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COMMUNICATIONS

Published on Web 12/16/2005

A Ratiometric Fluorescent Viscosity Sensor

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Variations in fluid viscosity are linked to a variety of functions and diseases both at the cellular level (e.g., membrane and cytoplasmic viscosity changes in cell signaling modulation)[1] and at the organismal level (e.g., blood, plasma, or lymphatic fluid viscosity changes in diabetes, hypertension, infarction, and aging).[2] It has been proposed that monitoring of biofluid viscosity could provide a diagnostic tool for the detection of diseases.[3]

Since mechanical devices do not provide the spatial and temporal resolution needed, a new type of fluorescent-based viscosity sensors was developed.[4] These sensors are based on a class of environment-sensitive fluorescent dyes that are characterized by a viscosity-dependent emission quantum yield.[4,5] The chemical structure of these dyes contains an electron donor unit (such as a nitrogen atom) in conjugation with an electron acceptor unit (such as a nitrile). Upon photoexcitation, the two units can rotate relative to each other in a manner that is dependent on the viscosity of their environment. Representative examples of such fluorescent rotors are 9-(*dic*y-ano*v*inyl)julolidine (DCVJ, **1**) and 2-*c*yano-3-(4-di*m*ethylaminophe-nyl)*a*crylic acid *m*ethyl ester (CMAM, **2**) (Figure 1).[5] Their viscosity-dependent fluorescent quantum yield is described by the Förster−Hoffmann equation (eq 1).[6]

$$\log I = C + x\log\eta \qquad (1)$$

I = fluorescent emission intensity
η = solvent viscosity
x = dye-dependent constant
C = conc. and temp. constant

Fluorescent molecular rotors have been used for viscosity studies that are performed by steady-state fluorescence through emission intensity measurements. This method suffers, however, from drawbacks arising from changes of the fluid optical properties and fluctuations in dye concentrations. An additional disadvantage is that a calibration curve is needed for the absolute determination of viscosity.[5] As a consequence, changes in fluid properties and dye concentration may cause erroneous readings.

We hypothesized that a dual dye composed of two distinct fluorescent units, one providing an internal intensity reference and the other acting as a viscosity sensor, would create a ratiometric sensing system, thus overcoming the above disadvantages. Dividing the sensor emission intensity by the reference emission intensity would yield a normalized intensity that should not only eliminate some of the fluid- and concentration-related artifacts but also provide a means to quantify viscosity by an internal reference. To test this hypothesis, we synthesized a compound **4** in which the CMAM motif was coupled with 7-*m*ethoxy*c*oumarin-3-*c*arboxylic *a*cid (MCCA, **3**). We chose MCCA as the donor fluorophore in order to induce excitation of the rotor moiety (CMAM) via Resonance Energy Transfer (RET).[7] We envisioned that, due to its viscosity-independent quantum yield, MCCA could be used as both the internal reference and the RET donor. The latter event could then excite the CMAM motif, resulting in a viscosity-dependent emission of the rotor. The linker was chosen to maintain a distance between the chromophores in the same range as the Förster distance[7a] to allow considerable energy transfer to the acceptor combined with measurable donor emission.

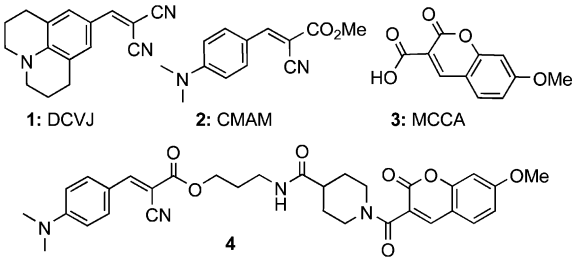
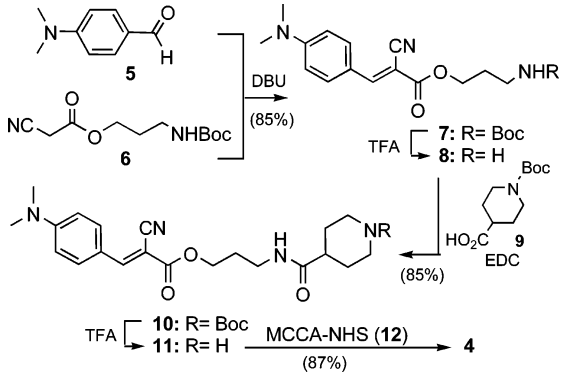


Figure 1. Structures of fluorescent dyes.

