Synthesis and Photostability of Unimolecular Submersible Nanomachines. Toward Single-Molecule Tracking in Solution-S1

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1. Synthetic Methods
All glassware was oven-dried overnight prior to use. Reagent grade dichloromethane (CH₂Cl₂) was distilled from calcium hydride (CaH₂) under N₂ atmosphere. All reactions were carried out under a nitrogen atmosphere unless otherwise noted. All other chemicals were purchased from commercial suppliers and used without further purification. Flash column chromatography was performed using 230-400 mesh silica gel from EM Science. Thin layer chromatography (TLC) was performed using glass plates pre-coated with silica gel 40 F₃₅ 0.25 mm layer thickness purchased from EM Science. ¹H NMR and ¹³C NMR spectra were recorded at 400, 500 or 600 and 100, 125 or 150 MHz, respectively. Chemical shifts (δ) are reported in ppm from tetramethylsilane (TMS). FTIR spectra were recorded using a FTIR infrared microscope with ATR objective with 2 cm⁻¹ resolution.

**Cy5-COT-acid (8).** An oven-dried 250 mL round-bottom flask equipped with a stir bar was charged with amine 4¹⁶ (1.30 g, 5.97 mmol), acid ⁵¹⁷ (2.54 g, 7.17 mmol), and EDC (1.49 g, 7.76 mmol) to which were added CH₂Cl₂ (70 mL). The mixture was stirred at ambient temperature for 21 h and concentrated under vacuum. The residue was purified by column chromatography (silica gel, 10% MeOH in CHCl₃) to afford the crude COT-amide 6 as a red oil, which was used in the next step without further purification. An oven-dried 100 mL round-bottom flask equipped with a stir bar was charged with acid 7 (0.878 g, 2.48 mmol) and malondialdehyde bis(phenylimine) monohydrochloride (0.642 g, 2.48 mmol) to which were added Ac₂O (7 mL).
The reaction mixture was heated in an oil bath at 120 °C for 30 min. The mixture was cooled to room temperature and a solution of COT-amide 6 (1.38 g, 2.48 mmol) in pyridine (14 mL) was added. The mixture was stirred at ambient temperature for 2 h and concentrated under vacuum. The residue was dissolved in CHCl₃ (100 mL) and washed with H₂O (30 mL). The organic layer was dried with Na₂SO₄ and concentrated under vacuum. The residue was purified by flash chromatography (silica gel, 15% MeOH in CHCl₃) to yield 8 as a blue solid (1.20 g, 29 %): ATR FT-IR 2930, 2870, 1700, 1650, 1560, 1540, 1480, 1450, 1370, 1330, 1220, 1130, 1090, 1070, 990 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 8.32 (br s, 1H), 8.03 (br s, 1H), 7.92 (td, J₁ = 13.0, J₂ = 5.6 Hz, 2H), 7.43–7.33 (m, 4H), 7.32–7.18 (m, 2H), 7.12 (dd, J₁ = 15.4, J₂ = 7.9 Hz, 2H), 6.76 (t, J = 12.5 Hz, 1H), 6.33 (d, J = 13.6 Hz, 1H), 6.21 (d, J = 13.6 Hz, 1H), 5.76-5.65 (m, 6H), 5.58 (s, 