Synthesis and use of an in-solution ratiometric fluorescent viscosity sensor

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A procedure for the synthesis of a ratiometric viscosity fluorescent sensor is described in this protocol. The essential requirement for the design of this sensor is the attachment of a primary fluorophore that has both a viscosity-independent fluorescence emission (coumarin dye shown in blue) and an emission from a fluorophore that exhibits viscosity-dependent fluorescent quantum yield (*p*-amino cinnamonitrile dye shown in red). The use of sensor 1 in viscosity measurements involves solubilization in a liquid of interest and excitation of the primary fluorophore at $\lambda_{ex} = 360$ nm. The secondary fluorophore is simultaneously excited via resonance energy transfer. The ratio of the fluorescent emission of the secondary over the primary fluorophore provides a fast and precise measurement of the viscosity of the solvent. The synthesis of compound 1 using commercially available materials can be completed within 5 d.

INTRODUCTION

In biological systems, viscosity plays an important role from the microscopic (cellular) to the systemic level. Examples are the viscosity of the cell membrane[1–4] and viscosity changes of blood plasma[5–7]. At all levels, in biological systems as well as in microfluidic devices, 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[8–10].

Scope and limitations of existing mechanical methods for measuring fluid viscosity

Viscosity is usually measured on a bulk scale by exposing the fluid under test to shear forces. The resistance against the shear force, caused by the internal friction of the fluid, can be measured and is related to viscosity. Precisely, the ratio of shear stress to shear rate is defined as the viscosity. Instruments include the falling ball viscometer (where a steel sphere is pulled toward the bottom of the tube by gravity), the capillary viscometer (where the fluid has to pass a capillary, driven by gravity) and the cone-and-plate viscometer (where a cone rotates inside a cup filled with fluid). In all cases, the measurement is time consuming, typically on a scale from 2 to 20 min, requires thorough cleaning of the instruments and may be adversely affected by solid precipitates in the fluid or proteins being deposited on the instrument walls. These properties preclude fast serial measurements.

Fluorescence-based methods for measuring fluid viscosity

Fluorescence-based methods provide good accuracy combined with high spatial and temporal resolutions[11]. However, fluorescence-based methods are often qualitative. In the case of fluorescence-based viscosity sensing, indirect measurements are most frequently employed. One examples is fluorescence anisotropy[12] using a dye that is excited by polarized light; here, diffusional rotation changes the polarization plane and the loss of emission polarization can be related to viscosity. Fluorescence recovery after photobleaching is a method where a laser pulse destroys the dye in a defined small spot; diffusion of unbleached dye into the spot causes fluorescence recovery with a time constant that is governed by viscosity[13]. Polarity-based sensing with polarity probes, such as LAURDAN, that change their emission wavelength in the presence of polar molecules such as water is also possible, as water is hypothesized to diffuse into the cell membrane at lower viscosities[14]. However, the relationship between water content and viscosity has not been fully explored, and other viscosity-sensing methods may help further clarify this relationship.

Fluorescent molecular rotors as viscosity sensors

Fluorescent molecular rotors, a subgroup of fluorophores capable of forming twisted intramolecular charge-transfer (TICT) states, are characterized by a viscosity-dependent quantum yield Φ_F (ref. 15). This property is accurately described by a power law relationship, $\Phi_F = C \times \eta^x$, where η is the solvent viscosity, x is the dye-dependent constant (typically, $x \approx 0.6$) and C is the proportionality constant. To measure the viscosity, the quantum yield would have to be determined. To eliminate the constant C, the dye system needs to be calibrated using fluids with known viscosity. Once the system has been calibrated, molecular rotors can measure fluids ranging from 1 to over 1,000 mPa s with high precision, a range that few single mechanical instruments cover. In steady-state fluoroscopy, emission intensity is proportionally related to Φ_F but also depends on the dye concentration, fluid absorption properties, excitation intensity and emission light collection efficiency. These experiment- and instrument-specific parameters can be eliminated using ratiometric sensors. A fluoroscopic measurement system employing the ratiometric principle has been presented[16]. With this specialized instrument, the effects of dye concentration and fluid scattering could be compensated for. A more elegant solution that does not require specialized instrumentation would be to have a fluorescent viscosity sensor that contains an additional fluorophore with a constant quantum yield as an internal reference[17].

Design of a ratiometric fluorescent viscosity sensor

The limitations of the fluorescent molecular rotors summarized above led to the design of a ratiometric sensor. The design is based on covalent attachment of two fluororescent dyes, a primary

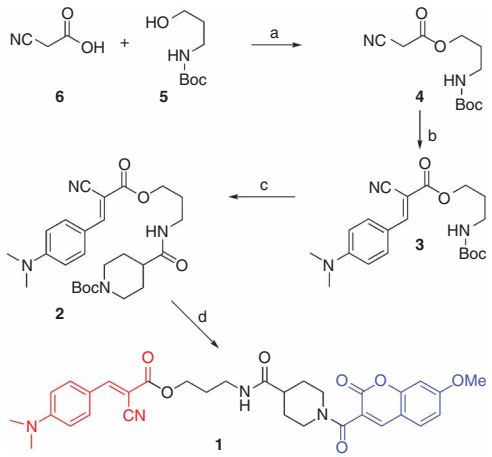

Figure 1 | Synthesis of sensor 1. (a) 1.0 eq. 5, 1.7 eq. EDC, 1.7 eq. 6, CH_2Cl_2, 6 h, room temperature; (b) 1.0 eq. 4-dimethylamino-benzaldehyde (7), 1.0 eq. DBU, THF, 11 h, room temperature; (c) TFA/CH_2Cl_2, 30 min, room temperature; then 1.2 eq. N-Boc-isonipecotic acid (8), 1.0 eq. EDC, 1.2 eq. DMAP, CH_2Cl_2, DMF, 12 h, room temperature; (d) TFA/CH_2Cl_2, 30 m, room temperature; then 1.0 eq. 7-methoxycoumarin-3-carboxylic acid-N-succinimidyl ester (9), 2.0 eq. DIEA, 0.1–1.0 eq. DMAP, CH2Cl2, 4 h, room temperature.

