Molecular rotors—fluorescent biosensors for viscosity and flow

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Viscosity is a measure of the resistance of a fluid against gradients in flow (shear rate). Both flow and viscosity play an important role in all biological systems from the microscopic (*e.g.*, cellular) to the systemic level. Many methods to measure viscosity and flow have drawbacks, such as the tedious and time-consuming measurement process, expensive instrumentation, or the restriction to bulk sample sizes. Fluorescent environment-sensitive dyes are known to show high sensitivity and high spatial and temporal resolution. Molecular rotors are a group of fluorescent molecules that form twisted intramolecular charge transfer (TICT) states upon photoexcitation and therefore exhibit two competing deexcitation pathways: fluorescence emission and non-radiative deexcitation from the TICT state. Since TICT formation is viscosity-dependent, the emission intensity of molecular rotors depends on the solvent's viscosity. Furthermore, shear-stress dependency of the emission intensity was recently described. Although the photophysical processes are widely explored, the practical application of molecular rotors as sensors for viscosity and the fluid flow introduce additional challenges. Intensity-based measurements are influenced by fluid optical properties and dye concentration, and solvent–dye interaction requires calibration of the measurement system to a specific solvent. Ratiometric dyes and measurement systems help solve these challenges. In addition, the combination of molecular rotors with specific recognition groups allows them to target specific sites, for example the cell membrane or cytoplasm. Molecular rotors are therefore emerging as new biosensors for both bulk and local microviscosity, and for flow and fluid shear stress on a microscopic scale and with real-time response.

Introduction

In biological systems, viscosity plays an important role on microscopic (*e.g.*, cellular) levels up to systemic levels. On all levels, viscosity-related investigations strongly depend on the availability of methods that allow detection of viscosity changes on a microscopic scale and with very short response times.

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Fluorescence-based methods advertise themselves, because they meet the two demands of high spatial and temporal resolution.

Cellular biomechanics are primarily determined by the cell membrane, the cytoskeleton, and the cytoplasm. While the cytoskeleton can be imagined as a relatively rigid framework, both the cytoplasm and the cell membrane have viscoelastic properties that change in various states of disease. The cell membrane plays a particularly important role, since its viscosity influences the activity of membrane-bound proteins.[1,2] Consequently, changes in membrane viscosity have been linked with alterations in various physiological processes in the cell, particularly in conjunction with various disease states.[3–6] Examples include atherosclerosis,[7] cell malignancy,[2] hypercholesterolemia,[8] and diabetes.[9,10] Increased viscosity of red blood cell and platelet membranes has been observed in diabetic patients, and the viscosity change has been proposed to contribute to the reduced ability of the insulin receptor (a membrane-bound protein) to undergo aggregation.[9,10] On the other hand, decreased membrane viscosity in leukocytes of patients with Alzheimer's disease has been postulated to facilitate aggregation of the amyloid precursor protein (a transmembrane protein), a fragment of which is deposited in the brain as an insoluble plaque.[11] Patients with liver disorders, including alcoholism-based diseases, showed higher erythrocyte membrane viscosity, which correlates highly with the severity of liver dysfunction.[12] Moreover, increased membrane viscosity in leukocytes has been connected to the aging process.[13] These established cases constitute only a few examples of an enormous body of literature on this important topic that is the relationship between cell membrane viscosity and disease.

The effects of cytoplasmic viscosity are widely unexplored. This may be attributed to the relatively difficult measurement methods available. However, it has been shown that increased cytoplasmic viscosity impairs the development of oocytes[14] and that cellular activation of cardiomyocytes is associated with changes in the micro-viscosity of the cytoplasm protein matrix.[15] Measurements of microparticle motion suggest that cytoplasmic viscosity also plays a role in smoking and lung macrophage activation.[16,17] In the cytoplasm, magnetic microparticles were predominantly used to obtain information on viscoelastic properties.[18–20] Clearly, the use of magnetic microparticles demands the use of very expensive equipment. The observation of the particles is time-consuming, thus limiting temporal resolution, and the interaction between the particles and the cellular environment may also cause measurement artefacts. These limitations may be one of the main reasons why only limited studies on the effects of cytoplasmic viscosity are available.

On a non-microscopic (systemic) scale, the viscosity of blood and its constituents plays an important role. Blood viscosity is one determinant of blood flow. Information about the distribution of blood viscosity in the circulation is particularly relevant for the shear stress mechanotransduction to the endothelium, which regulates the shear-stress-dependent production of vasoactive mediators[21] such as prostacyclin and nitric oxide, and modulates apoptosis.[22] Also, plasma (more generally, extracellular fluid) viscosity regulates cellular and biochemical functions in red blood cells and liver cells, which are responsible for the lipoprotein metabolism.[23,24] In this context, information on plasma viscosity is a key element in understanding the magnitude of the stimulus to which both endothelium and red blood cells are exposed. Changes in plasma viscosity have been observed in conjunction with various diseases and are mostly associated with altered protein levels.[25–27] Examples include infections and infarction,[25] hypertension,[28] and diabetes.[29] Protein-induced hyperviscosity may lead to further complications,[26] such as elevated risk of atherosclerosis.[30] Furthermore, one of the adverse effects of smoking is elevated plasma viscosity,[31,32] which may be the link between cigarette consumption and cardiovascular disease. Non-pathologic conditions may also influence blood rheology, including increased plasma viscosity. Examples include bedrest (*e.g.* associated with prosthetic implants),[33] pregnancy,[34] and aging.[35] Viscosity changes in aging may also be attributed to indirect effects, such as age-related changes in habits (*e.g.*, increased smoking, lack of exercise).[36] It is debatable whether a direct cause-and-effect chain exists, as for example suggested by Ernst and Matrai,[32] or whether those changes are a secondary, merely accompanying effect. Irrespective of this debate, plasma viscosity has been proposed to be used as a diagnostic tool that allows early detection of diseases.[25]