1H), 4.03 (dt, J₁ = 16.6, J₂ = 7.7 Hz, 4H), 3.41 (br s, 4H), 2.48–2.32 (m, 8H), 1.86-1.74 (m, 8H), 1.72 (s, 12H), 1.55-1.49 (m, 4H); ¹³C NMR (100 MHz, CDCl₃) δ 176.16, 174.27, 173.51, 172.93, 172.30, 153.05, 152.62, 143.13, 141.80, 141.71, 140.95, 140.81, 133.87, 132.21, 131.84, 131.59, 131.39, 130.77, 128.76, 128.68, 126.66, 126.04, 125.33, 125.08, 122.16, 122.12, 110.79, 110.43, 103.92, 103.21, 49.26, 49.11, 44.25, 43.94, 39.55, 39.41, 35.88, 35.50, 34.41, 33.51, 28.02, 27.97, 26.87, 26.74, 26.14, 26.11, 25.07, 24.46; HRMS (ESI) m/z calculated for [M]+ C₅₀H₆₃N₄O₄ 783.4840, found 783.4860.
Cy5-COT-alkyne (9). An oven-dried 50 mL round-bottom flask equipped with a stir bar was charged with COT-Cy5-acid 8 (159.1 mg, 0.184 mmol), propargyl alcohol (32 µl, 0.552 mmol), EDC (45.9 mg, 0.239 mmol) and DMAP (1.1 mg, 0.009 mmol) to which were added CH₂Cl₂ (2 mL). The mixture was stirred at ambient temperature for 17 h and concentrated under vacuum. The residue was purified by flash chromatography (silica gel, 15% MeOH in CHCl₃) to yield 9 as a blue solid (141.2 mg, 85%): ATR FT-IR 3070, 2930, 1730, 1650, 1480, 1450, 1370, 1330, 1220, 1130, 1090, 1046, 988 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 8.39 (s, 1H), 8.08 (s, 1H), 7.85-7.76 (m, 2H), 7.41-7.34 (m, 4H), 7.28-7.21 (m, 2H), 7.13 (d, J = 8.0 Hz, 1H), 7.03 (d, J = 8.0 Hz, 1H), 6.86 (t, J = 12.6 Hz, 1H), 6.47 (d, J = 14.0 Hz, 1H), 6.26 (d, J = 13.2 Hz, 1H), 5.76-5.66 (m, 6H), 5.57 (s, 1H), 4.65 (d, J = 2.4 Hz, 2H), 4.09 (t, J = 7.6 Hz, 2H), 4.00 (t, J = 7.6 Hz, 2H), 3.44-3.39 (m, 4H), 2.48 (t, J = 2.5 Hz 1H), 2.47-2.37 (m, 8H), 1.86-1.70 (m, 8H), 1.71 (s, 6H), 1.70 (s, 6H), 1.55-1.49 (m, 4H); ¹³C NMR (100 MHz, CDCl₃) δ 173.88, 173.23, 173.06, 172.32, 172.09, 153.27, 152.62, 143.28, 141.88, 141.66, 140.96, 140.76, 132.27, 131.85, 131.55, 131.28, 130.69, 128.81, 128.63, 126.52, 125.85, 125.49, 125.03, 122.21, 122.12, 110.93, 110.22, 104.05, 102.94, 77.55, 74.92, 51.80, 49.36, 49.06, 44.43, 43.94, 39.52, 39.41, 35.92, 35.52, 33.50, 33.42, 28.07, 27.95, 26.93, 26.88, 26.18, 25.04, 24.26; HRMS (ESI) m/z calculated for [M]+ C₅₃H₆₅N₄O₄ 821.5000, found 821.5011.
**USN-2.** A 2 mL vial charged with diazide 10 (7.26 mg, 0.0129 mmol), cy5 dye 9 (27 mg, 0.0298 mmol), CuSO$_4$·5H$_2$O(s) (0.65 mg, 0.0026 mmol) and sodium ascorbate (1.28 mg, 0.0065 mmol) was sealed with a rubber septum cap. A well-degassed mixture of CH$_2$Cl$_2$ (0.07 mL) and H$_2$O (0.07 mL) was added to the vial, and the vial was shaken by a wrist-action shaking machine for 36 h. The mixture was partitioned between CH$_2$Cl$_2$ (5 mL) and H$_2$O (5 mL). The organic phase was dried over anhydrous MgSO$_4$, filtered, and the filtrate was concentrated under vacuum. The