fluorophore whose fluorescence excitation and emission are viscosity insensitive and a secondary fluorophore whose emission quantum yield is viscosity-dependent. To facilitate the viscosity measurements, the two dyes were selected in such a way as to allow excitation of the secondary fluorophore (acceptor) upon emission of the primary fluorophore (donor). Thus, they form a resonance energy transfer (RET) pair. To optimize the energy transfer from the donor to the acceptor, the two dyes need to be relatively close to each other. The successful design would allow excitation of the primary fluorophore and measurement of the emissions from both the primary and the secondary fluorophore. By dividing the emission of the secondary fluorophore using the reference value (emission of the primary fluorophore), we can eliminate factors related to turbidity, refractive index, absorption of the solvent and the concentration of the dye. Another consideration is the choice of donor excitation wavelength. Proteins and biofluids absorb strongly in the UV range, primarily at 280 nm (Trp). A ratiometric sensor would, therefore, ideally be excited at much longer wavelengths. The efficiency of resonance energy transfer depends on several factors, mainly the donor emission peak, which influences the spectral overlap between donor emission and acceptor excitation, and the refractive index of the medium. A more polar solvent will cause a bathochromic shift of the donor emission and thus a higher transfer efficiency. A higher refractive index decreases the Förster distance and also causes a higher transfer efficiency. Although these changes are small compared with the changes in the acceptor quantum yield, careful consideration of the environmental effects (choice of calibration fluids, mathematical correction for refractive index) will further improve the measurement results. This article describes the synthetic route of such a ratiometric viscosity sensor and its use in viscosity measurements.

Use of a ratiometric fluorescent viscosity sensor

In bulk fluids, a defined amount of the sensor (typically in the lower micromolar range) is dissolved in the fluid to be measured. In the case of highly viscous fluids, heating of the fluids may be helpful as elevated temperature reduces a fluid's viscosity, making exact pipetting easier. The solution is excited at a wavelength of 365 nm. If autofluorescence of the fluid plays a significant role, the excitation wavelength may be increased to 390 nm and emission intensities are determined at 402 nm (I_{REF}) and 481 nm (I_{ROTOR}). The ratiometric intensity I_R is determined as $I_R = I_{ROTOR}/I_{REF}$. If a calibration curve exists that relates intensity to viscosity in similar fluids (i.e., the constants C and x of the above equation are known), viscosity can be computed as $\eta = (I_R/C)^{1/x}$. On the other hand, relative changes over time or distributions of local microviscosity can be determined with sufficient accuracy from a known intensity–viscosity pair (η_1, I_1) as $\eta/\eta_1 = (I_R/I_1)^{1/x}$, where $x = 0.6$. Since the relationship between intensity and viscosity is a power law, a plot of the intensity over the viscosity in a double-logarithmic scale provides a straight line with slope x as calibration curve. In as similar way, microscopic images can be obtained using UV excitation (365 nm) with two bandpass emission filters and a CCD (charge coupled device) camera or with a beamsplitter device. The calibration fluids should be similar to the fluid under test with respect to the fluid type (e.g., alcohols, lipids) and to the solvent polarity as the calibration constants may differ between different types of fluids[11].

Experimental design

The synthesis of compound **1** is depicted in **Figure 1**. The first step is an esterification reaction between commercially available cyanoacetic acid (**6**) and t-butyl *N*-(3-hydroxypropyl)carbamate (**5**) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl (EDC) that forms compound **4** (typical yields higher than 60%). The second step is a condensation reaction of ester **4** with commercially available 4-dimethylamino-benzaldehyde (**7**) in the presence of 1,5-diazabicyclo[4.3.0]non-5-ene (DBU) that forms compound **3** (typical yields between 70 and 85%). The third step involves deprotection of the primary amine of **3** using trifluoroacetic acid (TFA) followed by coupling with *N*-Boc-isonipecotic acid (**8**) to form amide **2** (typical yields between 50 and 85%). The last step includes removal of the Boc group of **2** using TFA and coupling of the resulting amine with 7-methoxycoumarin-3-carboxylic acid-*N*-succinimidyl ester (**9**) to produce sensor **1** (typical yields greater than 90%).

The ratiometric dye system **1** is used by dissolving a small amount of **1** in the fluid to be examined. Under 360 nm excitation, an emission scan from 400 to 550 nm is performed, and the emission intensity at 482 nm (rotor peak) is divided by the emission at 402 nm reference peak. The resulting ratiometric intensity needs to be calibrated by the intensities of a similar fluid with known viscosity (equivalent to the calibration of mechanical instruments).