Conventional methods to measure viscosity and flow

To date, conventional (*i.e.*, mechanical) methods to measure viscosity, such as the capillary viscometer, the falling ball viscometer, and the rotational viscometer have been used exclusively. Typically, fluid volumes between 1 ml and 5 ml are used, and the actual measurement process requires between 1 minute and 5 minutes. For the measurement of blood plasma, a specialized capillary viscometer (Harkness Viscometer) recommended by the International Committee for Standardization in Haematology[37] allows to measure sample sizes as low as 500 µl within one minute. 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 η and the velocity gradient (*i.e.*, the shear rate) between layers of different velocities. In all cases, the relatively high amount of sample fluid and the slow measurement process preclude real-time viscosity measurements in small samples or localized regions. Finally, mechanical viscometers are affected by proteins adhering to the surfaces of the instrument. This not only requires scrupulous cleaning between measurements, but may also introduce another source of error through protein deposition during the measurement process.

In all cases described in this section, measurement procedures would benefit from the rapid response time and high spatial resolution of fluorescent probes. In fact, fluorescence-based methods to estimate local viscosity have been introduced. The two most widespread methods are fluorescence anisotropy, a technique that employs polarized light for fluorescence excitation, and fluorescence recovery after photobleaching (FRAP). Typical anisotropy fluorophores, such as DPH (1,6-diphenyl-1,3,5-hexatriene), can only be excited by light waves parallel to the excitation dipole of the molecule. Emission occurs again in the plane of the dipole. During the excited lifetime, the molecule may rotate, changing the polarization plane. This rotation depends on the viscosity of the medium. Consequently, the approximate microviscosity of the membrane can be determined by fluorescence measurements using polarized light.[38] FRAP is based on fluorophore diffusivity in a two-dimensional layer such as the cell membrane. When a small spot of the cell membrane is photobleached by a strong,

focused laser pulse, diffusion leads to an exponential recovery of the fluorescence intensity. A model has been provided to derive local microviscosity from the diffusion constant.[39–41] Besides the necessity of specialized equipment (*i.e.*, a two-laser confocal microscope with fast shutters) and the challenging nature of the measurement process, limitations in time-resolution is another important disadvantage: in one representative report, a minimum time increment of 1.7 s is given.[40] A small spot diameter leads to poorer recovery measurement; therefore, the size of the bleaching spot limits the spatial resolution. Finally, the bleaching pulse introduces energies up to 1 MW cm^{-2}, which are sufficient to generate free radicals by photolysis and local heating with consequent protein damage.[42] Particularly, heat-related protein crosslinking reduces dye diffusivity, leading to artifactually increased viscosity readings.

The fluid velocity of flowing fluids is usually inhomogeneous. The spatial gradient of the velocity is called shear rate. The product of shear rate and viscosity yields shear stress, which is the primary determinant of the forces related to the flow. Apart from numerical computations, fluid flow is primarily measured noninvasively using Doppler ultrasound, laser Doppler techniques, or magnetic resonance imaging.[43] A popular alternative is particle tracking.[44] All of these methods are limited spatially because of the relatively low image resolution and the small particle size. Also, these methods measure fluid flow, but not fluid shear stress.

These considerations have led to the exploration and development of a special group of fluorescent viscosity-sensitive probes, referred to as molecular rotors, which are characterized by their ability to twist around a single bond. This twisting motion is influenced by the viscosity of the environment and, in turn, influences fluorescence emission.

Photophysical principles of molecular rotors

Molecular rotors are a special subgroup of TICT fluorophores where deexcitation from the TICT state occurs nonradiatively, that is, without photon emission.[45] Most commonly, molecular rotors are derived from the group of *p*-(dialkylamino)-benzylidenemalonitriles. Fig. 1 shows the chemical structure of two of the most commonly used molecular rotors, 9-(dicyanovinyl)-julolidine (DCVJ) and 9-(2-carboxy-2-cyano)vinyl julolidine (CCVJ). TICT formation takes place by photoinduced electron transfer from the julolidine nitrogen to one of the nitrile groups with subsequent intramolecular rotation around the julolidine–vinyl bond. The physical principle, which leads to viscosity-sensitivity, is steric hindrance of intramolecular rotation governed by the molecular-free volume of the solvent. For this reason, the fluid is not sheared, and no mechanical movement of the fluid takes place. The intramolecular rotation is a function of the free volume of the microenvironment of the probe. If intramolecular rotation is inhibited by high viscosity (low free volume) of the microenvironment, the balance of relaxation shifts towards higher radiative relaxation rates. In other words, fluorescence intensity increases with increased viscosity of the solvent.

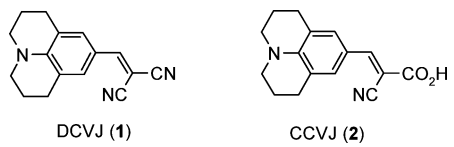


Fig. 1 Structure of two commercially available molecular rotors, 9-(dicyanovinyl)-julolidine (DCVJ, **1**) and 9-(2-carboxy-2-cyano)vinyl julolidine (CCVJ, **2**). The intramolecular charge transfer takes place between the nitrogen (left) as electron donor and the nitrile group (right).

Twisted intramolecular charge-transfer complexes, that is, fluorescent molecules that undergo a twisting motion in the excited state, have been investigated for more than 40 years.[46] The TICT hypothesis, which is still valid today, was first formulated by Z. R. Grabowsky in 1973.[47] Förster and Hoffmann examined some triphenylamine dyes with TICT characteristics and determined the power-law relationship between quantum yield and viscosity both analytically and experimentally.[48] Several recent studies show the interesting properties of molecular rotors in biological studies. For example, molecular rotors used as probes in polymerizing environments exhibit an increased fluorescence intensity as the polymerization progresses.[49] In biological systems, this principle was applied to examine the assembly processes of tubulin[50] or for the G–F transformation of actin.[51,52] In phospholipid bilayers, molecular rotors have been used to probe the transition from the gel to the liquid-crystal phase[53] and as a general microviscosity probe.[54] Interestingly, unlike many environment-sensitive probes, molecular rotors can be used independent of the polarity of the environment: emission intensity is influenced by the viscosity, whereas polarity changes are reflected in a small shift of the peak emission wavelength.[55,56] In contrast to other probes that have been used to relate fluorescence properties to cell membrane viscosity, such as LAURDAN,[57,58] PRODAN[59] or n-AS,[60] molecular rotors directly report microviscosity.