crude product was purified by flash chromatography (silica gel, 2% to 15% MeOH in CH$_2$Cl$_2$). The blue solid was dissolved in a 1:1 mixture of CH$_3$CN:H$_2$O (4 mL) and NH$_4$PF$_6$ (104.32 mg, 0.64 mmol) was added for the ion exchange step. The organic phase was removed by vacuum and the solid was filtered, collected and washed with H$_2$O (5 mL × 5) and then with CH$_2$Cl$_2$ (5 mL × 3). The CH$_2$Cl$_2$ was evaporated under reduced pressure and the solid was collected and dried to afford the desired USN-2 as a blue solid (23 mg, 71%): ATR FT-IR 2920, 2870, 1700, 1650, 1580, 1560, 1510, 1490, 1480, 1460, 1420, 1390, 1370, 1360, 1340, 1210, 1170, 1130, 1090, 1040, 1020, 994 cm$^{-1}$; $^1$H NMR
(600 MHz, CD3CN) δ 8.14-8.02 (m, 4H), 7.99 (br s, 1H), 7.86–7.81 (m, 2H), 7.78 (d, J = 8.2 Hz, 1H), 7.55-7.51 (m, 4H), 7.51–7.46 (m, 4H), 7.44–7.35 (m, 4H), 7.30-7.20 (m, 8H), 7.16 (d, J = 8.0 Hz, 2H), 6.99 (dd, J1 = 8.1, J2 = 1.8 Hz, 1H), 6.84 (ddd, J1 = 8.3, J2 = 6.8, J3 = 1.3 Hz, 1H), 6.76 (d, J = 8.5 Hz, 1H), 6.67-6.56 (m, 5H), 6.55-6.47 (m, 2H), 6.26-6.19 (m, 2H), 6.15 (dd, J1 = 13.7, J2 = 3.3 Hz, 2H), 5.87-5.60 (m, 12H), 5.55 (br s, 2H), 5.23-5.17 (m, 2H), 5.11-5.06 (m, 2H), 4.63 (t, J = 6.4 Hz, 2H), 4.36 (dt, J1 = 12.8, J2 = 6.3 Hz, 1H), 4.31 (dt, J1 = 13.4, J2 = 6.5 Hz, 1H), 4.23 (p, J = 6.8 Hz, 1H), 4.00 (q, J = 7.6 Hz, 4H), 3.94-3.85 (m, 4H), 3.74 (dd, J1 = 15.4, J2 = 6.2 Hz, 1H), 3.26-3.15 (m, 8H), 3.05 (t, J = 6.4 Hz, 2H), 2.71 (t, J = 6.1 Hz, 2H), 2.68 (d, J = 15.55 Hz, 1H), 2.28-2.20 (m, 10H), 2.13-2.08 (td, J1 = 7.4, J2 = 4.4 Hz, 4H), 1.76 (m, 4H), 1.72-1.64 (m, 28H), 1.66-1.59 (m, 4H), 1.58-1.51 (m, 4H), 1.42 (m, 4H), 1.36 – 1.28 (m, 6H), 0.70 (d, J = 6.8 Hz, 3H). 13C NMR (150 MHz, CD3CN) δ 174.00, 173.97, 173.72, 173.71, 173.42, 173.37, 173.03, 154.40, 154.26, 148.16, 147.67, 143.98, 143.22, 142.84, 142.80, 142.02, 141.95, 140.68, 138.11, 136.00, 134.74, 134.25, 133.63, 132.64, 132.37, 132.23, 131.67, 131.59, 131.17, 131.07, 130.15, 129.83, 129.14, 129.11, 128.75, 128.34, 128.31, 127.26, 127.18, 125.90, 125.65, 125.61, 125.55, 125.24, 125.06, 125.00, 124.74, 122.83, 122.12, 121.76, 111.63, 111.47, 103.71, 103.48, 87.35, 86.59, 82.81, 82.36, 57.89, 57.76, 49.83, 49.75, 49.21, 49.04, 44.49, 44.32, 39.93, 39.86, 39.60, 38.49, 36.19, 35.86, 33.96, 33.83, 29.78, 27.36, 27.16, 27.13, 26.61, 26.35, 25.55, 24.82, 21.69, 21.40, 19.17; HRMS (ESI) m/z calculated for [M-2PF6]1+ C141H156N14O8S 1102.5970, found 1102.5957.