MATERIALS

REAGENTS

- Silica gel (EMD Chemicals, Inc.; VWR, cat. no. EM-11567)
- Cyanoacetic acid (Sigma-Aldich, cat. no. 239976)
- EDC (Tanabe USA, Inc.)
- t-butyl *N*-(3-hydroxypropyl)carbamate (Sigma-Aldrich, cat. no. 416444)
- 4-dimethylamino-benzaldehyde (Sigma-Aldrich, cat. no. 156477)
- *N*-Boc-isonipecotic acid (Chess GmbH, cat. no. 1259)

- 7-methoxycoumarin-3-carboxylic acid, succinimidyl ester (Invitrogen, cat. no. M-1410)
- DBU (Sigma-Aldrich, cat. no. 139009)
- TFA (Acros Organics, cat. no. 13972)
- Anisole (Sigma-Aldrich, cat. no. 123226)
- 4-dimethylaminopyridine (DMAP; Sigma-Aldrich, cat. no. 107700)
- *N,N*-diisopropylethylamine (DIEA; Sigma-Aldrich, cat. no. D125806)
- Anhydrous *N,N*-dimethylformamide (DMF; Sigma-Aldrich, cat. no. 227056)
- Dichloromethane (DCM; Fisher, cat. no. D37)
- Hexanes (Fisher, cat. no. H292)
- Ethyl acetate (EtOAc; VWR, cat. no. EM-EX0240-3)
- Diethyl ether (ether; VWR, cat. no. EM-EX01855)
- Tetrahydrofuran (THF; Acros Organics, cat. no. 61045)
- Toluene (Acros Organics, cat. no. 34866)
- Methanol (MeOH; VWR, cat. no. EM-MX04903)
- Anhydrous magnesium sulfate (EMD Chemicals, Inc.; VWR, cat. no. EM-MX0075)
- Chloroform-D (Cambridge Isotope Laboratories, Inc., cat. no. 865-49-6)
- Ammonium molybdate tetrahydrate (Sigma-Aldrich, cat. no. 431346)
- Cerium (IV) sulfate (Sigma-Aldrich, cat. no. 359009)
- Concentrated sulfuric acid (Sigma-Aldrich, cat. no. 84716)
- Fluorescence-grade DMSO (Sigma, cat. no. 154938)
- Fluorescence-grade ethylene glycol (Sigma, cat. no. 293237)
- Fluorescence-grade glycerol (Sigma, cat. no. 191612)

EQUIPMENT

- Thin-layer silica gel plates (Bodman Industries, cat. no. 5715-7)
- Rotary evaporator (Buchi B-490) (see EQUIPMENT SETUP)
- Stir plate (Corning)
- Filter paper (Whatman qualitative circles)
- Chromatography columns: 45 × 4.5 cm, 30.5 × 2.5 cm (Chemglass)
- Argon gas
- Vacuum pump (Welch oil pump)
- Vacuum pump (Welch oil-less pump)
- Balloons (Sigma-Aldrich, cat. no. Z154970)
- Schlenk manifold
- Cotton
- UV lamp (254 nm)
- Rubber septa (14/20) (Chemglass; VWR, cat. no. 80062-438)
- Airline 14/20 adapter (Kontes, cat. no. 276000-0000)
- Single-use 20-gauge needles (BD Medical; VWR, cat. no. BD305176)
- Single-use 16-gauge needles (BD Medical; VWR, cat. no. BD305198)
- 100 µl syringe (Hamilton Co.)
- 1 ml Norm-Ject syringe (VWR, cat. no. 53548-000)
- 5 ml Norm-Ject syringe (VWR, cat. no. 53548-004)
- 10 ml Norm-Ject syringe (VWR, cat. no. 53548-006)
- 20 ml Norm-Ject syringe (VWR, cat. no. 53548-008)
- 50 ml Norm-Ject syringe (VWR, cat. no. 53548-010)
- 20 ml scintillation vials (Wheaton; VWR, cat. no. 66621-497)
- Jobin-Yvon Fluoromax-3 spectrofluorophotometer (see EQUIPMENT SETUP)
- 4 ml fluoroscopic-grade cuvettes (Fisher Scientific) ▲ **CRITICAL** All glassware and stainless steel needles are stored in a 60 °C oven (12 h) and then cooled to room temperature (23–25 °C) under vacuum or in a dessicator filled with anhydrous calcium sulfate before use. ▲ **CRITICAL** All reactions are performed under an argon atmosphere supplied via balloon. Explicit instructions for developing an argon atmosphere are provided in the protocol for the synthesis of ester **4** and were followed for all reactions.

REAGENTS SETUP

All solvents were purchased in an anhydrous form and used without any additional purification. Solvent mixtures are reported as volumetric ratios (vol/vol).

The cerium-ammonium molybdate (**CAM**) staining solution is prepared in a 1 l Erlenmeyer flask by dissolving 5.0 g (15.0 mmol) cerium (IV) sulfate and 25 g (20.2 mmol) ammonium molybdate tetrahydrate in 450 ml water. Then 50 ml concentrated H_2SO_4 at 0 °C is slowly added to this solution. The staining of TLC plates with CAM is performed by dipping the plate into the CAM stain, carefully removing the excess stain from the TLC plate with a paper towel and warming the TLC plate on a hot plate at 200 °C until charring is observed (approximately 10 s).

EQUIPMENT SETUP

Rotary evaporator Unless otherwise noted, the rotary evaporator is connected to a vacuum pump (20 mmHg) and its water bath operates at 35–36 °C.

Spectrofluorophotometer For measurement of the fluorescence spectra, set the excitation wavelength of a spectrofluorophotometer to 365 nm. Set the excitation and emission monochromator slits to a bandwidth of 5 nm. Scan the emission wavelength from 400 to 550 nm, allowing the emission detector to collect (integrate) light for at least 0.5 s per scan step.

PROCEDURE

Synthesis of ester 4

1| Transfer 2.0 g (23.55 mmol) cyanoacetic acid (**6**) into a dry 100 ml pear-shaped round-bottom flask containing an appropriately sized teflon-coated magnetic stir bar.
! **CAUTION** Cyanoacetic acid is a corrosive irritant.

2| Connect the flask to high vacuum through a three-way glass valve equipped with an appropriately sized ground glass joint. ('High vacuum' indicates a pressure range of 0.1–2 mmHg.)

3| Place a rubber septum tightly over the open valve outlet and evacuate the flask, including the valve outlets.

