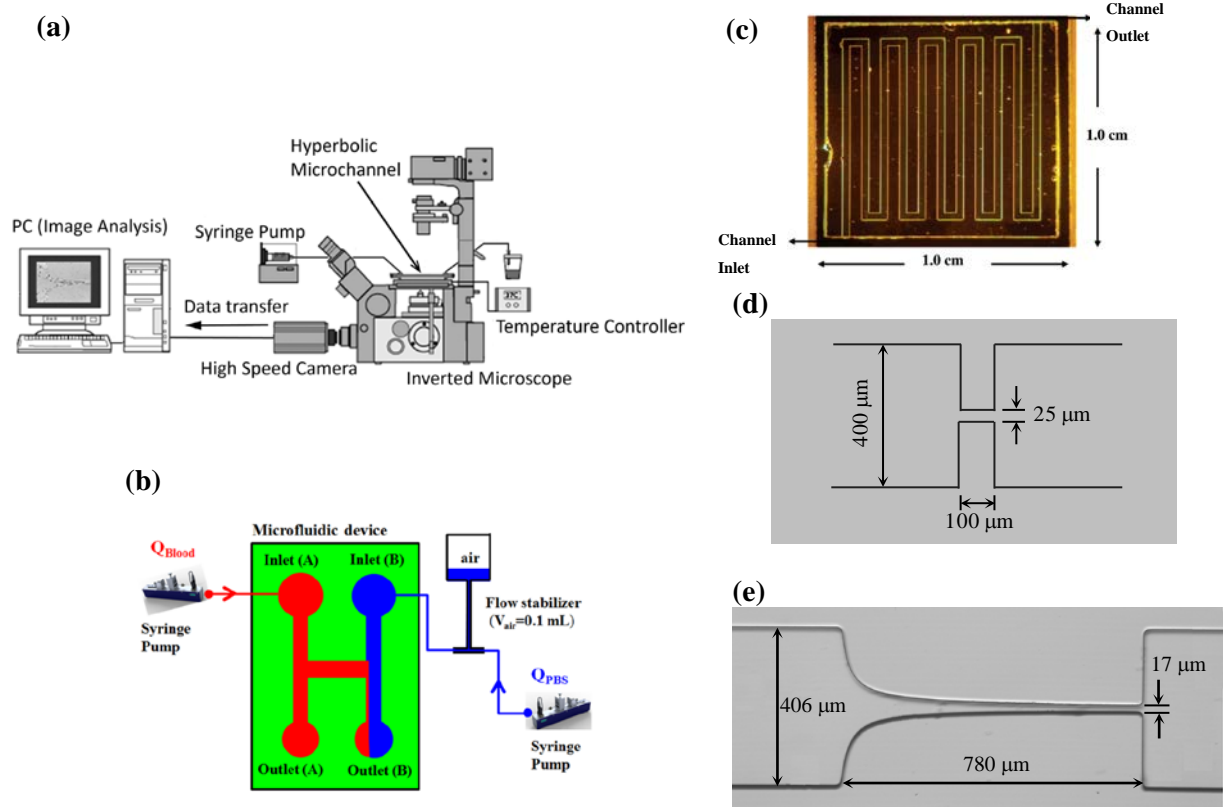


Figure 5. Microfluidic devices employed in blood rheological measurements. (a) The experimental set-up is typically composed of an inverted microscope on which the microfluidic chip is placed, a digital camera for recording images, a syringe pump for imposing a flow rate and a temperature controller for controlling the temperature of the fluid. Reprinted with permission from Yaginuma *et al.* (2013) (b) Microfluidic analogue of Wheatstone-bridge channel used by Kang and Lee (2013). Reprinted with permission from Kang and Lee (2013). Blood is fed to inlet A and a PBS solution to inlet B by means of syringe pumps. To ensure constant flow rates for both fluids, a flow stabilizer with 0.1 mL cavity volume was placed between the syringe pump and the channel inlet of PBS solution. (c) Microfabricated viscometer used by Srivastava *et al.* (2005). The channel, 340 μm wide and 36 μm in depth, is etched in glass and bounded to silicon and open to the atmosphere on the inlet and outlet. Reprinted with permission from Srivastava *et al.* (2005). Copyright (2015) American Chemical Society. (d) Sketch of the microfluidic channel composed of an abrupt contraction followed by an abrupt expansion used by Brust *et al.* (2013). The depth of the channel is 50 μm . (e) Optical