A strict mathematical relationship between viscosity η and quantum yield Φ exists, known as the Förster–Hoffmann-Equation (eqn (1)),

$$\log \Phi = C + x \log \eta \quad (1)$$

where C is a temperature-dependent constant[49] and x is a dye-dependent constant. This relationship has been derived analytically[48,49,54] and verified experimentally.[48,61] To illustrate this relationship, typical excitation and emission spectra for DCVJ in mixtures of ethylene glycol and glycerol are shown in Fig. 2. A higher glycerol content corresponds to a higher viscosity of the solvent. This leads to an increased emission intensity.

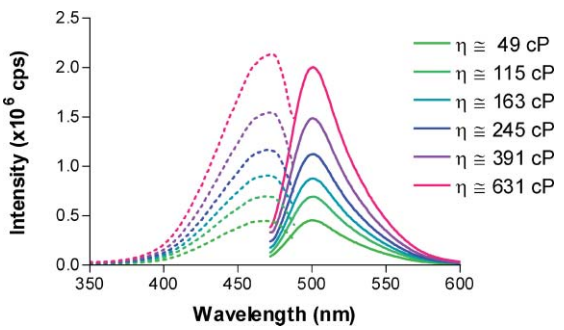


Fig. 2 Excitation (dashed lines) and emission (solid lines) spectra of DCVJ in different mixtures of ethylene glycol and glycerol. As viscosity increases with increased glycerol content, the fluorophore's quantum yield and, with it, the emission intensity increases.

If the absorbed light intensity I_{ab} is known, the emission intensity I_{em} and the quantum yield are directly and proportionally related through $I_{em} \propto I_{ab}\Phi$[62] For practical purposes, the dye concentration needs to be considered. At low concentrations, associated with negligible inner-filter effects, the dye concentration linearly increases I_{em}. The effective determination of C is possible, albeit impractical. However, relative changes in the viscosity of the environment can directly be determined by measuring two emission intensities I_1 and I_2 corresponding to the different viscosities η_1 and η_2. Under the assumption of constant temperature, constant absorption and negligible background light, eqn (2) can be obtained, eliminating the constant C.[63]

$$\frac{\eta_1}{\eta_2} = \left(\frac{\Phi_1}{\Phi_2}\right)^{\frac{1}{x}} = \left(\frac{I_1}{I_2}\right)^{\frac{1}{x}} \quad (2)$$

One intensity could be gained from a solvent with known viscosity. In that case, the only remaining unknown would be the second viscosity η_2, and eqn (2) can be solved for η_2. This approach corresponds to instrument calibration with known fluids.

Generalized structure and chemical modification

Since charge transfer takes place between the nitrogen electron donor and the nitrile electron acceptor, chemical modifications are possible that do not change the photophysical behavior but allow the attachment of different targeting or recognition elements. Fig. 3 shows the generic structure of a molecular rotor that can be thought of as the core (**3**), and two examples of modifications. **4** is a water-soluble alcohol with a monocyclic structure, whereas **5** is a triethyleneglycol ester with a tricyclic structure. In our experience the use of a monocyclic structure *versus* a tricyclic structure containing the electron-donating nitrogen does not change the viscosity-sensitive behavior, *i.e.* the adherence to eqn (1). Furthermore, the addition of various chains, alcohols, or other functional groups does not affect the viscosity-sensitive behavior.

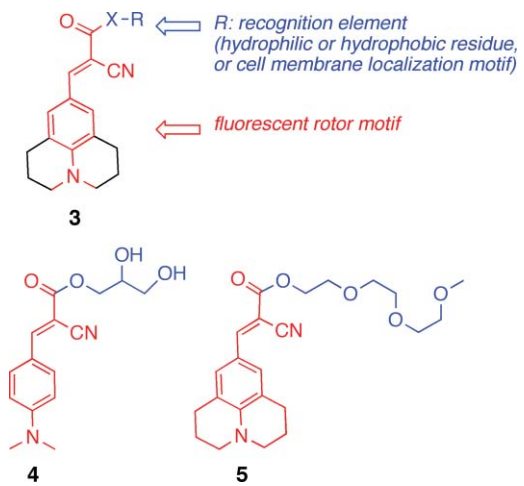


Fig. 3 Generic structure of a molecular rotor (**3**) and two hydrophilic derivatives (alcohol **4** and triethyleneglycol ester **5**).

Molecular rotors as viscosity sensors

The main advantages of fluorescence-based viscometry over mechanical measurements include small sample volumes needed to perform fluorescence measurements (microcuvettes typically have a volume of 100–250 µl) and the high speed of the readout. By using fixed wavelength filters, intensity can be measured within fractions of a second. In addition, molecular rotors allow the measurement of local microviscosity changes with the aid of epifluorescent microscopes.