4| Adjust the valve in such a way that the vacuum is pulled only in the flask and fill the septum-covered outlet with argon through a balloon attached to a 1 ml plastic syringe fitted with a disposable 16-gauge needle.

5| With the balloon attached, adjust the valve in such a way that the flask is opened only to the argon balloon.

6| Once the flask is filled with argon, repeat Steps 4 and 5 twice (total of three times).

7| With a gentle but steady pressure of argon gas, remove the flask from the three-way adaptor, rapidly seal with a septum and transfer the argon balloon from the adaptor to the flask. Clamp the flask over a stir plate.

8| Quickly weigh 4.49 g (23.6 mmol) EDC onto weighing paper and transfer to flask by removing the septum and pouring directly into the flask.
! **CAUTION** EDC is an irritant and repeated or prolonged exposure may cause allergic reactions in sensitive individuals.

9| With stirring, dissolve the solids by transferring 40 ml DCM in one portion using a syringe.
! **CAUTION** DCM is a skin irritant and has been reported to cause cancer in laboratory animals.

! CAUTION Heat will be generated in Step 9. If the reaction scale is increased, it is suggested that the flask be cooled to 0 °C by immersing it in an ice bath.

10| Using a syringe, add 2.35 ml (13.76 mmol) t-butyl *N*-(3-hydroxypropyl)carbamate (**5**) to the reaction solution dropwise over 3 min.

11| Wash the walls of the flask with 5 ml DCM.

12| Monitor the reaction progress using TLC. The reaction components can be visualized using CAM staining. R_f values developed in 40% EtOAc/hexanes are as follows: *N*-Boc-aminopropanol ($R_f \approx 0.1$), cyanoacetic acid ($R_f \approx 0$) and ester **4** ($R_f \approx 0.3$).

13| When aminopropanol is no longer observed by TLC (approximately 6 h), remove the stir bar from the flask and distill off the DCM by concentrating in a rotary evaporator.
? TROUBLESHOOTING

14| While the crude material is being concentrated, prepare a column for chromatography by pouring a hexane slurry of 60 g silica into a glass column measuring approximately 45 cm in height and 4.5 cm in diameter.

15| When condensation of solvent is no longer observed, remove the flask from the rotary evaporator and allow it to cool to room temperature.

16| Adsorb the crude material onto 12 g silica by mixing using a spatula in the reaction flask.

17| Transfer the silica into the chromatography column and begin elution with 20% EtOAc/hexanes (100 ml). Collect 50 ml fractions and switch to 40% EtOAc/hexanes (300 ml).

18| Follow the separation by TLC; the product should be visualized after approximately 250 ml of solvent has been passed through the column.
▲ CRITICAL STEP *N*-Boc-aminopropanol does not visualize well on TLC; it is therefore important to spot later fractions heavily to identify any unreacted aminopropanol.

19| Concentrate the fractions containing the product in a rotary evaporator, then further concentrate the clear oil under high vacuum at room temperature.
■ PAUSE POINT Product can be left for several days under argon in a −20 °C freezer.

Synthesis of ester 3

20| Charge a 100 ml pear-shaped round-bottom flask, equipped with a magnetic stir bar, with 1.11 g (4.58 mmol) ester **4**, 683 mg (4.58 mmol) 4-dimethylamino-benzaldehyde.
! CAUTION 4-dimethylamino-benzaldehyde is considered to be an irritant.

21| Create an argon atmosphere as in the synthesis of **4**.

22| Dissolve the reaction components in 30 ml anhydrous THF added via syringe.

23| Using a syringe, add 685 µl (5.32 mmol) DBU dropwise over 1 min.
! CAUTION DBU is corrosive.

24| Wash the walls of the flask with 5 ml THF.
▲ CRITICAL STEP Esters **3** and **4** are difficult to separate by silica chromatography; it is therefore important to allow enough time for complete conversion of ester **4**. The reaction components can be visualized using UV illumination or CAM staining. R_f values developed in 40% EtOAc/hexanes are as follows: dimethylamino-benzaldehyde ($R_f \approx 0.5$), ester **4** ($R_f \approx 0.3$, **4** has limited UV activity) and ester **3** ($R_f \approx 0.3$). If necessary, alternative reaction monitoring techniques such as ^{1}H NMR should be used.
■ PAUSE POINT Stir at room temperature overnight.

25| Remove the stir bar and concentrate in a rotary evaporator.
? TROUBLESHOOTING

26| While the crude material is being concentrated, prepare a column for chromatography by pouring a hexane slurry of 60 g silica into a glass column measuring approximately 45 cm in height and 4.5 cm in diameter.

27| When condensation of solvent is no longer observed, remove the flask from the rotary evaporator and allow it to cool to room temperature.

28| Adsorb the crude material onto 12 g silica by mixing using a spatula in the reaction flask.

29| Transfer the silica into the chromatography column and begin elution with 20% EtOAc/hexanes (500 ml). Once any unreacted aminobenzaldehyde has been eluted, change the solvent to 40% EtOAc/hexanes and collect 50 ml fractions until **3** is completely eluted (about 500 ml). This will require approximately ten fractions.

30| Concentrate the fractions containing **3** in a rotary evaporator, then further dry the yellow solid under high vacuum at room temperature.

31| Dissolve **3** in approximately 10 ml warm EtOAc.
■ **PAUSE POINT** Crystallize **3** by allowing the solution to sit at room temperature open to the atmosphere overnight.

32| Remove the mother liquors through a filter-tip pipette and dry the yellow solid by concentrating in a rotary evaporator followed by drying under high vacuum at room temperature.
■ **PAUSE POINT** Product can be left under argon in a −20 °C freezer for several days without alteration of purity.

