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A Reduction-Sensitive Fluorous Fluorogenic Coumarin

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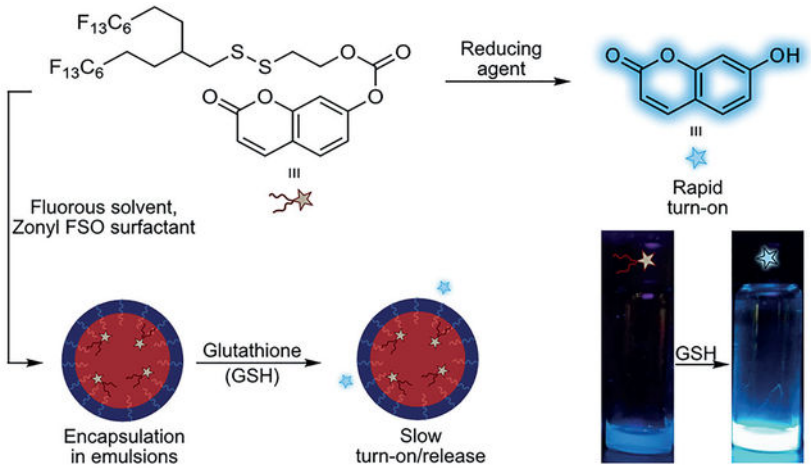
Abstract

Fluorophores that are sensitive to their environment are useful tools for sensing chemical changes and probing biological systems. Here, we extend responsive fluorophores to the fluorous phase with the synthesis of a reduction-sensitive fluorous-soluble fluorogenic coumarin. We demonstrate that this fluorophore responds to various reducing agents, most notably glutathione, a key biological reductant. The fluorous solubility of this probe allows for its encapsulation into two different fluorous nanomaterials: perfluorocarbon nanoemulsions and fluorous core-shell micelles. The fluorogenic coumarin allows us to study how efficiently these vehicles protect the contents of their interior from the external environment. In the presence of glutathione, we observe different degrees of release for micelles and emulsions. This understanding will help guide future applications of fluorous nanomaterials as drug delivery vehicles.

Graphical Abstract



Keywords

fluorous; fluorogenic; fluorofluorophore; perfluorocarbon nanoemulsions; fluorous micelles; glutathione responsive; coumarin

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[◊]These authors contributed equally to this work

Supporting Information

Supporting information for this article is available online at https://doi.org/10.1055/s-0039-1690770. Included are Figures S1–S8 and experimental procedures.

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Chromophores that undergo changes in their photo-physical properties in response to environmental or chemical perturbations are valuable tools to gain quantitative information in complex systems.[1–4] These probes are designed such that they either undergo a shift in $\lambda_{max,abs}$ or $\lambda_{max,em}$, deemed solvatochromic or ratiometric, respectively, or an enhancement in quantum yield, deemed fluorogenic (Figure 1A). Thousands of solvatochromic, ratiometric, and fluorogenic probes have been prepared to measure polarity changes,[1–5] quantify ion concentrations,[6–9] image cellular compartments,[10,11] detect metabolites,[12–14] sense chemical warfare agents,[15–18] etc. The medium of these probes can function in varies from gas phase to organic solvents to aqueous buffers; however, applications in the fluorous phase are scarce.

The fluorous phase, composed of molecules with high weight % fluorine in sp^3 C-F bonds, is orthogonal to aqueous and organic solutions. Distinct properties of the fluorous phase are extreme hydrophobicity and nonpolarizability, high gas content, narrow temperature window for the liquid phase, and decreased molecular motion.[19,20] These unique attributes have resulted in a variety of applications of highly fluorinated materials including nonstick coatings, artificial blood, chemical purification strategies, and self-assembled materials.[21–23] Despite the broad utility of perfluorinated materials, there are relatively few chromophores and fluorophores that are soluble in the fluorous phase.[24–27] One of these chromophores, 9-(α-perfluoroheptyl-β,β-dicyanovinyl)julolidine (**1**, Figure 1B), displays solvatochromic behavior and has been used to define the spectral polarity index, P_S, as a solvent polarity scale that calibrates perfluorocarbons and organics.[28] Other notable fluorous sensors and probes include a fluorous copper complex, which when combined with an organic chromophore and phase, enabled histamine sensing[29] as well as a fluorinated BINOL derivative for enantioselectivity measurements in the fluorous phase.[30]