However, the use of molecular rotors is associated with some challenges that are shared with most quantitative fluorescent methods. Although lifetime systems allow a good estimation of a fluorophore's quantum yield, lifetime fluorophotometers for lifetimes below 1 ns are not commonly used and are very expensive. It is therefore more practical to measure steady-state fluorescence. This method imposes the restriction to measure relative viscosity changes, because quantum yield is only one of several factors influencing steady-state emission intensity. The solvent may absorb excitation or emission light, or light may be scattered by small particles in turbid fluids. However, by measuring fluid absorption and scattering, these factors can be accounted for.[64] In addition, it is possible to account for dye concentration by measuring absorbance of the fluid at the dye's excitation wavelength (Fig. 4).[64,65]

$$\eta = \xi \cdot I^{\kappa}_{Em} \quad (3)$$

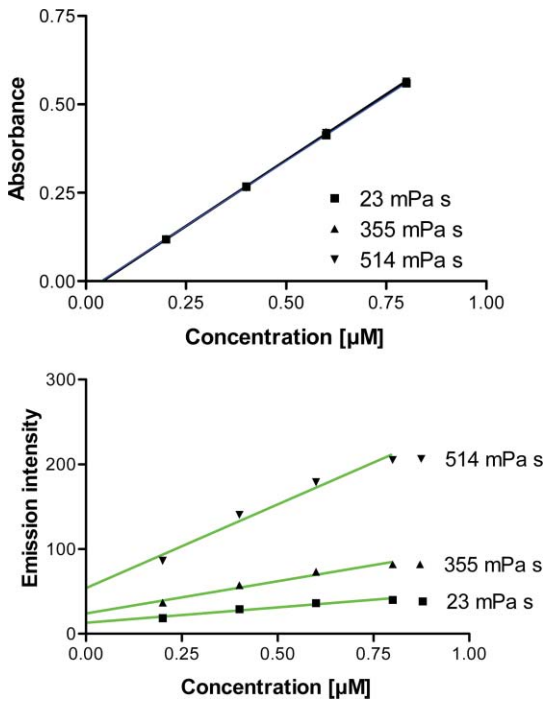


Fig. 4 Absorbance and emission intensity of the molecular rotor DCVJ in mixtures of ethylene glycol and glycerol as a function of dye concentration. While emission intensity increases both with viscosity and dye concentration, absorbance is dependent on the dye concentration alone and does not change with viscosity.

The emission intensity of a molecular rotor depends on both the concentration of the dye as well as the viscosity of the solvent (Fig. 4). Absorbance, however, is viscosity-independent and is only influenced by the dye concentration. By applying corrections for absorption, scattering, and dye concentration, it is possible to compute viscosity from emission intensity through eqn (3).

In eqn (3), I_{em} is the measured and corrected emission intensity, κ is the reciprocal of x in eqn (1), and ξ is a proportionality constant that depends on the above correction factors as well as on the instrument itself (such as excitation intensity, emission collection efficiency, amplifiers *etc.*). ξ needs to be determined by calibration with fluids of known viscosity.[65]

Molecular rotors, dissolved in fluids of different viscosities, exhibit a viscosity-dependent emission intensity. Moreover, the relationship between intensity and viscosity is that of a power-law relationship. In other words, the data points of intensity over viscosity, drawn in a double logarithmic scale, would lie on a straight line with the slope x (eqn (1)), as demonstrated in Fig. 5 in mixtures of ethylene glycol and glycerol.[61,66] It can be demonstrated that the power-law relationship holds true over more than three decades of viscosity.

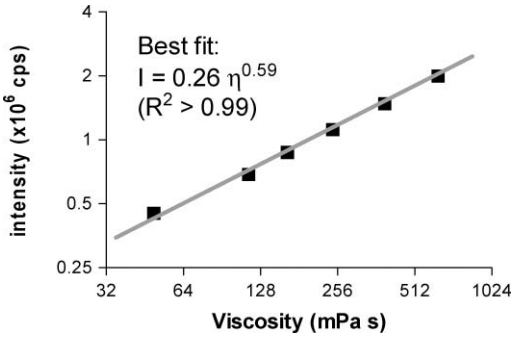


Fig. 5 Emission intensity of DCVJ in mixtures of ethylene glycol and glycerol (emission maxima from Fig. 2) drawn over the viscosity of the mixtures in a double-logarithmic scale. The data points lie on a straight line with slope 0.59.

Furthermore, a hydrophilic molecular rotor, 9-[(2-cyano-2-hydroxy carbonyl) vinyl]julolidine (CCVJ, Fig. 1) has been shown to change the emission intensity in aqueous colloid solutions, solutions of dextran, hetastarch, and pentastarch.[67] Therefore, molecular rotors can be used in fluids that contain viscosity-modulating macromolecules, such as starch molecules, which are several orders of magnitude larger than the molecular rotors themselves. In a similar way, hydrophilic molecular rotors can serve as viscosity probes in blood plasma and plasma expanders.[65,68] In a clinical setting, high-viscosity plasma expanders, such as Pentaspan (10% pentastarch in saline), are frequently prescribed to avoid blood hypoviscosity. In one study, we subjected mixtures of blood plasma and pentastarch solution to the fluorescent analysis. The emission intensity values of repeated experiments were very tightly grouped, leading to significant intensity differences between fluids that differed by only 0.3 mPa s.[68] In fact, there was no overlap between intensity values of any of the test fluids of different viscosity. This observation indicates that molecular rotors allow a measurement precision of 7% or better—a value comparable to conventional mechanical instruments. In a different study, we compared precision of fluorescent measurements to the measurement precision of a Brookfield DV-III+ computerized low-viscosity cone-and-plate rheometer.[65] Intersample variations were markedly higher when the samples were measured with a conventional cone-and-plate viscometer than when measured using fluorescence (Fig. 6). We found that backcalculating viscosity from fluorescence emission by using eqn (3) yielded an accuracy of about 5%.[65]

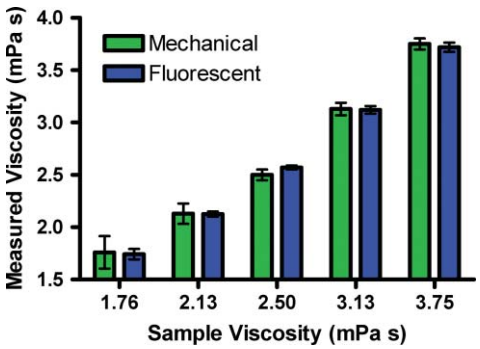


Fig. 6 Precision comparison between a mechanical cone-and-plate viscometer and viscosity calculated from fluorescent intensity measurements (mean and standard deviation for $n = 5$ independent experiments). Smaller standard deviation indicates a higher precision of fluorescent measurements than conventional mechanical measurements.

