A novel approach to blood plasma viscosity measurement using fluorescent molecular rotors

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Haidekker, Mark A., Amy G. Tsai, Thomas Brady, Hazel Y. Stevens, John A. Frangos, Emmanuel Theodorakis, and Marcos Intaglietta. A novel approach to blood plasma viscosity measurement using fluorescent molecular rotors. Am J Physiol Heart Circ Physiol 282: H1609–H1614, 2002; 10.1152/ajpheart.00712.2001.—Molecular rotors, a group of fluorescent molecules with viscosity-dependent quantum yield, were tested for their suitability to act as fluorescence-based plasma viscometers. The viscosity of samples of human plasma was modified by the addition of pentastarch (molecular mass 260 kDa, 10% solution in saline) and measured with a Brookfield viscometer. Plasma viscosity was 1.6 mPa·s, and the mixtures ranged up to 4.5 mPa·s (21°C). The stimulated light emission of the molecular rotors mixed in the plasma samples yielded light intensity that was nonoverlapping and of significantly different intensity for viscosity steps down to 0.3 mPa·s (n = 5, P < 0.0001). The mathematical relationship between intensity (I) and viscosity (η) was found to be η = (kI)n. After calibration and scaling the fluorescence based measurement had an average deviation versus the conventional viscometric measurements that was <1.8%. These results show the suitability of molecular rotors for fast, low-volume blood viscosity measurements achieving accuracy and precision comparable to mechanical viscometers.

BLOOD PLASMA IS AN IMPORTANT factor in physiology and disease. Blood plasma viscosity anomalies are associated with diseases such as diabetes, hypertension, infections, and infarctions (9, 13, 16, 17, 21). A direct cause-and-effect relationship has not been established between plasma viscosity and the development of these conditions; thus, clinical medicine has not provided a strong stimulus for the development of practical techniques for characterizing either blood or plasma viscosity. In transfusion medicine, however, the management and measurement of blood plasma viscosity is becoming a central issue affecting blood replacement with plasma expanders (20) and the development of artificial blood (10) consisting of colloidal solutions of modified hemoglobin.

Information about the distribution of blood viscosity in the circulation on the introduction of plasma expanders or artificial blood is particularly relevant in terms of shear stress mechanotransduction to the endothelium, which regulates the shear stress-dependent production of vasoactive mediators (2) such as prostacyclin and NO, and modulates apoptosis (4). In this context, information on plasma viscosity is a key element in understanding the magnitude of the stimulus to which endothelium is exposed, because shear stress is the product of vessel wall velocity gradient and local plasma/blood viscosity.

The measurement of viscosity of macromolecular solutions and particularly blood plasma requires mechanical tests. Standard mechanical viscometers include the capillary viscometer, where fluid is sheared by flow past the stationary inner wall of a capillary tube (15, 18), the falling-ball viscometer, and the rotational viscometer, where the test liquid is sheared between two surfaces, one fixed and one moving, with the torque being related to viscosity (15). For the measurement of blood plasma, a specialized capillary viscometer (Harkness Viscometer) has been recommended by the International Committee for Standardization in Haematology (1), which allows measurement of sample sizes as low as 0.5 ml within 1 min. However, reliable results cannot be obtained unless the effects of air/solution interfaces can be accounted for or controlled (3, 9), and the surfaces in contact with the test solutions are meticulously cleaned. These requirements complicate obtaining viscosity data on blood plasma in a clinical setting and limit the rate at which sequential measurements can be made if the same apparatus is used, if sample sizes of 3–4 ml are necessary due to the time required for separating red blood cells from plasma. Also, mechanical instruments are unable to assess microviscosity in microscopic environments or samples. Thus a potentially useful parameter for characterizing biological fluid properties is not available due to the lack of a suitable measurement technique/principle. A solution to this problem may be offered through the use of fluorescent molecules that are viscosity sensitive.

The viscosity of the mixture was modulated from pure plasma viscosity (1.6 mPa·s) to the approximate viscosity of full blood (4.3 mPa·s) in steps of ~30% (experiment A). Experiment B covers a smaller viscosity range (up to 3 mPa·s) with smaller steps of ~15%.

Those molecules, commonly referred to as fluorescent molecular rotors, belong to the group of twisted intramolecular charge-transfer complexes. Photoexcitation leads to an electron transfer from the donor group to the acceptor group. Relaxation can either occur through radiation (fluorescence) or intramolecular rotation (thermally induced nonfluorescent relaxation). The preferred relaxation mechanism, intramolecular rotation, is reduced in solvents with low free volume. Therefore, the quantum yield of a molecular rotor increases with decreasing free volume of the solvent (14). Free volume and viscosity are related (6), which links quantum yield directly to the viscosity of the microenvironment. The relationship between fluorescence quantum yield ($\Phi$) and the viscosity ($\eta$) of the solvent has been derived analytically (7, 12, 14) and experimentally (7, 11) and is known as the Förster-Hoffmann equation (7):

$$\log \Phi = C + x \log \eta$$

(1)

where $C$ is a temperature-dependent constant and $x$ is a dye-dependent constant. Two commercially available dyes in this group are 9-(dicyanovinyl)-julolidine (DCVJ) and 9-(2-carboxy-2-cyano-ethyl)-julolidine (CCVJ), the latter being soluble in aqueous solutions.