Synthesis of ester 2

33| Prepare a TFA solution by dissolving 100 µl anisole with approximately 4.9 ml DCM in a small vial. Then add approximately 5.0 ml TFA. Cap the vial and mix by gentle swirling.
! **CAUTION** TFA is corrosive, and anisole is a skin irritant.

34| Transfer 145 mg (0.405 mmol) **3** into a 25 ml pear-shaped round-bottom flask equipped with a magnetic stir bar and develop an argon atmosphere as in Steps 2–6.

35| Using a syringe, add 6 ml TFA solution (prepared in Step 33) at room temperature with stirring.

36| After 30 min, confirm complete deprotection using TLC. No compound migration should be observed in a TLC plate developed with 100% diethyl ether (for the R_f value of ester **3**, see Step 24).

37| Concentrate the reaction mixture in a rotary evaporator attached to a vacuum pump capable of achieving less than 10 mmHg.

38| Once the mixture has been concentrated, add 10 ml toluene and concentrate in the same manner.
! **CAUTION** Toluene is flammable.

39| Repeat Step 38 three more times (total of 4 concentrations with toluene). Perform the concentrations carefully as the crude amine-toluene solution bumps easily.
? **TROUBLESHOOTING**

40| Place the orange solid under high vacuum at room temperature.
▲ **CRITICAL STEP** Complete removal of TFA is necessary to increase the coupling efficiency. It is recommended that the TFA salt remain under high vacuum for a minimum of 6 h.

41| In a separate 10 ml pear-shaped flask containing a magnetic stir bar, mix 94 mg (0.490 mmol) EDC, 94 mg (0.409 mmol) *N*-Boc-isonipecotic acid (**8**) and 59 mg (0.484 mmol) DMAP.
! **CAUTION** *N*-Boc-isonipecotic acid is an irritant, and DMAP is a highly toxic irritant.

42| Create an argon atmosphere as in Steps 2–6 and cool to 0 °C in an ice bath.

43| Dissolve the reaction mixture in 0.7 ml DCM with stirring.

44| After stirring for 5 min at 0 °C, remove from the ice bath and stir at room temperature for 10 min.

45| While the reaction mixture is being stirred, prepare a solution of the crude amine formed in Step 40 by adding 0.7 ml anhydrous DMF under an argon atmosphere.
! **CAUTION** DMF is a toxic irritant.

46| Cool the reaction mixture of Step 44 to 0 °C using an ice bath.

47| Using a syringe, add the amine/DMF solution prepared in Step 45 dropwise over 5 min. Ensure complete transfer by washing the flask with 0.6 ml DCM.

48| Allow the ice bath to reach room temperature by letting the ice melt.

49| Monitor the reaction using TLC. Ester **2** can be visualized using UV illumination or CAM staining. R_f values developed in 100% diethyl ether are as follows: *N*-Boc-isonipecotic acid (**8**) ($R_f \approx 0.6$, I_2 stain only), ester **2** ($R_f \approx 0.15$, streaks).

▲ CRITICAL STEP *N*-Boc-isonipecotic acid co-spots with a side product. Therefore, reaction progress cannot be accurately judged by the disappearance of the acid spot. If necessary, alternative reaction monitoring techniques such as ^{1}H NMR should be utilized.
■ PAUSE POINT The reaction can be safely stirred at room temperature for longer than 12 h with no significant effect on yield.

50| Add 4 ml H_2O to the reaction, followed by 2 ml diethyl ether.
! CAUTION Diethyl ether is highly flammable.

51| Stir the mixture vigorously for several minutes, then transfer the organic layer into a 25 ml Erlenmeyer flask containing a small amount of anhydrous magnesium sulfate.

52| Extract seven times with 3 ml portions of diethyl ether, adding each organic extract to the flask containing magnesium sulfate.

53| Filter off the magnesium sulfate from the combined organic layers and concentrate the crude reaction mixture in a rotary evaporator.

54| While the crude material is being concentrated, prepare a column for chromatography by pouring a hexane slurry of 12 g silica into a glass column measuring approximately 35 cm in height and 2.5 cm in diameter.

55| Once concentrated, absorb the crude reaction mixture into the minimum amount of silica by stirring using a spatula and transfer to the chromatography column.

56| Elute with 50% EtOAc/hexanes (300 ml) until excess carboxylic acid and side product are off the column as shown by TLC.

57| Switch to 80% ether/hexanes and elute until **2** is off the column (300 ml).

58| Concentrate the fractions containing **2** in a rotary evaporator, then further concentrate the yellow solid under high vacuum at room temperature for 2 h.
▲ CRITICAL STEP The use of ether/hexanes as the eluent helps minimize the elution of any residual DMF. If DMF is observed in the product by NMR, trituration can be performed to purify **2**. Trituration can be performed with DCM and hexanes as described in **Table 1**.
■ PAUSE POINT Product can be left under argon in a −20 °C freezer for several days without alteration of purity.
? TROUBLESHOOTING

Synthesis of probe 1

59| Prepare a TFA solution by dissolving 100 µl anisole with approximately 4.9 ml DCM in a small vial. Then add approximately 5.0 ml TFA. Cap the vial and mix by gentle swirling.

60| Transfer 40 mg (0.083 mmol) **2** into a 15 ml pear-shaped round-bottom flask equipped with a magnetic stir bar and develop an argon atmosphere as in Steps 2–6.

61| Using a syringe, add 0.8 ml of the TFA solution at room temperature with stirring.

62| After 30 min, confirm complete deprotection using TLC. No compound migration should be observed in 100% diethyl ether (for the R_f value of compound **2**, see Step 49).

63| Concentrate the reaction mixture at no more than 45 °C in a rotary evaporator attached to a vacuum pump capable of achieving less than 10 mmHg.