Here, we contribute coumarin **2** (Figure 1C), the first fluorogenic fluorous soluble fluorophore. We chose coumarin as a scaffold for the development of a fluorogenic fluorous fluorophore due to literature precedent for solubilizing coumarin in perfluorocarbons[24,31–33] in addition to the large body of work demonstrating that coumarin fluorescence can be modulated by changes in substituents at the 7-position.[34–36] We envisioned that the hydroxyl group of 7-hydroxycoumarin could be alkylated with a chemically sensitive linkage connected to a fluorous tag. The branched fluorous tag imparts fluorous solubility to the starting fluorophore, while the chemically sensitive linker facilitates fluorescence turn-on. For our initial work, we chose a disulfide as the chemically sensitive linkage, which was connected to the coumarin via a self-immolating carbonate linker[37] to facilitate release of 7-hydroxycoumarin.

Coumarin **2** could be prepared in two steps from 7-hydroxycoumarin **3** (Scheme 1). First, treatment of 7-hydroxycoumarin with heterobifunctional linker **4**[38] provided **5**, which contains the fluorophore and self-immolating linker with an activated disulfide.[39] Displacement of pyridyl thiol from **5** was accomplished by reaction with branched fluorous thiol **6**[40] to generate fluorous disulfide coumarin **2** in 74% yield.[41] Fluorogenic coumarin **2** was stable for months when stored at 4 °C under nitrogen.

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We evaluated the response of coumarin **2** to a variety of reducing agents. We anticipated that disulfide cleavage via a reducing agent would lead to thiol **7**, which would spontaneously eliminate 1,3-oxathiolane-2-one (**8**) to release fluorescent **3** (Figure 2A). To test an initial panel of reducing agents, we dissolved **2** in acetone. Acetone was chosen as we have found it to be sufficient to solubilize many partially fluorinated compounds and it is miscible with water, allowing polar reducing agents to be analyzed. As can be seen in Figure 2B, upon addition of 10 mM β-mercaptoethanol (BME, dark blue), dithiothrietol (DTT, green), glutathione (GSH, purple), or tris(carboxyethyl phosphine) (TCEP, orange) suggested that efficient cleavage of the disulfide in **5** was readily obtained, with fluorescence levels becoming similar to that of free 7-hydroxycoumarin after treatment.[42] Importantly, if reagents that are not able to reduce disulfide bonds are added such as oxidized glutathione (Ox GSH, gray) or ethylene glycol (black), little fluorescence is observed.

Next, we moved to evaluating the fluorogenic nature of **2** in fluorous solvents. Coumarin **2** was dissolved in perfluorooctyl bromide (PFOB), methoxyperfluorobutane (MPFB), perfluorodecalin (PFD), and perfluorohexanes (PFH)[43] and partitioned with phosphate buffered saline (PBS). Minimal signal is observed until the addition of β-mercaptoethanol (BME), at which point robust emission from the aqueous layer is apparent (Figure 2C).

Of particular interest is the response to glutathione, as glutathione levels are very high intracellularly (5–10 mM) but low extracellularly (10 μM).[44,45] We further probed the turn-on kinetics of **2** under various concentrations of glutathione and found that when treated with micromolar concentrations of glutathione there was less than 10-fold turn-on over the course of an hour compared to the control (Figure 2D, orange, gray vs. black). When exposed to either 5 mM or 10 mM glutathione, the fluorescence increased over 100-fold within an hour (Figure 2D, red, blue vs. black). These data suggest that fluorogenic coumarin **2** is capable at differentiating biologically relevant glutathione levels.