This study was carried out to investigate the suitability of using molecular rotors derived from DCVJ and CCVJ in assessing the viscosity of blood plasma and plasma expanders.

**MATERIALS AND METHODS**

**Chemicals and instrumentation.** Human blood plasma was purchased from the San Diego Blood Bank. DCVJ and CCVJ were purchased from Helix Research (Springfield, OR). All other fluorescent compounds were synthesized as described earlier (8). Stock solutions of the probes were prepared at a concentration of 20 mM in fluoroscopy-grade dimethyl sulfoxide (Sigma). Plasma viscosity was changed by adding the clinical-grade high-viscosity plasma expander pentastarch (10% by weight, average molecular mass 260 kDa) in physiological saline solution (Pentastan, DuPont; Wilmington, DE). Fluorescence measurements were performed on a fluorospectrometer (model RF-1501, Shimadzu; Kyoto, Japan) using standard methylacrylate cuvettes (Fisher Scientific; Pittsburgh, PA). Viscosity was measured using a Brookfield DV-II+ cone-and-plate viscometer with CP-40 spindle (Brookfield; Middleboro, MA). Viscosity values were obtained at discrete shear rate settings of 75, 90, 150, 225, 450, and 750 s$^{-1}$ (10, 12, 20, 30, 60, 100 rpm). Viscosities measured at 60 rpm (450 s$^{-1}$) were used as a reference to avoid the apparent non-Newtonian behavior at low shear rates because this was not present beyond this shear rate. This shear rate allowed us to measure the viscosity of all the samples without exceeding the torque limitations of the instrument.

**Experiment of suitability of different probes.** Blood plasma was kept frozen and thawed at room temperature overnight for use. Coarse precipitates were eliminated by centrifugation at 180 g for 10 min. To ensure homogeneous and precise distribution of the fluorescent probe, we prepared prestained plasma by mixing 20 µl of the probe stock solution in 6 ml of plasma under vigorous stirring. For each probe, a series of mixtures of plasma with pentastarch solution (PS) was prepared according to Table 1, experiment A. Fluorescence emission spectra were acquired at an excitation wavelength of 440 nm, and the maximum intensity was determined, irrespective of the wavelength of the maximum. Immediately after the fluorescence measurement, the viscosity of the plasma/PS solution mixture was determined in the cone-and-plate viscometer as described above. All experiments, fluorescence measurement, and viscometry, were performed at a constant temperature of 21°C.

### Table 1. Mixture ratios of plasma and pentastarch solution used for high-viscosity range (experiment A) and for lower viscosity increment (experiment B)

<table>
<thead>
<tr>
<th>Plasma/Pentastarch Ratio, vol:vol</th>
<th>Prestained Plasma (67-nM probe), ml</th>
<th>Unstained Plasma, ml</th>
<th>Pentastarch, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100:0</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>80:20</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>60:40</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>40:60</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>20:80</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td><strong>Experiment B</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100:0</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>90:10</td>
<td>1</td>
<td>3.5</td>
<td>0.5</td>
</tr>
<tr>
<td>80:20</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>70:30</td>
<td>1</td>
<td>2.5</td>
<td>1.5</td>
</tr>
<tr>
<td>60:40</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Results

General behavior of molecular rotors in plasma. All probes dissolved in plasma without forming precipitates.

### Table 2. Changes of the relative intensity of tested probes with increasing viscosity of plasma/pentastarch mixture

<table>
<thead>
<tr>
<th>Viscosity, mPA/s</th>
<th>DCVJ</th>
<th>CCVJ</th>
<th>CCVJ-ME</th>
<th>CCVJ-EE</th>
<th>CCVJ-BE</th>
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</thead>
<tbody>
<tr>
<td>1.63</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2.07</td>
<td>1.23</td>
<td>1.23</td>
<td>1.42</td>
<td>1.25</td>
<td></td>
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<tr>
<td>2.63</td>
<td>1.51</td>
<td>1.47</td>
<td>1.77</td>
<td>1.62</td>
<td>1.48</td>
</tr>
<tr>
<td>3.34</td>
<td>1.78</td>
<td>1.86</td>
<td>2.49</td>
<td>2.33</td>
<td>2.19</td>
</tr>
<tr>
<td>4.25</td>
<td>2.14</td>
<td>2.24</td>
<td>3.40</td>
<td>3.21</td>
<td>3.09</td>
</tr>
<tr>
<td>Slope</td>
<td>0.79</td>
<td>0.85</td>
<td>1.25</td>
<td>1.23</td>
<td>1.18</td>
</tr>
</tbody>
</table>