64| Once concentrated, add 2 ml toluene and concentrate in the same manner.

65| Repeat Step 64 three more times (total of four concentrations). Perform the concentrations carefully as the crude amine-toluene solution bumps easily.

66| Place the residue under high vacuum at room temperature.
▲ CRITICAL STEP Complete removal of TFA is necessary to increase the coupling efficiency. It is recommended that the TFA salt remain under high vacuum for a minimum of 6 h.

67| Add a magnetic stir bar to the flask containing the crude TFA salt and develop an argon atmosphere.

68| Add 1 mg (0.008 mmol) DMAP and 25 mg (0.079 mmol) 7-methoxycoumarin-3-carboxylic acid, succinimidyl ester (**9**) as solids to the reaction flask.

69| Prepare a solution by dissolving 30 µl (0.17 mmol) DIEA in 0.7 ml DCM.

70| At room temperature, add the DIEA/DCM solution rapidly using a syringe. Wash the syringe and walls of the flask by adding an additional 0.5 ml DCM.

71| Stir at room temperature.

72| Monitor the reaction using TLC. The succinimidyl ester (**9**) has an R_f value of 0.3 in 100% ether and is easily visualized using UV illumination. The reaction is expected to be completed within 4 h.
▲ CRITICAL STEP Disappearance of **9** indicates a complete reaction and should occur within 4 h.
? TROUBLESHOOTING

73| Concentrate the reaction mixture in a rotary evaporator and absorb into the minimum amount of silica.

74| Prepare a column for chromatography by pouring a 5% MeOH/DCM slurry of 20 g silica into a glass column measuring approximately 35 cm in height and 2.5 cm in diameter.

75| Transfer the silica-absorbed reaction mixture from Step 73 on the chromatography column prepared in Step 74. Elute with 5% MeOH/DCM until **1** ($R_f \approx 0.2$ in 5% MeOH/DCM) is off the column. Probe **1** should be contained in fractions between 200 and 350 ml.

76| Concentrate in a rotary evaporator. Dissolve the solid with 20 ml toluene and concentrate in a rotary evaporator at 70 degrees. Dry further under high vacuum at room temperature for 2 h.

77| Dissolve in approximately 4 ml DCM and add EtOAc until turbidity is observed. Mix by gentle swirling to produce a clear solution.
■ PAUSE POINT Crystallize by allowing the flask to sit overnight open to the atmosphere.

78| Remove the mother liquors using a filter-tip pipette and dry the yellow solid by concentrating in a rotary evaporator followed by drying under high vacuum at room temperature for 2 h.
■ PAUSE POINT Probe **1** is stable for extended periods if stored at reduced temperature under inert gas.

Application of probe 1 as ratiometric viscosity sensor in sample fluids of different viscosity

79| Dissolve **1** at room temperature in fluorescence-grade DMSO at a concentration of 10 mM to obtain a concentrated stock solution. A slow vortex mixer is suitable to achieve complete dissolution. For a quick verification of proper fluorescence properties, dilute 1 µl of the stock solution with 1 ml DMSO and acquire a spectral emission scan from 375 to 550 nm with an excitation of 365 nm. The exact fluorometer settings are uncritical. The emission scan should show two distinct emission peaks.
■ PAUSE POINT The stock solution can be kept at 4 °C for up to 4 weeks.

80| Add 3 µl stock solution to 6 ml ethylene glycol and vortex thoroughly at slow speed to avoid air bubbles. Make sure that the ethylene glycol solution is homogeneously mixed. One milliliter of this fluid will remain unused.

81| Heat 12 ml glycerol in a boiling water bath. Heat reduces viscosity and makes the glycerol easier to pipette.

82| In five separate centrifuge tubes, add 1 ml prestained ethylene glycol to 4 ml glycerol; 1 ml ethylene glycol, stained with compound **1** as per Step 80, and 1 ml unstained ethylene glycol to 3 ml glycerol; 1 ml stained ethylene glycol and 2 ml unstained ethylene glycol to 2 ml glycerol; 1 ml stained ethylene glycol and 3 ml unstained ethylene glycol to 1 ml glycerol; and 1 ml stained ethylene glycol to 4 ml unstained ethylene glycol. The five fluids have a glycerol content of 80, 60, 40, 20 and 0%, respectively, with a resulting viscosity of 374, 165, 72, 32 and 14 mPa s.

83| Place the five centrifuge tubes on an inverting mixer and allow mixing for at least 2 h. At the same time, allow the fluids to equilibrate to room temperature.
▲ CRITICAL STEP Mixing of the fluids must be complete. The presence of visible streaks in the fluid indicates incomplete mixing.
■ PAUSE POINT The measurement-ready fluids are stable for at least 24 h at room temperature.

84| Pour 3.5 ml of each of the fluids into methacrylate fluoroscopic cuvettes.
▲ CRITICAL STEP Avoid trapping air bubbles when filling the fluoroscopic cuvettes.

85| Set the excitation wavelength of a spectrofluorophotometer to 365 nm. Set the excitation and emission monochromator slits to a bandwidth of 5 nm. Scan the emission wavelength from 400 to 550 nm, allowing the emission detector to collect (integrate) light for at least 0.5 s per scan step. Analyze the emission scans for emission maxima. Emission maxima should be found at approximately 402 and 481 nm. Obtain the intensities of the maxima and, for each individual fluid, compute the intensity ratios of the intensity at the 481 nm maximum to the intensity at the 402 nm maximum. Record the ratios.

86| Analyze the data points by plotting the $\log_{10}$ of the intensity ratios over the $\log_{10}$ of the viscosity. The double-logarithmic data points should lie on a straight line with a slope of 0.6. This verifies that emission intensity follows the relationship $\Phi_F = C \times \eta^x$, which was mentioned in the INTRODUCTION (**Fig. 2**).