It can be concluded that measuring viscosity in bulk fluids by using molecular rotors in solution is possible. Once calibrated, the fluorescence-based measurement process using molecular rotors can reach the precision and accuracy comparable with, or better than, conventional mechanical instruments.

One major advantage of fluorescent probes in general is their high spatial resolution and instantaneous response to their environment. These properties also apply to molecular rotors. In epifluorescent or confocal microscopes, changes of local micro-viscosity can be determined quantitatively by using molecular rotors. DCVJ is hydrophobic; therefore, DCVJ integrates into phospholipid bilayers and the cell membrane. DCVJ and related probes have been used to probe temperature-dependent viscosity changes and the phase transition in vesicles.[53] In a microscopic study, DCVJ was brought into the membrane of adherent confluent endothelial cells, where the fluorescence emission was used to report membrane viscosity changes under the influence of fluid shear stress.[66] We found that emission intensity decreases when the cells are exposed to shear stress (Fig. 7). Although the intensity change is statistically significant at shear stress levels as low as 0.6 Pa, the relative change is still surprisingly low. The reason for this behavior lies in the propensity of the hydrophobic DCVJ to bind to proteins.[69] Therefore, DCVJ is able to cross the cell membrane and migrate into the cell, where it causes fluorescence emission from the cytoplasmic area as well as from organelle membranes and nucleoles.[70] This cytoplasmic DCVJ is not exposed to viscosity changes in the cell membrane and therefore reduces the relative intensity change and the sensitivity simultaneously.

To increase sensitivity, we developed by chemical synthesis molecular rotors that integrate into the membrane as specific as possible. The basic reaction is based on CCVJ, where the carboxylic acid can be used to covalently attach long-chained alcohols through an ester bond. Among several hydrophobic derivatives that we tested, a CCVJ farnesyl ester (FCVJ, Fig. 8) proved most successful.[70] Farnesyl consists of three isoprenyl groups, and isoprenyl chains are known to aid localizing membrane-bound proteins.[71,72] By merit of the farnesyl group, FCVJ showed dramatically improved localization in the outer cell membrane and an about 20-fold increase of the relative emission intensity change in cells exposed to fluid shear stress.[70]

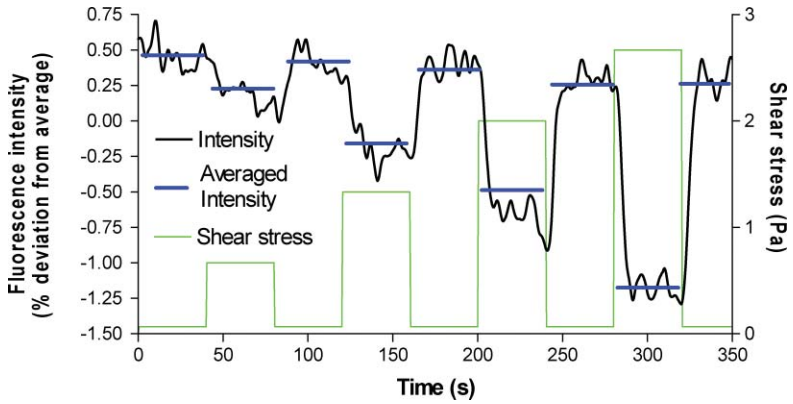


Fig. 7 Changes of membrane-bound DCVJ emission intensity in a confluent layer of endothelial cells exposed to fluid shear stress. A small, but statistically significant, decrease can be observed whenever the cells are exposed to shear stress, and the intensity drop is more pronounced at higher shear stress.

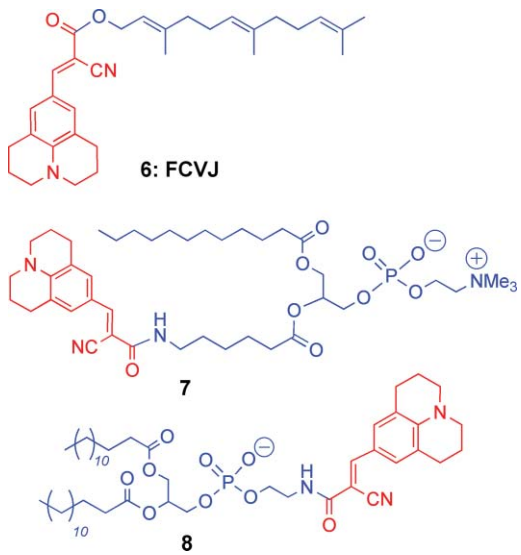


Fig. 8 Membrane-compatible probes CCVJ-farnesol ester (FCVJ, **6**) and the phospholipid-bound probes **7** and **8**.

Pursuing the same idea, we synthesized phospholipids with a molecular rotor moiety attached either to the head group or to one of its hydrophobic tails (Fig. 8).[73] We were able to demonstrate excellent membrane localization in both vesicles and adherent cells. The specific molecular rotor, covalently bound to the head group of a phospholipid (**8**), was markedly less sensitive to viscosity changes than the other phospholipid-based molecular rotors (generic structure **7**), and it showed no intensity change in vesicles where viscosity was influenced by temperature. In vesicles and artificial bilayers, those phospholipid-rotor molecules where the rotor was attached to one of the hydrophobic tails (*e.g.* compound **7**) showed excellent sensing performance. However, in cultured cells some of the compounds exhibited a certain cytotoxicity, most likely because they changed the membrane's phospholipid composition.