DCVJ, 9-(dicyanovinyl)-julolidine; CCVJ, 9-(2-carboxy-2-cyanovinyl)-julolidine; ME, methyl ester; EE, ethyl ester; BE, butyl ester. All probes show a distinct increase of the emission peak with increased viscosity. At the highest viscosity examined (plasma/pentastarch solution 20:80), intensity is twofold (DCVJ, CCVJ) to 3.5-fold (CCVJ-esters) higher than in pure plasma. Slope is computed from the logarithms of intensity and viscosity and represents the constant $x$ in Eq. 1.
tates, and blood plasma stained with the fluorescent probes exhibited typical spectral behavior with absorption maxima in the blue range (440–454 nm, depending on the probe) and emission in the green range (464–494 nm). Figure 1 shows typical excitation and emission spectra of CCVJ in blood plasma and a mixture of 40% plasma and 60% PS solution. All other spectra were similar with only minor shifts of the maxima, and all probes exhibited increased emission intensity when dissolved in fluids with higher viscosity as covered in detail in the following sections.

Viscosity-dependent emission intensity for all probes in plasma: PS solution mixtures. Table 2 gives an overview over the measured maximum intensities of the probes DCVJ, CCVJ, CCVJ-methyl ester, CCVJ-ethyl ester, and CCVJ-butyl ester, normalized by the intensity measured in pure blood plasma. Pure blood plasma and mixtures of 80% plasma with 20% PS solution, 60% plasma with 40% PS solution, 40% plasma with 60% PS solution, and 20% plasma with 80% PS solution were used. The intensity increase of fluorescence intensity in a high-viscosity mixture containing 80% PS solution over pure plasma is about twofold (with DCVJ and CCVJ) to 3.5-fold (with CCVJ esters).

Precision assessment of measurement method. To determine the repeatability of the measurement procedure, each sample of the matrix was divided into five aliquots, which were independently mixed with the probe CCVJ (Table 1, experiment A). Fluorescence was measured and variability was calculated according to Eqs. 2 and 3, which led to the calculation of standard deviations and coefficients of variability. To obtain values at lower viscosity increments, the experiment was repeated with modified mixture ratios as described in Table 1, experiment B, using both CCVJ and CCVJ-ME as probes. Figures 2 and 3 show the means ± SD of the five measurements for each mixture of plasma and PS solution. There is no overlap between the measured values, and all average values are significantly different with $P < 0.0001$ ($P = 0.0016$ for the last two bars). Coefficients of variability (standard deviation divided by the average within each column) ranged from 0.001 to 0.046.

Relationship between viscosity and intensity. Measured fluorescence data were compared with mechanically obtained viscosity. Pure blood plasma as well as mixtures of plasma and PS solution exhibited shear-rate-dependent viscosity (Fig. 4, A and B). Viscosity values for further data analysis were obtained at matched shear rates, 450 s$^{-1}$, which is the highest shear rate common to all measurements. Thus, for each sample, one data pair (viscosity, intensity) was obtained. The application of the Förster-Hoffmann equation (Eq. 1) to the data pairs leads to the calibration curve required to mathematically relate intensity and viscosity values. Data points of intensity over viscosity were plotted in double-logarithmic scale (Fig. 5, A and B), and the slope was determined using a least-squares fit. The following calibration equation to calculate $\eta$ from fluorescence intensity ($I$) was derived from Eq. 1

$$\eta = (\kappa \cdot I)^v$$

with the constants $v$ and $\kappa$ related to the constants in Eq. 1 through

$$v = \frac{1}{x}; \quad \kappa = 10^{-c}$$

Also, in Eq. 2 the measured $I$ was used, which is proportionally related to $\Phi$ but also depends on geometry, concentration, and incident light intensity. Empirical curve fitting yielded the constants $v = 1.18$ and $c = 0.20$.

![Graph](image-url)
\( \kappa = 7.2 \times 10^{-3} \) for the experiments with the high-viscosity range (Table 1, experiment A). For the experiments with the smaller viscosity increment (Table 1, experiment B), \( \nu = 0.77 \) and \( \kappa = 11.7 \times 10^{-3} \) were obtained for CCVJ and \( \nu = 0.78 \) and \( \kappa = 7.8 \times 10^{-3} \) for CCVJ-ME. The average deviation between the fluorescence-based results and measurements carried out by mechanical viscometry for the first set of experiments was 0.8% (range: 3.9 to 6.9%). For the second experiments at lower viscosity increments, the average deviation using CCVJ was 1.8% (range: 6.5 to 6.4%) compared with measured values. An average deviation of 1.5% (range: 7.2 to 4.8%) against measured values was found using CCVJ-ME. Correlation coefficients between mechanical and fluorescence-based viscosity values were \( r_1 = 0.987 \) for the first experiment (Table 1, experiment A), \( r_2 = 0.996 \) for the second experiment (Table 1, experiment B) with CCVJ, and \( r_3 = 0.998 \) for CCVJ-ME.