Scheme 1. Synthesis of Fluorescent Dye **4**



The synthesis of compound **4** is depicted in Scheme 1. Condensation of aldehyde **5** with β-cyanoester **6** produced compound **7** (85%). Deprotection of the primary amine of **7** followed by coupling with *N*-Boc-isonipecotic acid (**9**) afforded amide **10** (85%). Removal of the Boc group of **10** and coupling of the resulting amine with the succinimidyl ester of MCCA (**12**) formed compound **4**, a red water-insoluble oil, in 87% yield.

Figure 2 shows the fluorescent fingerprint[8] of dye **4** in ethylene glycol. Peak A corresponds to the fluorescence excitation and emission of MCCA. RET between the two fluorophores elicits peak B, while photon-excited emission from the CMAM moiety results in peak C. Peak B does not occur in a solution containing both of the unconjugated dyes.

The viscosity sensitivity of dye **4** was tested by measuring its fluorescent quantum yield in mixtures of ethylene glycol and glycerol (Figure 3).[5,9] Increased glycerol content is known to increase viscosity with only minimal changes of solvent polarity.[10] Glycerol contents of 40, 50, 60, 70, and 80% resulted in viscosities of 72, 109, 165, 248, and 374 mPa·s, respectively. As shown in Figure 3, both the direct rotor emission and the rotor emission through RET excitation exhibit viscosity sensitivity.

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10.1021/ja056370a CCC: $33.50

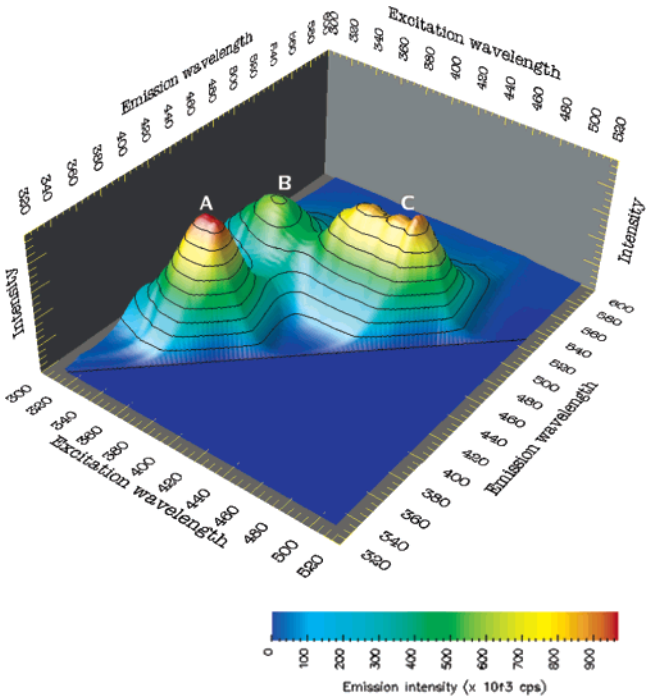

Figure 2. Fluorescent fingerprint of dye **4**. Peak A (λ_{ex} = 360 nm and λ_{em} = 402 nm) corresponds to the donor fluorescence peak (MCCA). Peak B (λ_{ex} = 349 nm and λ_{em} = 481 nm) corresponds to the RET, while peak C (λ_{ex} = 449 nm and λ_{em} = 486 nm) is emitted by CMAM.