Ratiometric dye systems

All molecular rotor dyes presented above suffer from the possible factors that can influence the measured emission intensity. While fluorescence-based measurements of bulk viscosity can be corrected by compensating for fluid absorption and dye concentration, a similar correction is much more difficult in the cell. Local dye concentrations in the cell are unknown and restrict measurements to relative viscosity changes according to eqn (2). We therefore conceived a dye where one molecular rotor is covalently coupled to a viscosity-insensitive reference dye. The desired ratiometric dye was designed to follow several specific criteria. First, a molecular rotor and a second fluorescent unit that is not viscosity-sensitive were to be covalently coupled. The second fluorescent moiety would serve as a reference and calibration intensity dye. Second, the two fluorescent units were to form a resonance energy transfer pair; therefore, a rigid linker would keep the units at a distance approximately equal to the Förster distance. Third, spectral overlap would have to be chosen so that the reference moiety acts as the resonance energy transfer donor. The design principle is shown in Fig. 9.

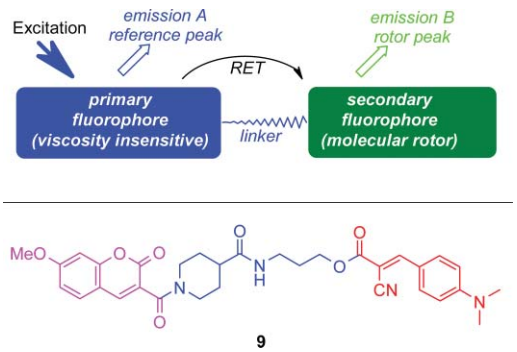
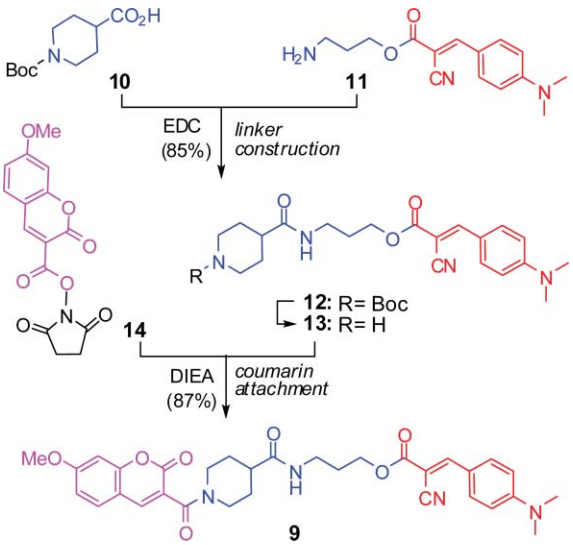


Fig. 9 Design principle and reduction to practice of a ratiometric dye system composed of a viscosity-insensitive coumarin RET donor and a molecular rotor as RET acceptor.

We recently presented[74] the first ratiometric dye system with coumarin as a reference fluorescent moiety (Fig. 9, compound **9**). Scheme 1 shows the basic synthesis pathway to obtain the ratiometric dye **9**, and detailed instructions for the synthesis of compound **9** can be found in ref. 75. The emission spectra of the coumarin-ratiometric dye when excited at 360 nm (coumarin excitation) in fluids of varying viscosity is shown in Fig. 10. It can clearly be seen that coumarin emission is viscosity-independent



Scheme 1 Synthesis of ratiometric probe **9**.

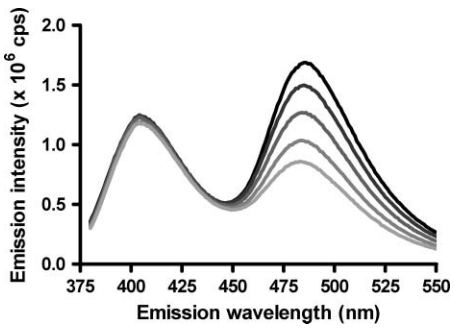


Fig. 10 Emission spectra of the ratiometric dye **9** excited at 360 nm. Shown are spectra in different mixtures of ethylene glycol with 40% to 80% v/v glycerol to modulate viscosity. RET takes place from the coumarin donor to the rotor acceptor. The rotor emission at 480 nm shows strong viscosity sensitivity whereas the reference emission at 402 nm remains viscosity-insensitive.

and can therefore be used as a reference. The rotor molecule is excited through resonance energy transfer, and its emission intensity was significantly increased with increased viscosity. The constant x in eqn (1), an indicator of the dye's sensitivity, was found to be $x = 0.55$ ($R2 = 0.995$, $P < 0.0001$). This value is very close to the known value of $x = 0.6$ for generic non-ratiometric molecular rotors such as DCVJ and CCVJ (Fig. 5). To further demonstrate the ratiometric principle, the viscosity series from Fig. 10 were repeated with different dye concentrations. The peak emission intensity is a function of fluid viscosity (determined by different mixture ratios of ethylene glycol and glycerol) where the dye concentration has been intentionally varied between samples (Fig. 11). Blue bars show the rotor emission which strongly depends both on viscosity and dye concentration, while the ratiometrically corrected intensity (green bars) indicates a dramatic reduction of the influence of dye concentration in spite of a very high dye concentration variations of a factor of eight.