● TIMING
Synthesis of ester 4 Steps 1–7: 5 min; Steps 8 and 9: 2 min; Step 10: 3 min; Steps 11–13: 6 h, Steps 14–16: 20 min; Steps 17 and 18: 30 min; Step 19: 40 min for rotary evaporation and an additional hour for high-vacuum drying

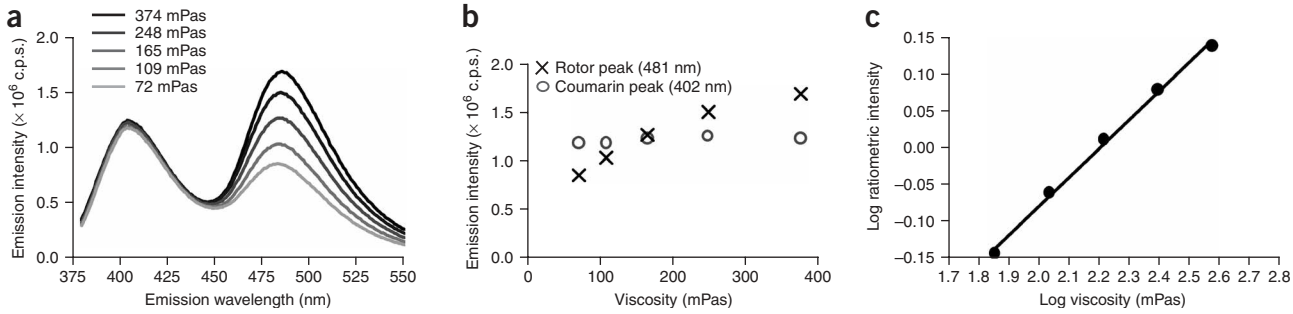

Figure 2 | Typical spectra and typical behavior of emission maxima in fluids of different viscosity (mixtures of ethylene glycol and glycerol). The emission spectrum shows a distinct peak at 402 nm from coumarin emission and another peak at 486 nm from the rotor emission (**a**). Only the rotor emission peak is viscosity dependent, whereas there is no significant ($P = 0.19$) correlation between coumarin intensity and viscosity (**b**). The ratio of the rotor emission peak to the coumarin peak, drawn over the solvent viscosity in a double-logarithmic scale, lies on a straight line (**c**). This confirms that the relationship between intensity and viscosity is described by the equation $\Phi_F = C \times \eta^x$, where x is the slope of the best-fit line.

Synthesis of ester 3 Steps 20–22: 10 min; Steps 23 and 24: 2 min; Step 25: 11 h; Steps 26 and 27: 20 min.; Steps 28 and 29: 10 min; Step 30: 40 min for rotary evaporation and an additional hour for high-vacuum drying; Step 31: 10 min to dissolve and at least 8 h to crystallize; Step 32: 1.5 h

Synthesis of ester 2 Step 33: 5 min; Steps 34 and 35: 35 min; Step 36: 3 min; Steps 37–39: 1.5 h; Step 40: minimum of 6 h; Step 41: 10 min; Steps 42–45: 20 min; Step 46: 6 min; Steps 47–49: up to 12 h; Steps 50–52: 35 min; Steps 54 and 55: 15 min; Steps 55–57: 25 min; Step 58: total of 2 h

Synthesis of probe 1 Step 59: 5 min; Steps 60 and 61: 35 min; Step 62: 3 min; Steps 63–65: 1.5 h; Step 66: minimum of 6 h; Steps 67 and 68: 7 min; Steps 69 and 70: 5 min; Step 71: 4 h; Step 72: 3 min; Step 73: 15 min; Step 74: 5 min; Step 75: 30 min; Step 76: 4 h; Step 77: 5 min to dissolve and up to 12 h to crystallize; Step 78: 2 h

Fluid viscosity measurement Step 79: 1 min; Step 80: 2 min; Step 81: 10 min (can be started before Steps 79 and 80); Steps 82 and 83, 2.5–3 h; Step 84, 2 min; Step 85, 10 min depending on the instrument scanning speed; Step 86, typically 10 min

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reasons	Solution
13	Poor yield of **4**	Insufficient reaction time, impure or wet reagents	Allow longer reaction time (approximately 12 h) Adding a catalytic amount of DMAP (10%) should facilitate activation of the carboxylic acid and decrease the reaction time Moisture will make the reaction slow; repeat with high-quality dry reagents and rigorously develop the argon atmosphere Maintain the prescribed concentration
25	Slow formation of **3**	Moisture	Repeat the reaction with high-quality dry reagents and rigorously develop the argon atmosphere Maintain the prescribed concentration
39	Poor yield or slow formation of **2**	Residual acid from Boc deprotection or slow coupling	Repeat the reaction with high-quality dry reagents and rigorously develop the argon atmosphere Pressures developed by high-vacuum pumps are highly variable and dependent on a multitude of factors. Ensure the pump is in good working condition and all joints are sealed Rigorously remove the residual TFA from the crude amine by additional azeotropic distillations with toluene on a rotary evaporator operating at 40 °C or by increasing the time under high vacuum Preactivation of the carboxylic acid in the reaction is important in minimizing side reactions; ensure the prescribed procedure is followed Maintain the prescribed concentration