**DISCUSSION**

The principal finding of this study is that the viscosity-dependent fluorescence of molecular rotors can be used to measure blood plasma viscosity, thus providing a new method for viscosity measurement in a field where mechanical methods to measure viscosity (capillary viscometer, falling ball viscometer, rotational viscometer) have been used exclusively. All mechanical methods have in common that the fluid is subjected to shear forces, and the resistance of the fluid to these forces (internal friction) is measured. The internal friction of a fluid is proportional to the dynamic viscosity \( \eta \) and the velocity gradient (shear rate) between layers of different velocities.

Fluorescent molecular rotors, used as viscosity probes, rely on a different mechanism than the application of shear forces. They provide two modes of relaxation: fluorescence and nonradiative intramolecular rotation. The latter is dependent on the free volume of the environment, which in turn is related to its viscosity. Through the free-volume theory of viscosity, photophysical measurements using molecular rotors become independent from mechanical forces. The fluid under observation is not sheared during the measurement, and protein-surface interactions that may lead to artifacts at low shear rates do not affect the result. Further advantages over mechanical measurements include small sample volumes needed to perform fluorescence measurements (microcuvettes typically have a volume of 200–250 \( \mu l \)) and the high speed of the readout: with the use of fixed wavelength filters, intensity can be measured within fractions of a second.

Molecular rotors provide a measurement of viscosity for conditions at zero shear rate (zero flow); therefore, the viscosity data are applicable solely if the fluid has Newtonian behavior. It is generally accepted that plasma is a Newtonian fluid as indicated by the studies of Cokelet (3), Harkness (9), and Reinhart et al. (18),
Shear rates above 250 s\(^{-1}\) indicate that it exhibits shear thinning. Our own measurements show that plasma is Newtonian at shear rates above 250 s\(^{-1}\), the shear rate found in blood vessels under normal conditions. At lower shear rates our mechanically measured viscosity appears to increase slightly, a behavior that is qualitatively similar for plasma and mixtures of plasma and colloidal plasma expanders and that is probably due to the added force needed to deform the protein layer at the air-liquid interface.

The viscosity measurements derived from molecular rotors obtained with plasma samples at different concentrations and mixtures with colloids to obtain different viscosities are linearly related to the viscosity measured in a conventional mechanical viscometer operated in the range of 450 s\(^{-1}\). Molecular rotors show the same difference in viscosity between different plasma samples as that shown by a mechanical viscometer used in the Newtonian range of plasma viscosity. Therefore, in principle for the samples used in this study the viscosity at zero shear stress is quantitatively and linearly related to the viscosity in the Newtonian regime for plasma. Consequently, measurements with the rotors are representative of conventional measurements, when the rotors are calibrated against a sample that can be measured by both mechanical and fluorescent methods.

The mathematical relationship between viscosity and quantum yield (thus, under constant excitation conditions, between viscosity and measured emission intensity) has been established experimentally and theoretically. The precision within the experiments of this study was similar to that of mechanical viscosity measurements under routine conditions. Scatter between similar experiments was of the order of 1–2%, and precision was never worse than 7.5%. These small deviations between fluorescence and mechanically based viscosity data suggest that the method is suitable for most applications. There are problems, however, that influence precision, mainly temperature, fluid turbidity and dye concentration. Viscosity and the intrinsic relaxation rate of the dye (which is one determinant of the quantum yield) are temperature-dependent functions; therefore, temperature control is more important than for mechanical measurements. Ideally, the sample cuvette should be temperature controlled, which is feasible due to the low amounts of volume used. Fluid turbidity, common in blood plasma samples, strongly affects fluorescence through absorption of excitation and emission light. Optimized cuvette geometries, such as a narrow rectangular cross section and offsetting the angle of excitation light, may solve this problem. Dye concentration linearly affects emission intensity; therefore, the precision of the measurement depends on the precision of dye delivery. This was the main reason to use prestained plasma in this study, because aliquots of 1 ml are easier to produce than aliquots of 10 \(\mu\)l or less. A solution would be to measure absorption simultaneously to emission. Because absorption is not a function of viscosity, it should be dependent only on dye concentration and therefore provide a measurement standard. Another approach would be the simultaneous calibration with samples of known viscosity.

In summary, this study shows that fluorescent molecular rotors allow the measurement of biofluid viscosity through different means than through shearing of the fluid and that the measurement results are comparable to mechanical measurements in both precision and accuracy.

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