The ratiometric dye will be particularly advantageous in environments where local concentration may vary, such as the cell membrane. Once again, the design of membrane and cytoplasmic compatible ratiometric dyes is still under investigation. We expect

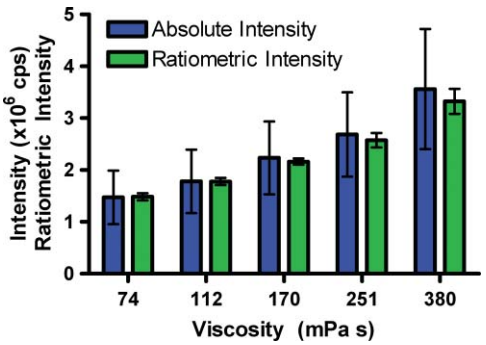


Fig. 11 Demonstration of the ratiometric principle to compensate for major variations in dye concentration. Blue bars show the peak emission of the rotor moiety in mixtures of ethylene glycol and glycerol at dye concentrations ranging from 2.5 μM to 20 μM ($n = 5$, shown: mean intensity and SD). Since peak intensity strongly depends on dye concentration, a huge variation of the intensity values can be observed. Green bars show peak rotor emission divided by peak coumarin emission for the same samples. Variation between the samples is dramatically reduced in spite of the high range of concentrations used.

that suitable ratiometric dyes will be an advantageous tool for the investigation of the role of viscosity (membrane and cytoplasm) in physiologic processes of the cell. On the bulk scale, the ratiometric molecular rotor will provide an important tool to investigate blood plasma hyper- or hypoviscosity and its relation to disease. Although it is unknown where the established molecular rotor DVCJ locates in the cell membrane, it can be speculated that it is localized closely to the phospholipid head groups, similar to DPH,[60] because it has been shown that the membrane dielectric constant can be probed by the peak wavelength shift of a molecular rotor.[54] Protein binding properties and membrane localization of the ratiometric dye systems are unknown at this time. It is conceivable, though, that ratiometric systems with low protein affinity can be found, similar to non-ratiometric molecular rotors with low protein affinity.[69] Probes of this type, likely characterized by multiple -OH or -COOH groups, would be excellently suited for cytoplasmic viscosity measurement. On the other hand, ratiometric dye systems attached to lipophilic chains (*e.g.* isoprenyl chains) will be preferably localized in the cell membrane. Local concentrations in protein-rich areas need yet to be examined.

Molecular rotors as flow sensors

Hydrophilic molecular rotors, that is, those molecular rotors that contain polar groups (carboxylic acid, alcohol, or *n*-ethylene glycol) exhibit an increase of the emission intensity in polar fluids (*e.g.*, water, alcohols) under shear stress.[76] Other dyes, such as fluorescein (which is not a molecular rotor) or DCVJ (which does not have a polar functional group) do not exhibit this behavior. In a simple experiment, a 10 μM solution of CCVJ in ethylene glycol was stirred by using a magnetic stir bar (Fig. 12). Under moderate stirring, a 20% increase of emission intensity was observed. This increase was not accompanied by any wavelength shift.

In a more complex system where flow could be controlled more accurately with a syringe pump, we determined that the intensity increase depends on both viscosity and fluid velocity in a nonlinear fashion. Particularly, a saturation effect exists where a further increase of flow does not measurably increase emission

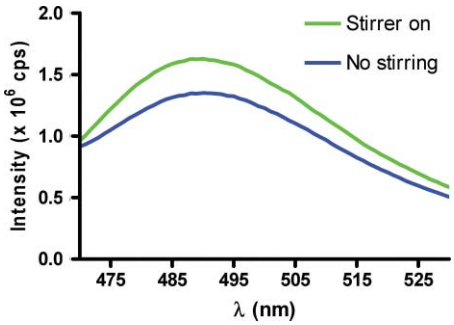


Fig. 12 Basic experiment to demonstrate shear sensitivity of molecular rotors. In a fluoroscopic cuvette, a solution of CCVJ in ethylene glycol is either stirred or at rest. Stirring causes an approximately 20% intensity increase, but no emission peak shift.

intensity (Fig. 13). This saturation point depends on the fluid used. Presently, no theory exists that could explain why molecular rotors would exhibit such a behavior. An empirical curve fit has yielded eqn (4),[76]

$$\Delta I = \Delta I_{\max} \cdot \left(1 - \exp\left(-\frac{\eta}{\eta_c}\right)\right) \cdot \left(1 - \exp\left(-\frac{v}{v_c}\right)\right) \qquad (4)$$

where the intensity increase ΔI follows both fluid velocity v (with a characteristic constant velocity v_c) and viscosity η (with a constant characteristic viscosity η_c) in an exponential-association fashion with very high correlation.

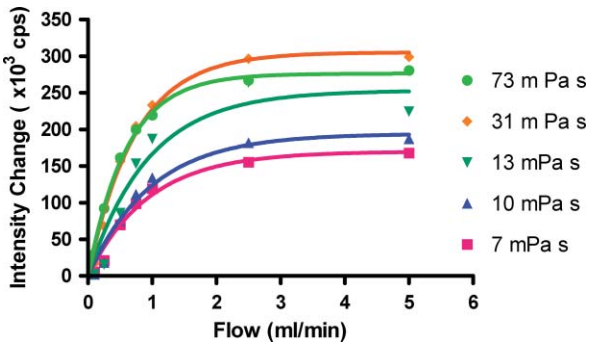


Fig. 13 Intensity increase of CCVJ in ethylene glycol in dependency of flow rate and viscosity. High flow rates as well as high-viscosity fluids lead to a saturation where increased flow does not further increase emission intensity.

Molecular rotors are extraordinarily sensitive flow sensors with the ability to sense fluid velocities of 0.5 mm s^{-1} and less. Only very expensive instrumentation can match this sensitivity. Furthermore, molecular rotors, as discussed above, have high spatial and temporal resolution. It is therefore possible to observe flow patterns in flow chambers or other fluidic systems (examples in Fig. 14 and 15). In circular tubes, Poiseuille flow follows a parabolic velocity profile where the flow is fastest in the center of the tube and slowest near the walls. The emission intensity, acquired with an optical fiber, followed closely the expected parabolic profile (Fig. 14). The flow chamber (Fig. 15) exhibits a high fluid velocity in the narrow channel at the top. The flow velocity decreases as the fluid enters the wider basin. Consequently, the area of highest fluorescence intensity is the narrow entrance channel. In the basin itself, the intensity in some areas is not

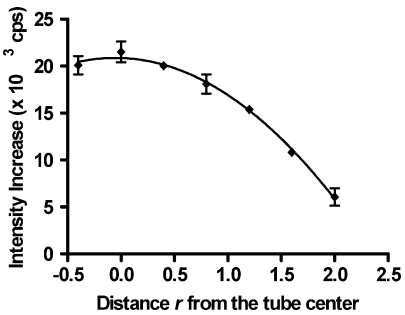


Fig. 14 Parabolic fluid velocity profile acquired by positioning a fiberoptic tip at different radial positions in a 6 mm diameter glass tube (mean ± SD, $n = 3$).

elevated above background fluorescence (top right). In these areas, the flow is stagnant. Since the spatial and temporal resolution of a flow sensing system using molecular rotor fluorescence is limited by only the image acquisition apparatus, microscopic images of very high resolution are possible to obtain.