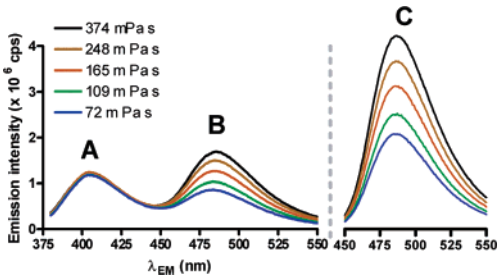

Figure 3. Emission spectra of probe **4** in mixtures of ethylene glycol and glycerol. Peaks A, B, and C correspond to MCCA (reference), RET, and CMAM emissions, respectively. Peaks A and B were acquired at λ_{ex} = 360 nm, while peak C was acquired at λ_{ex} = 444 nm. Only peaks B and C are viscosity-dependent.

As stipulated by eq 1, the data points of rotor emission intensity over solvent viscosity, drawn in a double-logarithmic scale, would lie on a straight line with a slope of x. For this class of dyes, a slope of $x = 0.6$ can be expected.[6] Indeed, the rotor emission maxima exhibited the expected behavior for peaks B ($x = 0.55$, $R^2 = 0.997$) and C ($x = 0.52$, $R^2 = 0.995$), while the MCCA reference peak had a slope of only $x = 0.04$ ($R^2 = 0.98$) (Figure 4). The minute increase of the reference peak is most likely due to small changes of refractive index of the solvent. Since refractive index changes affect both the rotor and the reference emission values, the ratiometric system eliminates this error.

To verify the ability of dye **4** to compensate for concentration fluctuations, we prepared mixtures of ethylene glycol and glycerol with concentrations of **4** ranging from 2.5 to 20 μM. The emission intensity of the rotor increased not only with increased solvent viscosity but also with increased dye concentration. Nonetheless, the ratio of the CMAM peak to the MCCA peak (reference) remains within a very tight range of values (Figure 4).

In conclusion, we report herein the first ratiometric viscosity sensor. The chemical design involves covalent attachment of a primary fluorophore (MCCA), having a constant emission quantum

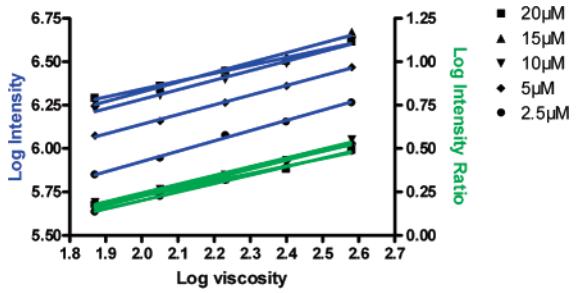

Figure 4. Reduction in the influence of dye concentration using ratiometric measurements. Blue lines correspond to CMAM emission in mixtures of ethylene glycol and glycerol (dye concentration 2.5−20 μM). Green lines correspond to the CMAM emission divided by the MCCA emission. Ratiometric measurement dramatically reduces variations between samples despite the high range of concentrations used.

yield, to a secondary fluorophore with a viscosity-sensitive quantum yield (CMAM). The high quantum yield of MCCA permits the excitation of CMAM via RET while still maintaining sufficient emission for reference computation. Such a dye can be probed with a single excitation source with two emission channels, allowing fast ratiometric measurements of fluid viscosity. The covalent link between the dye pair is fundamental to achieve RET as well as to ensure that local dye concentrations are identical even when preferential binding sites exist. Viscosity sensitivity covers a wide range of viscosities from 1 to 400 mPa·s, and no mechanical device would be capable of covering a comparable range. The emission ratio widely eliminates influences of refractive index and dye concentrations, allowing fast and accurate measurements of fluid viscosity.

Acknowledgment. Financial support by the NIH (1R33 018399) and the UCSD is gratefully acknowledged.

Note Added after ASAP Publication. After this paper was published ASAP on December 16, 2005, the structure of compound **4** was corrected in Figure 1, the Supporting Information, and the table of contents. The corrected version was published ASAP December 20, 2005.

Supporting Information Available: Synthesis and characterization of compound **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA056370A