One application of **2** is as a probe to measure the response of fluorous nanomaterials to intra- and extracellular conditions. Towards this end, we compared the properties of **2** encapsulated in two different delivery vehicles: (i) droplets of fluorous solvent stabilized in water, (i.e., perfluorocarbon nanoemulsions) and (ii) fluorous core-shell micelles. Our group is interested in exploiting the orthogonal nature of the fluorous phase to create advanced nanotherapeutics.[24,40,46–48] An important component of fluorous nanotherapeutics is the ability for fluorous-tagged molecules to be protected inside the biologically inert and nontoxic fluorous core.[22,49] Fluorogenic coumarin **2** allowed us to directly test if cargo loaded into the center of the perfluorocarbon nanoemulsions or micelles sense the surrounding environment.

Perfluorocarbon nanoemulsions containing **2** were prepared by first solubilizing **2** in PFOB (982 μM) and then combining with PBS to make a biphasic solution of 10 vol% fluorous oil. A commercially available nonionic fluorosurfactant, Zonyl FSN-100 (**9**), was added to a surfactant loading of 2.8 wt% in PBS (Figure 3A). Emulsions were then formed through ultrasonication at the liquid–liquid interface for 90 s at 0 °C. This procedure resulted in weakly fluorescent emulsions with a diameter of 185 nm and a polydispersity of 0.13 [Figures S2, S4 in the Supporting Information (SI)]. To prepare micelles, Zonyl FSN-100

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was dissolved in a stock solution of MilliQ water at 2.8 wt% (far above the critical micelle concentration), and **2** was added via an acetone stock, yielding weakly fluorescent 6 nm micelles with a polydispersity of 0.04 (Figures S3, S4 in the SI).[50]

To probe the role of these delivery vehicles in protecting the fluorogenic dye from the surrounding environment, nanomaterials were treated with no, low (e.g., extracellular, 0.1 mM), or high (e.g., intracellular, 10 mM) levels of glutathione in PBS. The fluorescence was then monitored over 48 h at 25 °C (Figure 3B and S4, S5 in the SI). As expected, when encapsulated in either micelles or nanoemulsions, **2** shows reduced turn-on. After a 12 h incubation in 10 mM GSH at 25 °C, micelles and emulsions show similar levels of cargo protection, having 79% and 83% less fluorescence than the free dye, respectively. After 48 h, micelles and emulsions exhibit 85% and 70% less release than the free dye, indicating that, at room temperature, micelles show delayed release kinetics over time compared to emulsions. However, after 48 h at physiological temperature (37 °C), similar levels of protection are observed for the two vehicles (Figures S6–S8 in the SI). As seen in Figure 3C which compares 48 h fluorescence measurements at 25 °C or 37 °C, the micelles and emulsions both effectively reduce fluorescence by roughly 40% compared to free dye, although there is more significant turn-on of **2** at elevated temperatures. Together, these data demonstrate that encapsulation of a fluorogenic dye within the perfluorocarbon droplets or fluorous micelles effectively shields cargo from environmental stimuli. Yet, different turn-on kinetics are observed for the two vehicles, suggesting opportunities for engineering slow release of drugs by tuning the fluorous delivery vehicle.

In summary, we have prepared the first fluorogenic fluorous probe. This probe is soluble in a range of solvents and readily responds to reducing agent with over 100-fold turn-on. By incorporating **2** into either perfluorocarbon-in-water nanoemulsions or Zonyl FSN-100 stabilized micelles, turn-on can be reduced through protection of the encapsulated fluorogenic dye from the exterior environment. Over time, however, leakage dynamics of these delivery vehicles allow for the turn-on to be modulated. An understanding of the release dynamics for these systems is of interest for future applications within delayed or sustained delivery of therapeutic payloads.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Biographies

Prof. **Ellen Sletten** received her BSc in chemistry from Stonehill College in 2006 and PhD in chemistry in 2011 from UC Berkeley. Prof. Sletten's graduate work was performed in Carolyn Bertozzi's Laboratory and involved the optimization and development of bioorthogonal chemistries. Upon graduation, Prof. Sletten performed postdoctoral studies in Timothy Swager's group at MIT, where she worked on dynamic fluorous materials. Prof. Sletten began her independent career as an assistant professor in the Department of Chemistry and Biochemistry at UCLA in late 2015. The Sletten group focuses on methods to control and detect chemistries in living systems and their work has been recognized with an Alfred P. Sloan Award and NIH New Innovator Award.