Conclusion

In conclusion, molecular rotors are a special subgroup of TICT-forming fluorescent dyes that are characterized by nonradiative decay from the TICT state. Therefore, a high rate of TICT formation will lead to a low quantum yield and low emission intensity. Since the TICT formation rate is determined by the solvent's viscosity, molecular rotors are fluorescent probes for viscosity with real-time response and high spatial resolution. Chemical synthesis allows the modification of molecular rotors to target specific sites, such as the cell membrane or aqueous partitions. A rigid mathematical relationship (eqn (1)) between viscosity and intensity exists that allows backcalculating the viscosity from emission intensity, provided that the calibration fluids are known. This relationship holds over three orders of magnitude. Therefore, fluorescence-based viscosity measurement is faster and more precise than conventional, mechanical viscometry. In addition, some molecular rotors can be used to probe flow with similarly high resolution and real-time response time. The sensitivity of molecular rotors as flow sensors meets or exceeds any mechanical instrument. Future work will include several thrust areas. Viscosity sensitivity may be exploited in two ways: with the help of a specialized instrument, rapid bulk viscosity measurement can be performed, ideally based on disposable sets. Such an instrument needs the ability to compensate for fluid optical properties,[64] but it would allow rheological tests to be performed with an accuracy comparable to mechanical instruments, but with higher precision and dramatically higher speed. For cell signaling and drug studies on the cellular level, emphasis will be put on the development of membrane- and cytoplasm-compatible ratiometric probes. Lastly, exploitation of the flow-sensitive behavior requires the determination of the exact underlying mechanism at the molecular level. From this point, instruments for flow measurement or feedback flow control could be developed. In summary, the development of molecular rotors promises a new approach to measuring viscosity in large series (*e.g.* in a clinical setting) or on microscopic scales, and it may well provide an emerging new tool in basic science and diagnostics.

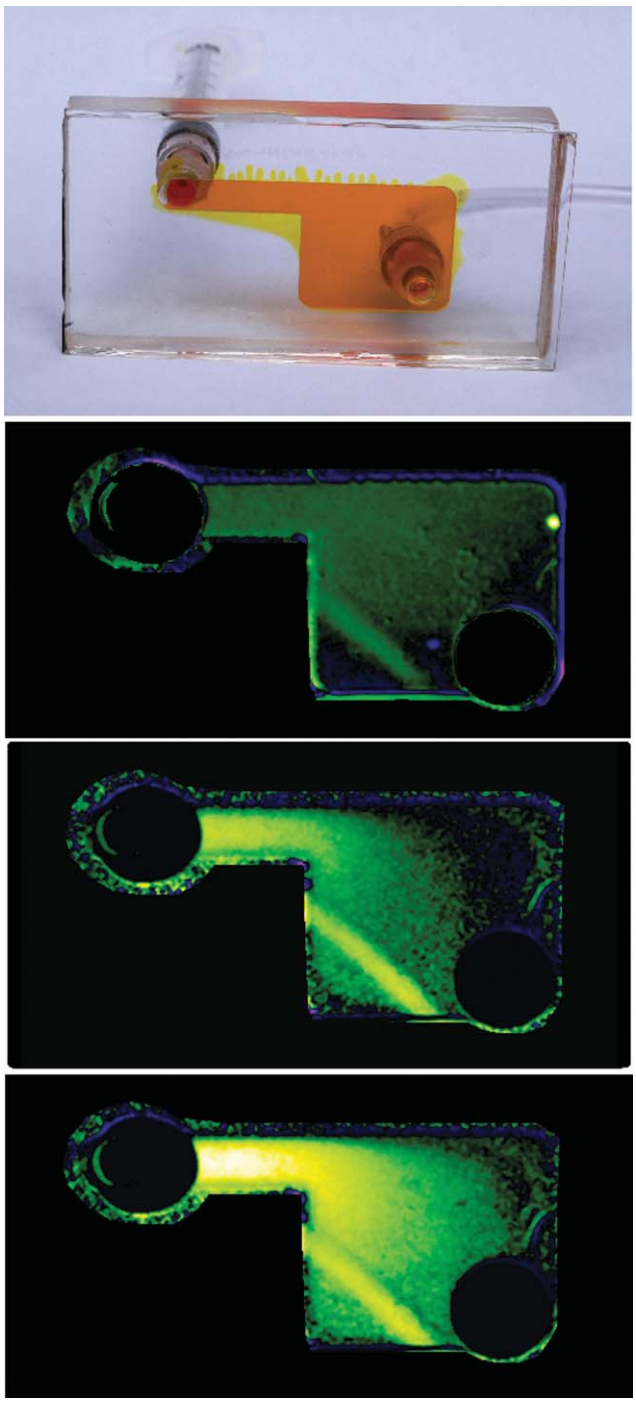


Fig. 15 Fluorescence patterns in a flow chamber where flow enters a wide basin from a narrow channel (top). The photos below show the fluorescence emission intensity, corrected for no-flow intensity (difference image) for flow rates of 250 l min^{-1}, 500 l min^{-1}, and 1000 l min^{-1} (top to bottom). The relationship between fluid velocity and intensity can clearly be seen.

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