micrograph of the hyperbolic-shaped contraction followed by an abrupt expansion channel similar to that employed by Yaginuma *et al.* (2013). The depth of the channel is 60 μm . The same type of contraction was also used in the work of Lee *et al.* (2009) for investigating RBC deformability.

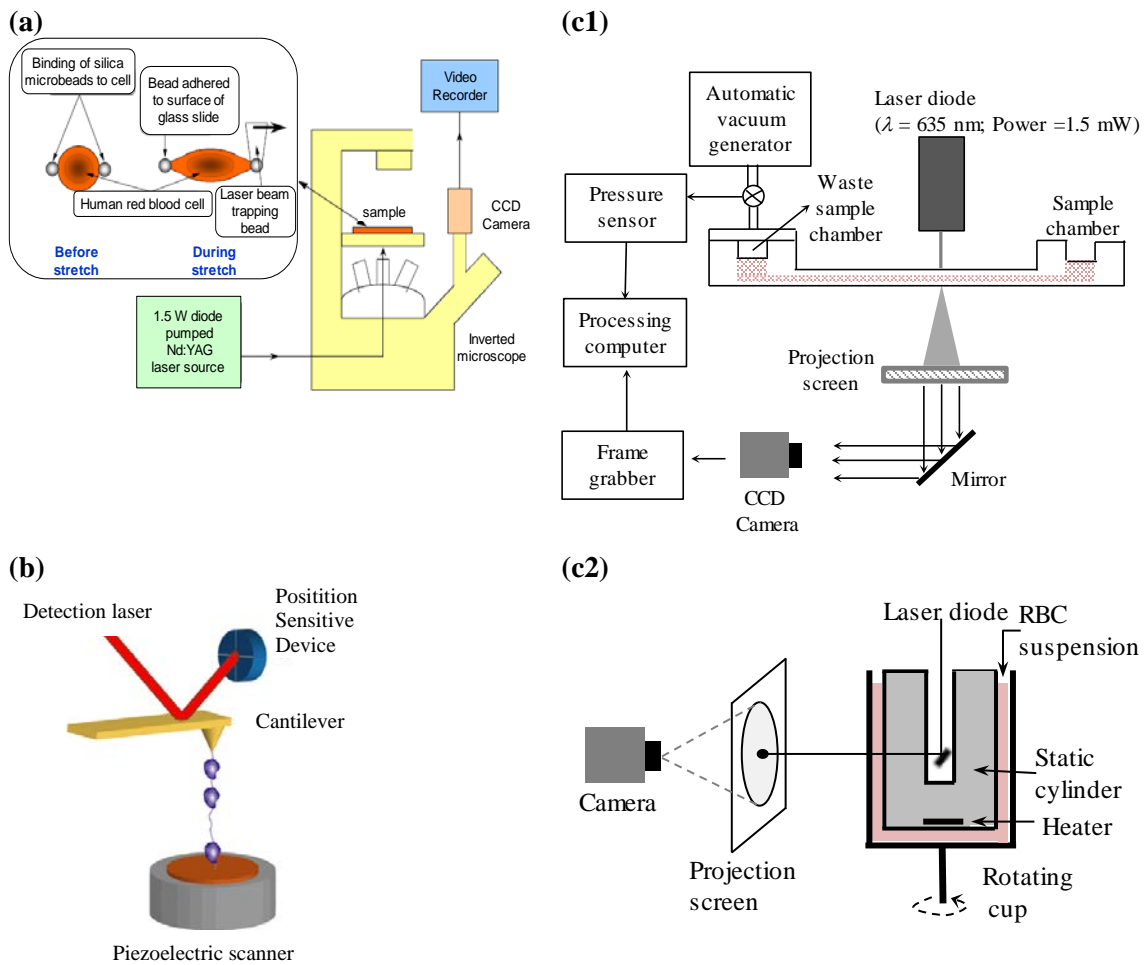


Figure 6. (a) Schematics of an optical tweezers set-up. Two microbeads are bound to the RBC at opposite points, one bead is anchored to the surface of a glass slide and the other can be trapped using the laser beam and the force is applied through the movement of the laser beam. Reprinted with permission from Dao *et al.