Rachael Day received her BSc in chemistry from Dordt University in 2016 and is currently pursuing her PhD in Chemical Biology in Prof. Sletten's group at UCLA where she is studying the interactions of perfluorocarbon nanoemulsions with biological systems.

Margeaux Miller received her BA in chemistry from Barnard College in 2016. She is currently a PhD student in Prof. Ellen Sletten's group studying the synthesis of perfluorinated molecules and their applications in chemical biology.

Daniel Estabrook received his BSc in chemistry from the University of Massachusetts, Amherst in 2016. He is currently a PhD student at the University of California, Los Angeles within Prof. Ellen Sletten's group developing custom polymeric surfactants for functionalizable nanoemulsions.

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(39). 2-Oxo-2H-chromen-7-yl [2-(Pyridin-2-yldisulfaneyl)ethyl] Carbonate (5) To activated disulfide 4 (600 mg, 1.64 mmol, 1.00 equiv) was added 7-hydroxy coumarin 3 (318 mg, 1.97 mmol, 1.20 equiv), DMF (31 mL, 0.05 M), and DMAP (23 mg, 0.20 mmol, 0.12 equiv). To the solution was added DIPEA (340 μL, 1.97 mmol,1.20 equiv). The solution was stirred for 21 h at room temperature and then concentrated. The crude material was purified by column chromatography (silica gel, 35% → 50% EtOAc/hexanes). Extensive drying provided the product as a white amorphous solid (427 mg, 1.14 mmol, 70% yield). R_f= 0.17 (in 40% EtOAc/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.49 (ddd, J = 4.8, 1.8, 1.0 Hz, 1 H), 7.71–7.62 (m, 3 H), 7.50 (d, J = 8.5 Hz, 1 H),7.21 (d, J = 2.2 Hz, 1 H), 7.16–7.08 (m, 2 H), 6.41 (d, J = 9.6 Hz, 1H), 4.55 (t, J = 6.4 Hz, 2 H), 3.16 (t, J = 6.4 Hz, 2 H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 160.3, 159.3, 154.8, 153.3, 152.6, 150.0, 142.8, 137.2, 128.8, 121.2, 120.3, 117.8, 117.0, 116.5, 110.0, 66.7, 36.9. HRMS (ESI): *m/z* calcd for C + 17H_{14}NO + 5S_2 $[M + H]^+$: 376.0313; found: 376.0307.$_f{}^1{}_3{}^{13}{}_{3142}{}^+$

