Reversibly Photoswitchable Dual-Color Fluorescent Nanoparticles as a New Tool for Live Cell Imaging

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Experimental Methods

Reagents. All reagents and solvent were purchased from Aldrich Chemical Co. and used as received except stated otherwise. Monomer 2, 2'-N-isopropylacrylamide (NIPAM) and polymerization initiator 4, 4'-azobis (4-cyano-valeric acid) (ABVA) were recrystal lized from n-hexane and methanol, respectively. Styrene (St, 99%) was distilled from CaH_2 under reduced pressure.

Synthesis of 5-(1,3-dihydro-3,3-dimethyl-6-nitrospiro[2H-1-benzopyran-2,2'-(2H)-indole])ethyl acrylate (SP). 5-(1,3-dihydro-3,3-dimethyl-6-nitrospiro[2H-1-benzopyran-2,2'-(2H)-indole])ethyl acrylate (SP) was synthesized according to procedures described in the literature (Raymo F. M.; Giordani, S. *J. Am. Chem. Soc*. **2001**, *123* (19): 4651-4652 and Zhu M.-Q.; Zhu L.Y.; Han J. J.; Wu W.W.; Hurst J.K.; Li A. D. Q. *J. Am. Chem. Soc*. **2006**, *128*, 4303-4309). Yield is 31%. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.14 and 1.35 (3H each, s, $C(CH_3)_2$), 3.53 (2H, m, $COOCH_2$), 4.36(2H, m, NCH_2), 5.87 and 6.14 (2H and 1H, m, CH_2=CH), 6.36 (1H, d, CCH=C*H*Ph), 6.78 (1H, d, 7'-H of indoline), 6.87 (1H, d, CC*H*=CHPh), 6.95 (2H, m, , 4' and 5'-H of indoline), 7.17 (1H, d, 6'-H of indoline), 7.20 (1H, m, 4-H of benzopyran), 8.0 (2H, m, 5 and 7-H of benzopyran).

Synthesis of bis-N, N'-(2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl) perylenetetracarboxylic diimide-acrylate conjugate (PA). Bis-N, N'-(2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl) perylenetetracarboxylic diimide was synthesized according to a procedure reported earlier **(**Wang W.; Li L.-S.; Helms G.; Zhou H.-H.; Li A. D. Q. *J. Am. Chem. Soc*., **2003**, *125* , 1120 -1121). In a typical procedure, a mixture of bis-N, N'-(2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl)perylenetetracarboxylic diimide (0.2 mmol) and triethylamine (0.6 mmol) was dissolved in 20-ml anhydrous CH_2Cl_2 and cooled in an ice bath under an argon atmosphere. Acryloyl chloride (1 mmol) was added dropwise while stirring and the mixture at room temperature for additional 24 hours. The accumulated triethylamine hydrochloride salt was removed by filtration, the filtrate was rotoevaporated to dryness, and the residue was purified by preparative silica gel chromatography (solvent: CH_2Cl_2: CH_3OH; 20:1, v/v), yielding a dark- red solid (42%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.55-3.78 (36H, m, CH_2OCH_2), 3.88 (4H, t, OCH_2-C*H*$_2$N), 4.28 (4H, m, $COOCH_2$), 4.48 (4H, t, CH_2N), 5.8, 6.12 and 6.4 (2H, 2H and 2H, q, CH_2=CH), 8.48 (4H, d, Ar), 8.6(4H, d, Ar).

Characterization. ^{1}H NMR spectra were recorded in $CDCl_3$ using a JEOL JNM-AL300 instrument. A JEOL 1010 transmission electron microscope (TEM) operated at 100 kV was employed to obtain TEM images. The microscope sample was prepared by placing a

drop of the polymer dispersion on a carbon-coated Cu grid, followed by solvent evaporation at room temperature. Fluorescence spectra were recorded with SPEX Fluorolog-3-21 spectrometry with excitation at 420 nm (slit width: 3mm, 450 W Xe lamp). Spiropyran-merocyanine interconversions were performed using a 1.5 kW Xe lamp whose output was focused and passed through aqueous $CuSO_4$ and Schott UG 11 (light transmission between 260 nm and 390 nm; 2.8 x 10^{-9} Einstein) or Schott OG 515 glass filters (light transmission at $\lambda > 515$ nm; 8-10 x 10^{-10} Einstein).

Emulsion polymerization. Emulsion polymerization was carried out according to the literature procedure with minor modifications (Zhu M.-Q.; Zhu L.Y.; Han J. J.; Wu W.W.; Hurst J.K.; Li A. D.Q. *J. Am. Chem. Soc*. **2006**, *128*, 4303-4309) as shown in Table 1.

Table 1. Materials feed ratio for emulsion polymerization and the size of the obtained polymer nanoparticles.