* (2003). (b) Schematics of the AFM technique. The deflection of a pre-functionalized cantilever is measured by the displacement of a low power laser reflected on a Position Sensitive Device (PSD). The piezo stage is retracted along the axial direction and the stretching of the molecule is equal to the distance between the AFM tip and the sample surface. Reprinted with permission from Neuman and Nagy (2008). (c) Comparison between the RheoScan-D (c1) and LORCA (c2) systems. The RheoScan-D employs a channel 4 mm wide and 40 mm long with a depth of 200 μm and analyses RBC deformability whereas the LORCA system can measure the aggregation and deformability of the RBC in a Couette cell.

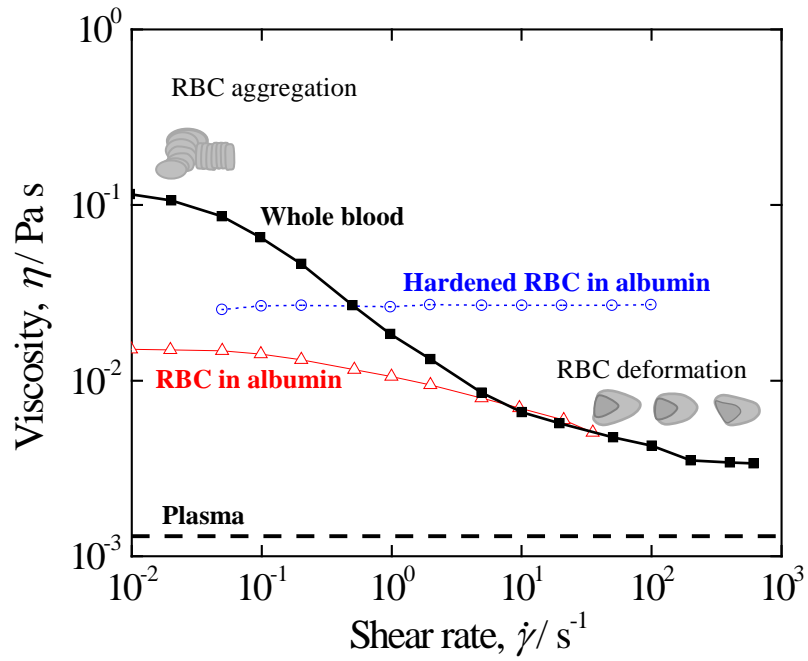
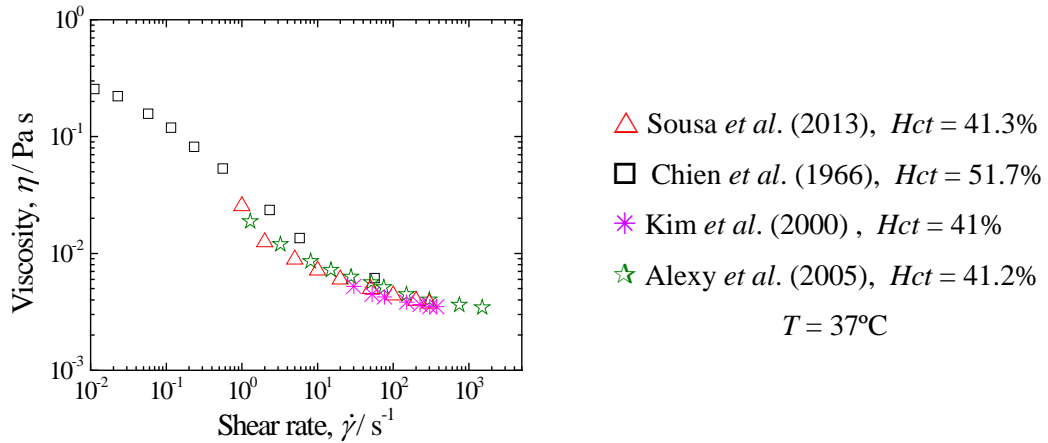
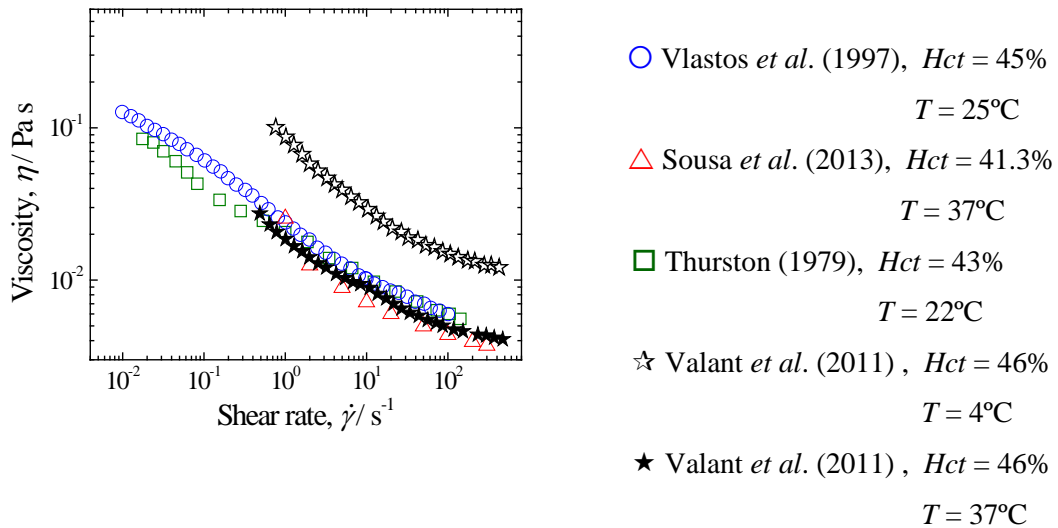