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(41). Fluorous Fluorogenic Coumarin 2To fluorous thiol 6 (120 mg, 0.158 mmol, 1.10 equiv) was added 5 (54 mg, 0.14 mmol, 1.0 equiv) and $CHCl_3$ (2.9 mL, 0.05 M). The solution became pale yellow within 5 min and became more yellow over time. The reaction was stirred at room temperature for 18 h. The crude product was evaporated onto silica from $CHCl_3$ and purified by column chromatography (silica gel, 20% → 40% → 60% EtOAc/hexanes) to afford the product as a white solid (107 mg, 0.105 mmol, 75% yield). The average yield across three reactions was 74%. R_f= 0.66 (in 40% EtOAc/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.69 (dd, J = 9.6, 0.6 Hz, 1 H), 7.50 (d, J = 8.5 Hz, 1 H), 7.22 (d, J = 2.3 Hz, 1 H), 7.14 (dd, J = 8.5, 2.3 Hz, 1 H), 6.42 (d, J = 9.6 Hz, 1 H), 4.55 (t, J = 6.5 Hz, 2 H), 3.04 (t, J = 6.5 Hz, 2 H), 2.79 (d, J = 6.1 Hz, 2 H), 2.19–2.05 (m, 4 H), 1.90 (h, J =6.3 Hz, 1 H), 1.84–1.67 (m, 4 H). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 160.3, 154.8, 153.3, 152.8, 142.8, 128.8, 117.7, 117.1, 116.5, 110.0, 66.8, 43.2, 36.8, 36.7, 28.2 (t, J_{CF} = 22.6 Hz), 23.0 (t, J_{CF} = 31.4 Hz). ^{19}F NMR (282 MHz, $CDCl_3$): δ = −80.6 to −80.9 (m), – 114.2 to −114.5 (br s, 4 F), −121.9 (br s, 4 F), −122.8 (br s, 4 F), – 123.3 (br s, 4 F), −125.9 to −126.3 (m, 4 F). HRMS (ESI): *m/z* calcd for C_{30}H F + 20 26O_5S_2 $[M + H]$: 1019.0415; found: 1019.0405.$_{33}f{}^1{}_3{}^{13}{}_3$CFCF$^{19}{}_{33052}$

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(43). Although 2 was soluble in fluorous solvent, there was aggregation seen via a hypsochromic shoulder in the absorbance spectra in all solvents tested (Figure S1 in the SI).

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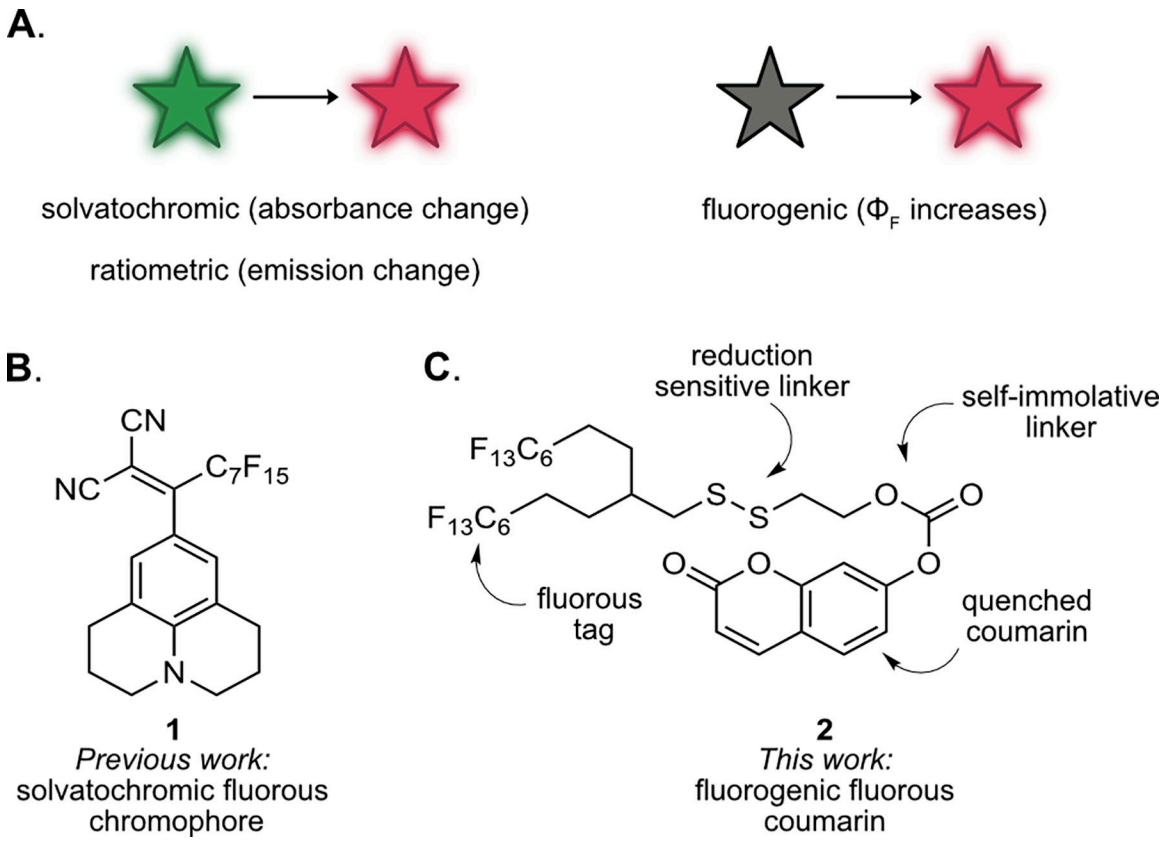