USN-3. A 2 mL vial charged with diazide 11 (8.55 mg, 0.0144 mmol), cy5 dye 9 (30 mg, 0.0330 mmol), CuSO₄·5H₂O(s) (0.72 mg, 0.0029 mmol) and sodium ascorbate (1.42 mg, 0.0072 mmol) was sealed with a rubber septum cap. A well degassed mixture of CH₂Cl₂ (0.1 mL) and water (0.1 mL) was added to the vial, and the vial was shaken by a wrist-action shaking machine for 36 h. The mixture was partitioned between CH₂Cl₂ (5 mL) and H₂O (5 mL). The organic phase was dried over anhydrous MgSO₄, filtered, and the filtrate was concentrated under vacuum. The crude product was purified by flash chromatography (silica gel, 2% to 15% MeOH in CH₂Cl₂). The blue solid was dissolved in a 1:1 mixture of CH₃CN:H₂O (4 mL) and NH₄PF₆ (117.36 mg, 0.72 mmol) was added for the ion exchange step.
The organic phase was removed by vacuum and the solid was filtered, collected and washed with H$_2$O (5 mL x 5) and then with CH$_2$Cl$_2$ (5 mL x 3). The CH$_2$Cl$_2$ was evaporated under reduced pressure and the solid was collected and dried to afford the desired USN-3 as a blue solid (24 mg, 67%). ATR FT-IR (neat) 2930, 2870, 1700, 1650, 1560, 1510, 1480, 1450, 1390, 1370, 1330, 1220, 1170, 1130, 1090, 1040, 1020, 992 cm$^{-1}$; $^1$H NMR (600 MHz, CD$_3$CN) δ 8.14-8.02 (m, 4H), 7.99 (br s, 1H), 7.73-7.67 (m, 2H), 7.65 (d, $J = 8.3$ Hz, 1H), 7.58 – 7.52 (m, 2H), 7.51-7.45 (m, 5H), 7.44-7.36 (m, 5H), 7.31-7.22 (m, 7 H), 7.21-7.11 (m, 4 H), 7.02-6.96 (m, 1H), 6.70 (dd, $J_1 = 8.1$, $J_2 = 1.8$ Hz, 1H), 6.66-6.56 (m, 4H), 6.56-6.46 (m, 2H), 6.30 (d, $J = 1.8$ Hz, 1H), 6.23 (d, $J = 13.8$ Hz, 2H), 6.16 (dd, $J_1 = 13.8$, $J_2 = 3.4$ Hz, 2H), 5.95-5.57 (m, 12H), 5.55 (br s, 2H), 5.23-5.14 (m, 4H), 4.66-4.59 (m, 2H), 4.46-4.36 (m, 2H), 4.07-3.97 (m, 5H), 3.90 (t, $J = 7.5$ Hz, 4H), 3.80 (dd, $J_1 = 11.08$, $J_2 = 3.55$ Hz, 1H), 3.21 (br s, 8H), 3.13 (dd, $J_1 = 11.5$, $J_2 = 3.1$ Hz, 1H), 3.05 (t, $J = 6.34$ Hz, 2H), 2.77 (t, $J = 6.36$ Hz, 2H), 2.34-2.20 (m, 10H), 2.13-2.08 (m, 4H), 1.80-1.73 (m, 4H), 1.71-1.64 (m, 28 H), 1.64-1.59 (m, 4H), 1.57-1.51 (m, 4H), 1.45-1.39 (m, 4H), 1.36-1.28 (m, 6H), 0.71 (d, $J = 6.7$ Hz, 3H). $^{13}$C NMR (150 MHz, CD$_3$CN) δ 173.09, 172.83, 172.80, 172.52, 172.49, 172.13, 153.50, 153.37, 143.08, 142.32, 142.07, 141.93, 141.91, 141.12, 141.06, 137.95, 137.84, 135.42, 135.35, 135.16, 134.09, 133.35, 131.74, 131.48, 131.33, 131.26, 131.13, 130.69, 130.20, 130.14, 129.91, 129.66, 128.87, 128.24, 128.21, 127.62, 127.52, 127.38, 126.36, 126.31, 125.70, 125.02, 124.75, 124.65, 124.34, 124.18, 124.13, 123.32, 121.94, 121.12, 119.90, 110.74, 110.58, 102.81, 102.59, 86.74, 85.64, 85.18, 81.18, 57.01, 56.97, 48.93, 48.86, 48.29, 48.20, 43.59, 43.42, 38.96, 38.71, 36.32, 35.29, 34.96, 33.06, 32.93, 31.68, 28.88, 26.47, 26.45, 26.24, 25.71, 25.45, 24.66, 23.92, 23.90, 20.80, 20.47, 17.73; HRMS (ESI) m/z calculated for [M-2PF$_6$]$^{+2}$ C$_{141}$H$_{156}$N$_{14}$O$_{8}$S$_{2}$ 1118.5831, found 1118.5815.
2. Monitoring of half rotation of the motor