Figure 7. Shear viscosity of blood as a function of shear rate for normal human blood, normal red blood cells in 11% albumin-Ringer solution and red blood cells hardened by glutaraldehyde in 11% albumin-Ringer solution. All suspensions contain 45% (v/v) of RBC. The plasma and the suspension of rigid erythrocytes in plasma show a Newtonian rheological behavior in shear, whereas RBC aggregation and deformation lead to a shear-thinning shear viscosity. The differences in the curves highlight the effects of RBC aggregation and deformation. Adapted from Chien (1970).



(a)



(b)

Figure 8. Viscosity of human blood as a function of shear rate obtained experimentally in different works. (a) $T = 37\text{ }^{\circ}\text{C}$; (b) $T = 4\text{--}37\text{ }^{\circ}\text{C}$. Blood viscosity data obtained by Alexy *et al.* (2005) was measured from 44 donors at a mean hematocrit of 41.2% using an automated scanning capillary viscometer (RheologTM). Chien *et al.* (1966) employed a coaxial cylinder viscometer; Kim *et al.* (2000) used a scanning capillary tube viscometer; Sousa *et al.* (2013) and Valant *et al.* (2011) used a shear rheometer (Physica MCR301, Anton Paar) with a serrated plate-plate geometry and a double gap cell, respectively; Thurston (1979) and Vlastos *et al.* (1997) employed a Couette-type viscometer, Contraves LS 30 and Contraves LS 40, respectively.