Figure 1.

(A) Schematic of chromophores sensitive to their environment. (B) Solvatochromic fluorous fluorophore from ref. 28. (C) Fluorogenic fluorous coumarin presented herein.

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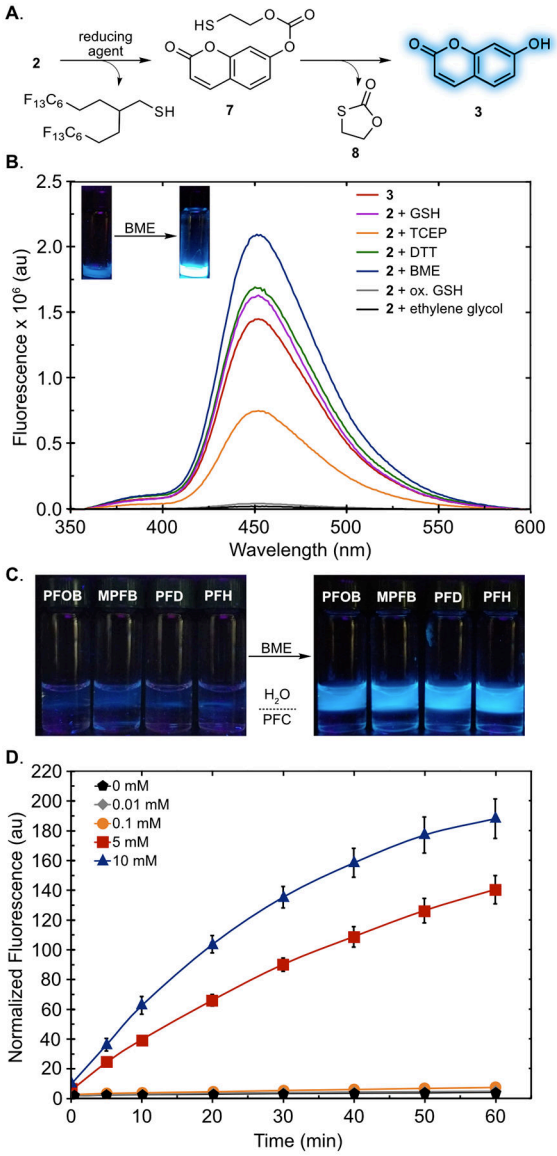

Figure 2.

(A) Cleavage of disulfide bond in the presence of reducing agent to release a free thiol that rapidly undergoes self-immolation to give fluorescent 7-hydroxycoumarin. (B) Fluorescence spectra of **2** (49.1 μM, 1:1 acetone/PBS pH 7.4) after 30 min treatment with 10 mM: glutathione (GSH, purple), tris(carboxyethylphosphine) (TCEP, orange), dithiothreitol (DTT, green), β-mercaptoethanol (BME, blue), oxidized glutathione (Ox. GSH, gray), and ethylene glycol (black). 7-hydroxycou-marin **3** (49.1 μM in 1:1 acetone/PBS) is shown in red. Inset: Long-wave UV image of **2** in 1:1 acetone/PBS before and after the addition of 10 mM β-mercaptoethanol (BME). (C) Long-wave UV images of **2** (0.05 mg in 0.75 mL solvent partitioned against 0.75 mL PBS pH 7.4) Perfluorooctyl bromide (PFOB), methoxyperfluorobutane (MPFB), perfluorodecalin (PFD), perfluorohexane (PFH) before and after reduction with 10 mM BME. (D) Time-dependent turn-on of **2** (19.1 βM, 1:1 acetone/PBS pH 7.4) in the presence of intra- (5–10 mM) and extracellular (0–0.1 mM)