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reasons	Solution
58	Trituration problems	Films instead of solids are formed	Rigorously dry the compound under high vacuum. Add the absolute minimum amount of DCM necessary to solubilize the material (about 1 ml) and then add hexanes (about 1 ml) until no further precipitation of the products is observed
72	Poor yield or slow formation of 1	Residual acid from Boc deprotection or slow coupling	Repeat the reaction with high-quality dry reagents and rigorously develop the argon atmosphere. Confirm the succinimidyl ester is of good quality by comparing its spectroscopic data to that of the known compound Rigorously remove the residual TFA from the crude amine by additional azeotropic distillations on a rotary evaporator or increasing the time under high vacuum Additional DIEA may be added to neutralize residual TFA Maintain the prescribed concentration
79–86	Coumarin peak (402 nm) too low	RET is too high, and couramin is unable to emit, because all energy is transferred to the rotor	Heat the DMSO solution at 95 °C until the desired RET efficiency is obtained. Verify by spectroscopy (Step 79). This may require several hours. Alternatively, Dissolve **1** in ethylene glycol (mg/ml) and heat at 95 °C until the desired RET efficiency is obtained. This may require several hours. Upon cooling to room temperature desired RET level should be maintained. If necessary, extract **1** with diethyl ether after dilution with water

ANTICIPATED RESULTS

Typical yields

Typical yield of individual reactions producing **4** will be greater than 60%; **3**, between 70 and 85%; **2**, between 50 and 85%; and **1**, greater than 90%.

Analytical data

Ester 4

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.65 (bs, 1H), 4.27 (t, J = 6.4 Hz, 2H), 3.48 (s, 2H), 3.22 (m, 2H), 1.88 (m, 2H) 1.43 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.0, 155.9, 113.0, 79.4, 64.2, 36.9, 28.8, 28.3, 24.7; HR-EI-MS calculated for $C_{11}H_{18}N_2O_4$ $[M]^+$ 242.1261, found: 242.1256.

Ester 3

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.06 (s, 1H), 7.94 (d, J = 9.2 Hz, 2H), 6.69 (d, J = 9.2 Hz, 2H), 4.82 (bs, 1H), 4.34 (t, J = 6.0 Hz, 2H), 3.26 (m, 2H), 3.11 (s, 6H), 1.93 (m, 2H), 1.44 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.4, 156.0, 154.8, 153.6, 134.5, 119.3, 117.5, 111.5, 93.4, 79.2, 63.4, 40.0, 37.3, 29.0, 28.4; HR-EI-MS calculated for $C_{20}H_{27}N_3O_4$ $[M]^+$ 373.1996, found: 373.1990.

Ester 2

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.07 (s, 1H), 7.92 (d, J = 9.2 Hz, 2H), 6.69 (d, J = 9.2 Hz, 2H), 6.18 (bs, 1H), 4.34 (t, J = 5.8 Hz, 2H), 4.11 (bs, 2H), 3.41 (q, J = 6 Hz, 2H), 3.11 (s, 6H), 2.73 (bs, 2H), 2.34 (dt, J = 11.4, 3.6 Hz, 1H), 1.94 (p, 6 Hz, 2H), 1.78 (m, 2H), 1.63 (dt, J = 12.0, 4.0 Hz, 2H), 1.44 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.7, 164.2, 154.8, 154.5, 153.6, 136.4, 134.1, 118.9, 117.8, 111.4, 110.8, 92.4, 79.2, 64.2, 42.8, 39.9, 36.6, 28.4, 28.2; HR-FAB-MS calculated for $C_{26}H_{36}N_4O_5$ $[M]^+$ 484.2680, found: 484.2678.

Probe 1

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.07 (s, 1H), 7.93 (d, J = 8.8 Hz, 2H), 7.86 (s, 1H), 7.43 (d, J = 8.4 Hz, 1H), 6.87 (dd, J = 8.8, 2.4 Hz, 1H), 6.81 (d, J = 2 Hz, 1H), 6.71 (d, J = 9.2 Hz, 2H), 6.29 (m, 1H), 4.67 (m, J = 13.2 Hz, 1H), 4.36 (t, J = 5.2 Hz, 2H), 3.88 (s, 3H), 3.65 (m, J = 13.6 Hz, 1H), 3.44 (q, J = 5.8 Hz, 2H), 3.22 (m, J = 12.0 Hz, 1H), 3.13 (s, 6H), 2.93 (m, J = 12.0 Hz, 1H), 2.53 (dt, J = 12.0, 4.0 Hz, 1H), 1.95 (m, 3H), 1.85-1.74 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.3, 164.4, 163.8, 163.6, 158.3, 156.0, 155.2, 153.9, 143.1, 134.3, 129.6, 121.6, 119.1, 118.2, 113.2, 112.0, 111.6, 100.6, 92.3, 65.0, 55.9, 46.7, 42.6, 41.5, 40.0, 37.3, 29.0, 28.5, 28.4; HR-FAB-MS calculated for $C_{32}H_{34}N_4O_7$ $[M]^+$ 586.2422, found: 586.2428.

Fluorescence data

The emission peaks generated by coumarin and by the rotor (at 402 and 486 nm, respectively) should be distinctly identifiable (**Fig. 2a**). In fluids of very low viscosity (e.g., methanol), the peak at 486 nm may be very weak. The coumarin peak may

change with refractive index of the fluid as well as some absorption properties, whereas the rotor peak predominantly changes with the viscosity of the fluid (**Fig. 2b**). In fluids of known viscosity, the rotor emission intensities, drawn over the viscosity in a double-logarithmic scale, should lie on a straight line with a slope of approximately 0.6. To eliminate the fluid properties and dye concentration fluctuations, the rotor emission must be divided by the coumarin emission. These values show a similar behavior to the rotor emission peaks alone (**Fig. 2c**). A curve such as the one depicted in **Figure 2c** may be considered a calibration curve. The ratiometric emission intensity of the dye **1** in a fluid of unknown viscosity can be converted into a viscosity value using the calibration curve.

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