Example	Surfactant Tween 20 (g)	NIPAM (g)	St (g)	SP (mg)	PA (mg)	Initiator (mmol)	Particle Size (nm)
1	0.05	0.4	0.7	5	0.01	0.01	110
2	0.05	0.4	0.7	5	0.05	0.01	56
3	0.1	0.1	1.0	2	0.1	0.01	50
4	0.05	0.4	0.7	0	0.05	0.01	60

Delivery of dual color nanoparticles into live cells. About $5x10^5$ human embryonic kidney (HEK-293) cells were grown on each 35mm polylysine coated glass bottom culture dish (MatTek Corp.) with 1 ml DMEM (Dulecco's Modified Eagle Medium) containing 10% FBS (Fetal Bovine Serum). Before each experiment, cells were washed with DMEM without FBS. Then, 1 pmol dual color nanoparticles in 50 μL DMEM and 10 μL Lipofectamine™ 2000 (Invitrogen) in 50 μL DMEM were mixed and the mixture was subsequently diluted to 500 μl using DMEM. The mixture was added to the cell culture and cells were imaged immediately. After 1 hour, cells were washed with 1 ml DMEM again, and 1 ml fresh DMEM containing 10% FBS was added to sustain cell survival, and live cell imaging continued at room temperature. After 6 hours, cells were retrieved to the CO_2 incubator and cultured overnight. After 16 hours, the cell was imaged again.

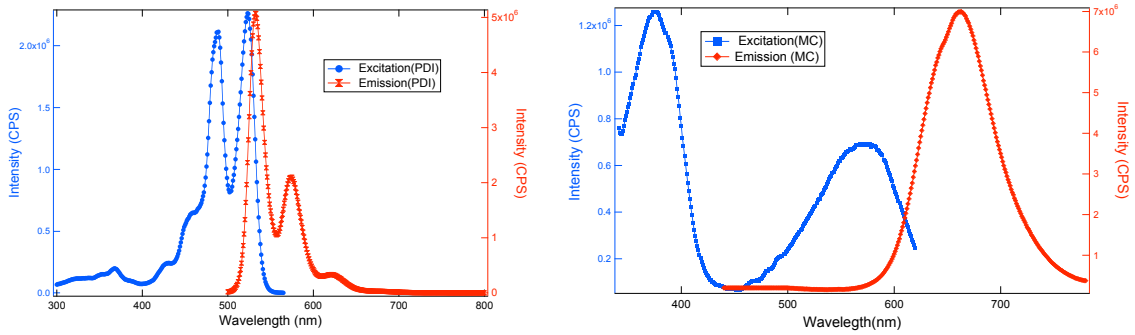
Live cell imaging.

Cells were imaged using Zeiss Axiovert 200 inverted microscope equipped with a liquid-nitrogen cooled CCD detector (Princeton Instruments, Roper Scientific). Firstly, cells were photographed under white light illumination. Then a 488-nm argon ion laser was used to image the green fluorescence and a 510±40 nm bandpass green filter was used for collecting green fluorescence; Afterward, a hand held UV lamp (365 nm) were used to photochemically converted the spiro-nanoparticles to mero-nanoparticles by illuminating cells for 10 sec, and subsequently, a 590-nm long pass red filter was used when red fluorescence was imaged under 488nm argon ion laser. The cycle of white

light-488-365 nm was repeated 3 times and dual color fluorescent nanoparticles showed the same green and red fluorescence pattern as shown in Figure 3.

Single particle spectroscopy. Samples were first brought into focus using wide field nanoparticle imaging. After a new area on the cover glass was moved into place and illuminated with 365-nm light for 3-4 s, the 488 nm line from an argon ion laser was directed into the back port of the microscope. Prior to entering the microscope, the laser beam was collimated and expanded to slightly overfill the back aperture of the objective with parallel rays to achieve a diffraction-limited spot.

To collect single particle spectra, an image of the nanoparticles was first obtained by scanning a small area (typically 10 x10 μm) of the sample over the diffraction limited laser spot using the nanopositioner. Emission from this type of scan was directed to the bottom microscope port and onto the APD. The resulting image showing individual particles (bright spots) was used to park the laser on a specific nanoparticle for spectral acquisition. The entire scanning and image generation routine was achieved using custom LabVIEW software. The typical step size and dwell (integration) times for each pixel were 200 nm and 100 ms, respectively. Once a specific nanoparticle was chosen, the light (emission) train was switched to the spectrometer/CCD, this time using the spectrometer's grating instead of the mirror. Spectra were acquired through WinSpec/32 software using a typical dwell time of 1-2 s. Acquisition continued until the fluorescence emission was no longer discernible from the baseline. Typical laser powers used for single particle spectral acquisition were 800 W/cm^2 at the cover glass surface. However, this power elicits much faster red-to-green fluorescence switching for the spectrometer/CCD to capture the spectrum and therefore, the laser power was lowered to 0.4 kW/cm^2 in order to show down the gradual changes from red fluorescence to green fluorescence for each single nanoparticle. The reverse process is too fast to be resolved even when using a low power UV lamp.



Supporting Figure 1. Spectra of fluorescence excitation (blue color) and emission (red color) of the two dyes used in the preparation of polymer nanoparticles: perylene diimide (PDI: circles and double triangles) and spiropyran-merocyanine (SP-MC: squares and diamonds).