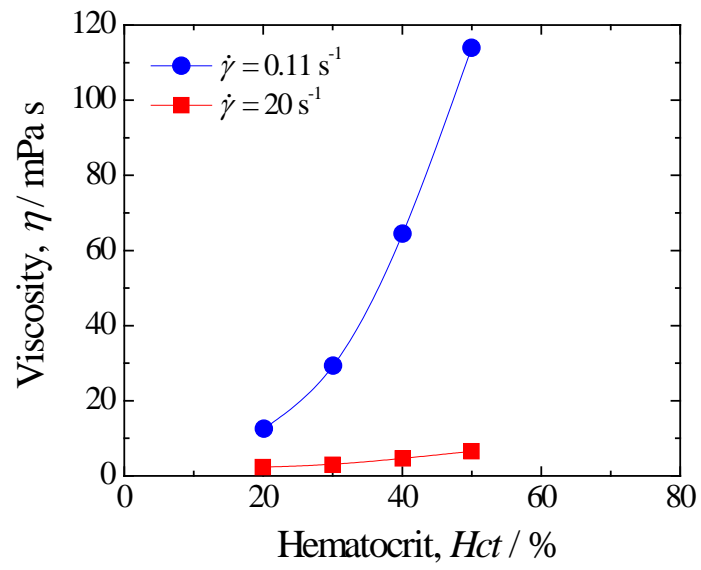


Figure 9. Effect of hematocrit on the viscosity for normal human RBC suspended in autologous plasma at two different values of shear rate. Adapted from Cokelet and Meiselman (2007).

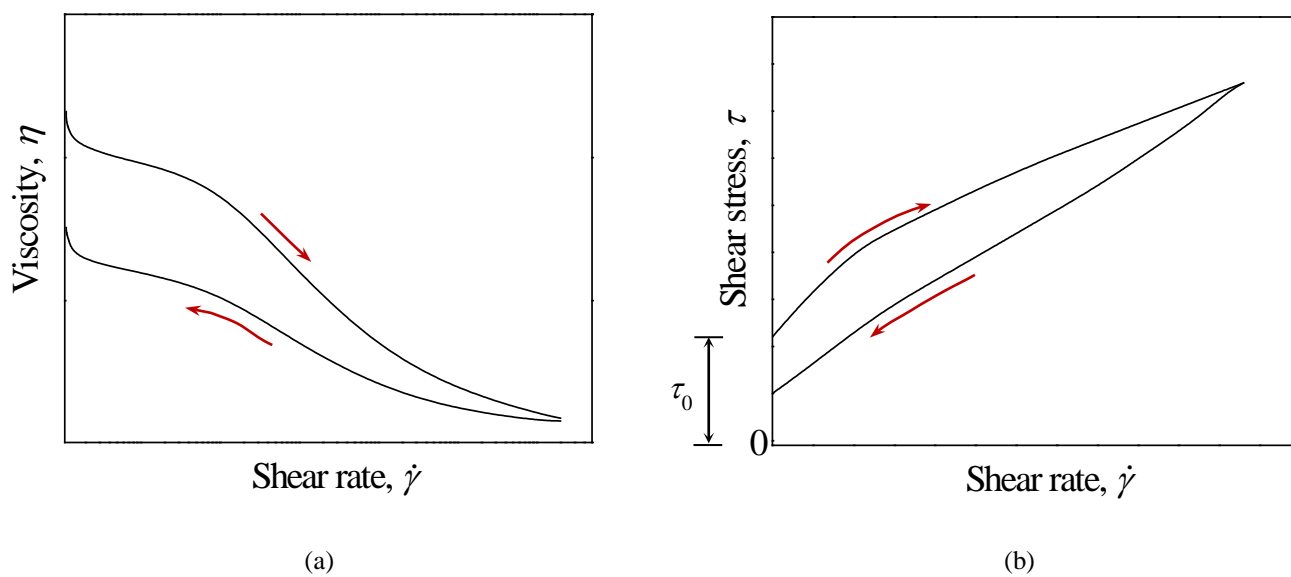


Figure 10. Typical thixotropic hysteresis loop observed in the curve of the shear viscosity as a function of shear rate (a) and in the curve of shear stress as a function of shear rate (b). The fluid yield stress (τ_0) is also illustrated in (b).