A NMR tube was charged with 0.7 mL of a 1 mM solution of USN-3 in CD$_3$CN. The $^1$H-NMR spectrum of the sample was recorded using a Bruker AVANCE III HD 600 MHz High Performance Digital NMR. Then the sample was excited at 365 nm for 1 h using a UVGL-25 lamp (4 W) and the $^1$H-NMR spectra was recorded. The sample was heated in a water bath at 60 °C for 1 h, then the $^1$H-NMR spectra was recorded. The yields of the conversion were calculated using the integration values of the methyl group (Me).

3. Measurement of optical properties

UV/visible spectra were recorded on a Shimadzu UV-2450 spectrophotometer using spectroscopic grade CHCl$_3$. The fluorescence emission spectra were obtained on a PerkinElmer LS50B instrument using CHCl$_3$ solutions open to air. All fluorescence spectra were corrected. Excitation was done at 630 nm wavelength.

![Figure S1](image)

**Figure S1.** (A) UV/vis absorption spectra and (B) Fluorescence spectra of 1.0 µM solutions of USN-1, USN-2, and USN-3 in CHCl$_3$. Excitation was done at 630 nm.
The fluorescence quantum yields were determined by the comparative method of Williams et al.\textsuperscript{18} and using Zinc phthalocyanine as a reference, $\Phi_F = 0.30$\textsuperscript{19} in benzene, $\lambda_{\text{exc}} = 630$ nm. For the purpose of the quantum yield measurement, the solutions were bubbled with high purity N$_2$ for 20 min immediately before the fluorescence spectrum was recorded. The absorbance was measured afterwards.

$$\Phi_x = \Phi_{st} \left( \frac{Grad_x}{Grad_{st}} \right) \left( \frac{\eta_x^2}{\eta_{st}^2} \right)$$

4. Emission time extension analysis

Data was acquired using an Olympus IX81 microscope adapted for laser-fluorescence microscopy. A 637nm CW laser diode (Coherent, OBIS 637LX) was used as an excitation source. Excitation light was focused on the back aperture of a 100x oil immersion objective (Olympus, UPlanSApo 100x/N.A 1.4) with an average excitation power density of $\sim 150 \frac{W}{cm^2}$ in the sample plain. Sample fluorescence was collected by the same objective and imaged through a polychroic beamsplitter (Chroma, ZT473/532/637 rpc-xt890) and emission filter (Semrock, 679/41 Brightline) onto an electron multiplying charge coupled device (EMCCD) camera (Andor, iXon Ultra 897) with single-molecule detection sensitivity.

USNs were dissolved in DMSO and deposited on clean coverslips for single molecule imaging. Prior to use, coverslips were cleaned with hydrochloric and nitric acids (1:2 HCl:HNO$_3$ v/v) and washed repeatedly with acetone, ethanol and deionized water. All measurements were carried out at room temperature under ambient conditions. Upon excitation, surface bound USNs were detected as fluorescent spots in the field of view (80 x 80 µm$^2$). Time lapse movies were acquired for several hundred USNs ($N = \sim 700$) in order to assess their photostability (500 frames, 50 ms/frame). The first ten frames of each movie were intensity averaged (Figure S2)
and individual USNs were located by 2D Gaussian fitting of the isolated fluorescent spots. After localization, time traces of fluorescence intensity were calculated by taking the average intensity of a 3 x3 pixel window around the center of each fitted Gaussian. In order to account for the uneven excitation intensity, a local background subtraction was performed for each molecule by taking the average intensity of a 7 x7 pixel contour window around the fitted Gaussian (Figure S3). An intensity threshold was set in order to extract and sum the “on” (fluorescent state) and “off” (non-fluorescent state) times for the individual USNs (Figure S4). Data analysis was carried out using custom MATLAB code.

![Figure S2. Representative field of view. Average image of first ten frames.](image)

Figure S2. Representative field of view. Average image of first ten frames.
**Figure S3.** 2D Gaussian fits define the regions for analysis. The green contours are the fit envelopes, the black squares are the intensity detection windows, and the red squares are the contour for local background substraction.

**Figure S4.** Example for on-time and off-time analysis of the time traces. The green area represents the “on” level where the molecule is emitting, and the red area represents the “off” level.
Figure S5. Pie plot comparison of fluorescence total ($\tau_{on}$). A substantial increase on $\tau_{on}$ was achieved by using cy5-COT fluorophores in USN-2.

Figure S6. Histogram comparison of total ($\tau_{on}$). Each histogram distribution was fitted to an exponential decay law and the average decay times were calculated. A simple average over all total ($\tau_{on}$) was calculated as well and presented as mean total $\tau_{on}$. 
Cy5-COT-alkyne-9