concentrations of glutathione: (0 mM, black hexagon; 0.01 mM, gray diamond; 0.1 mM, orange circle; 5 mM, red square; 10 mM, blue triangle).

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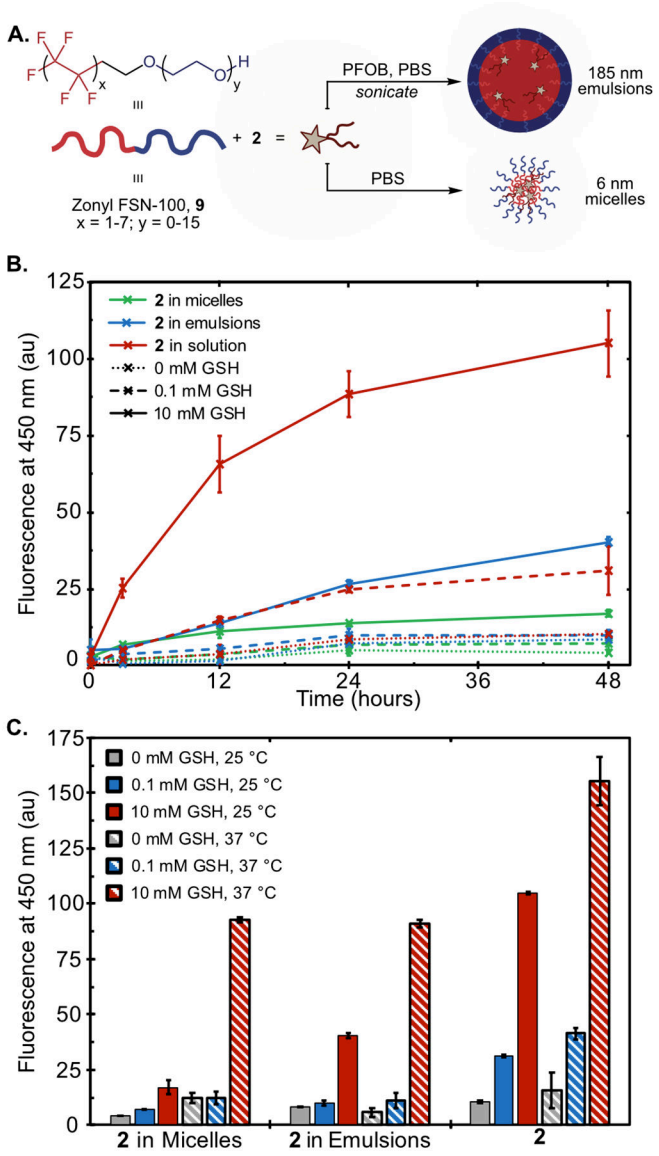

Figure 3.

(A) Fluorogenic coumarin **2** was loaded into either perfluorooctylbromide-in-water emulsions or micelles formed from fluorosurfactant **9**. See Figures S2, S3 (SI) for size analysis of emulsions and micelles, respectively. See Figures S4, S6 (SI) for emission spectra of emulsions and micelles containing **2** at 25 °C and 37 °C, respectively. (B) Emulsions and micelles were exposed to no, low (0.1 mM, extracellular), or high (10 mM, intracellular) levels of glutathione (GSH) in PBS, and fluorescence was monitored over 48 h at 25 °C. See Figure S5 (SI) for inset. (C) Fluorescence turn-on of **2** in emulsions, micelles, or free in solution after 48 h at both 25 °C and 37 °C. See Figures S7, S8 (SI) for fluorescence traces over time at 37 °C.