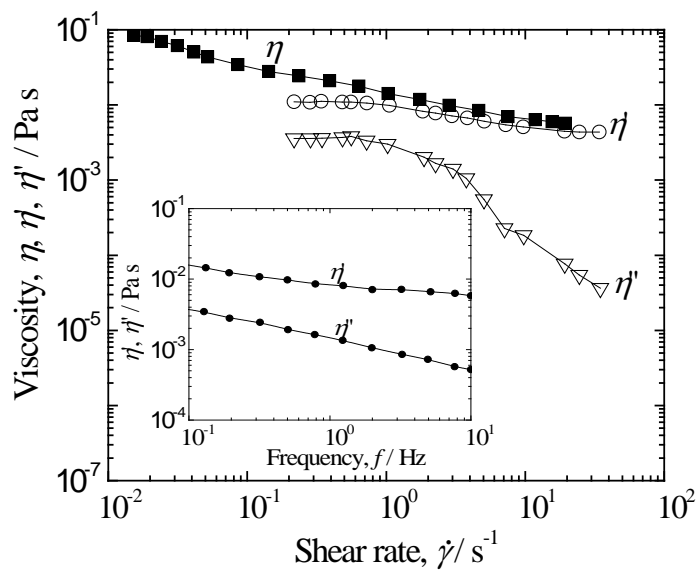


Figure 11. Steady shear viscosity and viscous and elastic components of the complex viscosity as a function of shear rate at a frequency of oscillation, $f = 2$ Hz. In the inset, viscous and elastic components of the complex viscosity are presented as a function of frequency of oscillation. Adapted from Thurston (1979, 1996).

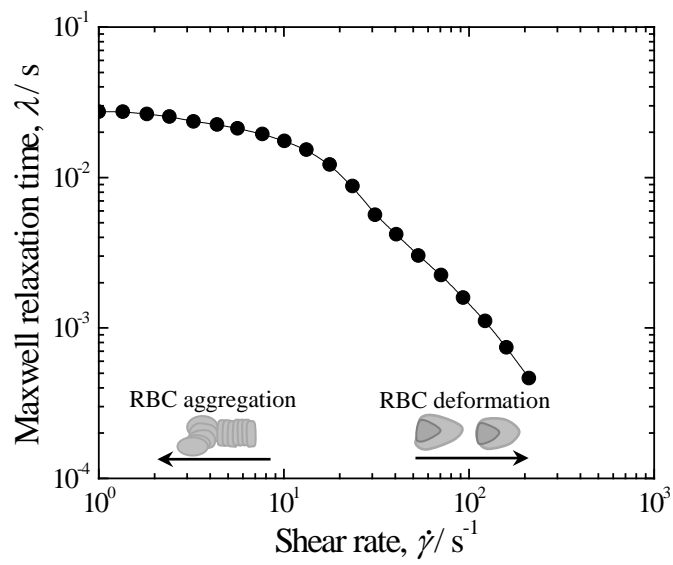


Figure 12. Maxwell relaxation times for $Hct = 44\%$, at $f = 2$ Hz and $T = 22^\circ\text{C}$. Adapted from Thurston and Henderson (2007).

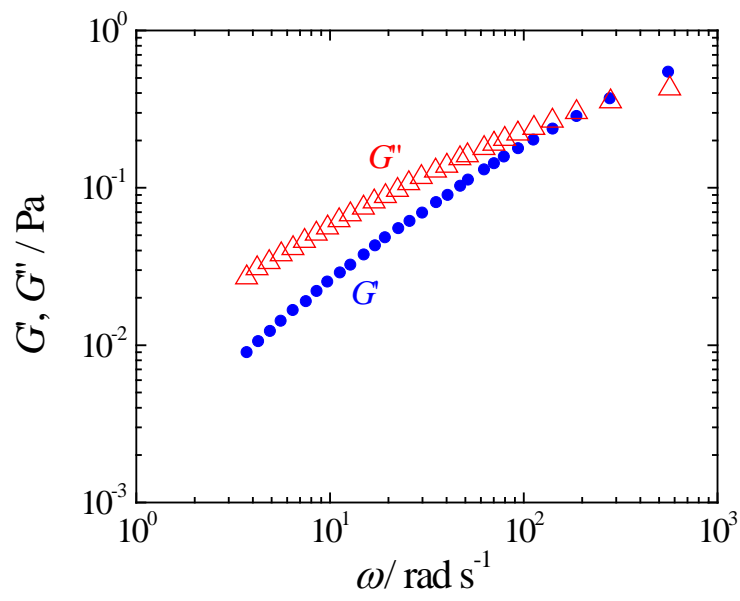


Figure 13. Storage (G') and loss (G'') moduli as a function of the angular frequency measured using passive microrheology for $Hct = 44\%$ and $T = 27\text{ }^\circ\text{C}$. Reproduced with permission from Campo-Deaño *et al.* (2013).

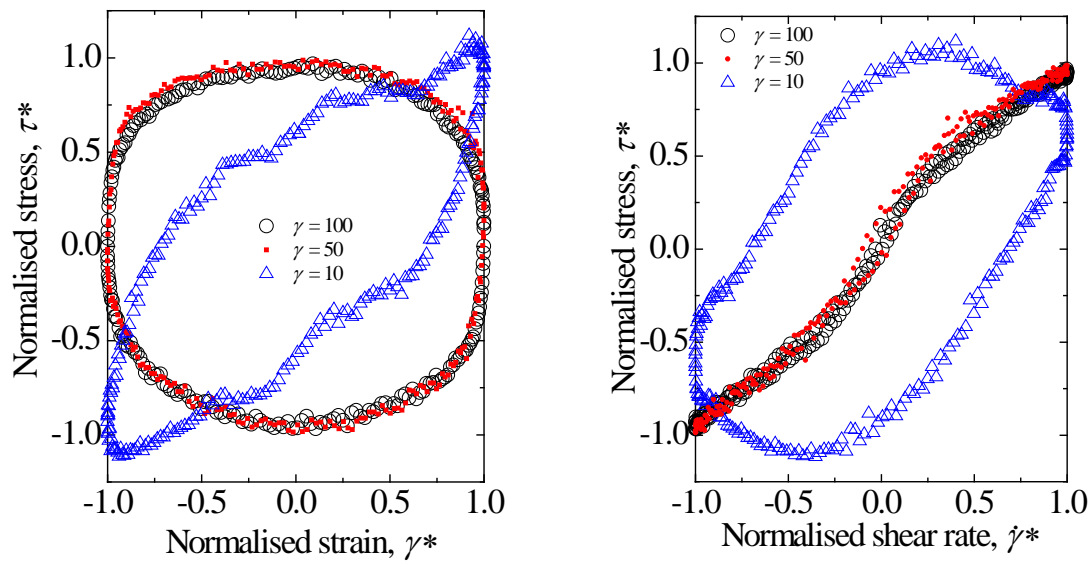


Figure 14. Lissajous-Bowditch plots of shear stress vs. strain and shear stress vs. shear rate at strain amplitudes $\gamma = 10, 50$ and 100 and for a constant angular frequency, $\omega = 0.1 \text{ rad s}^{-1}$. Reproduced with permission from Sousa *et al.* (2013). The authors employed a Fourier transform analysis to describe the results and normalized the data presented in the Lissajous-Bowditch plots using the first harmonic of the Fourier series.