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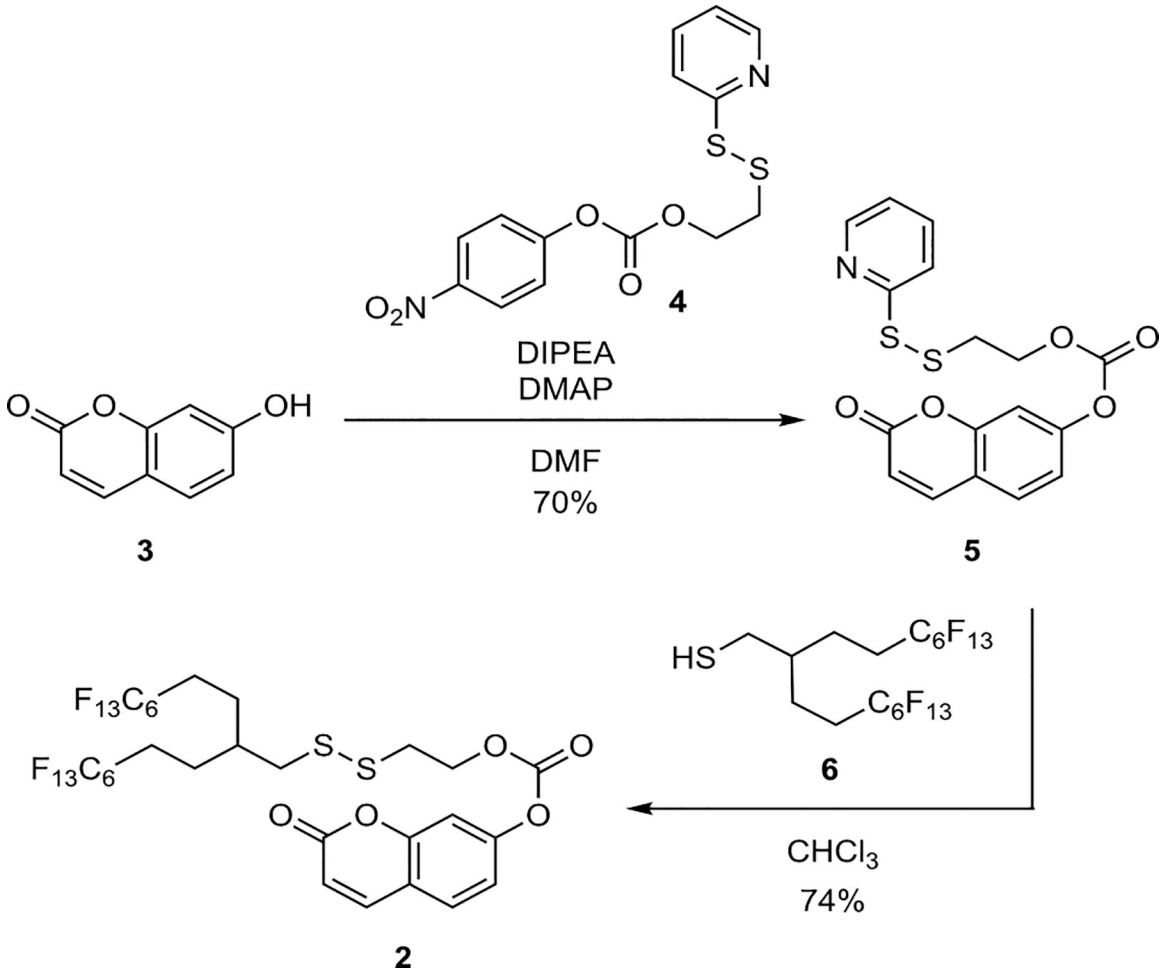

Scheme 1.
Synthesis of fluorous fluorogenic coumarin **2**