Supporting Information

A Nanocrystal–based Ratiometric pH Sensor for Biological Applications

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**Experimental Procedures**

**Materials and Methods.** Poly(amido amine) generation 1 amine terminated dendrimer, with 8 terminal amines, was purchased from Dendritech. Trioctylphosphine oxide (90%, TOPO), cadmium oxide (CdO), 1-ethyl-3,3’-dimethylaminopropylcarbodiimide (EDC), *N*-hydroxysuccinimide (NHS), decylamine, and *N*,*N*’-dimethylformamide (DMF) were purchased from Sigma–Aldrich. 1-Tetradecylphosphonic acid (98%, TDPA), *n*-hexylphosphonic acid (HPA), dimethylcadmium (CdMe₂), and selenium shot were purchased from Alfa Aesar. Trioctylphosphine (TOP) was obtained from Strem Chemicals. Bovine serum albumin (BSA), Fraction V, bis(trimethylsilyl)sulfide [(TMS)₂S] and diethylzinc (ZnEt₂) were obtained from Fluka. SNARF–5F 5 (and –6) carboxylic acid was purchased from Molecular Probes, a division of Invitrogen. All materials were used as purchased, except for TOPO, which was purified through vacuum distillation, and diethylzinc, which was passed through a 0.2 μm syringe filter before use. Glass microslides were obtained from Vitrocom. Broadband filters and dichroics were obtained through Chroma Technologies. Float–a–lyzer tubing equipped with 500 Da MWCO membranes were purchased from Spectra–por. Centrifugal tubes equipped with 50000 Da MWCO filters were purchased from Millipore.

**Synthesis of CdSe / CdZnS Nanocrystals.** CdSe NCs overcoated with alloyed CdZnS were prepared by a modified literature method.¹ ² Briefly, CdO (0.128 g, 1.0 mmol), TDPA (0.418 g, 1.5 mmol) and TOPO (6 g, 15.5 mmol) was loaded into a degassed 3-neck flask and heated to 320 °C. Upon generation of a clear, colourless homogenous solution, the temperature was lowered to 270 °C and 1.5 M TOPSe (5 mL, 7.5 mmol, prepared from dissolving 5.92 g Se powder into 50 mL of TOP) was rapidly injected into the flask. The resulting solution was heated at 220 °C until the first absorption feature of the core CdSe NCs was 470 nm. The NCs were subsequently overcoated by injecting a hexane solution of the bare CdSe (prepared by size selected precipitation from the original growth solution) into a degassed solvent of TOPO (10 g, 25.9 mmol) and HPA (0.4 g, 2.4 mmol). The hexane was removed *in vacuo* at 80 °C from the CdSe cores, after to which decylamine (0.25 mL, 1.3 mmol) was added and stirred for 2 h. Using a syringe pump, two separate solutions of (TMS)₂S in 5 mL TOP and a 90:10 molar ratio of ZnEt₂ and CdMe₂ in 5 mL TOP were slowly dripped into the CdSe solution at 130 °C over the course of 2 h. Reagent doses were chosen to yield a ~3 monolayer shell on the bare CdSe NCs, using known methods.² The quantum

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yield of the NC sample was found to be $\Phi = 58\%$ in hexanes after one precipitation, with a FWHM = 32 nm. The lowest energy absorption peak was at 508 nm.

**Synthesis of Dihydrolipoic Acid–modified Poly(amide amine) (DHLA–PAMAM).**

Generation 1 poly(amide amine) [PAMAM] (1.60 g, 1.13 mmol) was dissolved in DMF. Approximately 2 equiv. of dihydrolipoic acid (DHLA, 0.467 g, 2.24 mol), prepared by literature methods, was added to a DMF solution containing EDC (1.29 g, 6.72 mmol) and NHS (0.774 g, 6.73 mmol). The DHLA solution was subsequently mixed with the PAMAM solution and stirred overnight. Removal of DMF in vacuo resulted in a pale amber coloured oil. The ligand was purified through dialysis with Spectra–por dialysis float–a–lyzer membranes equipped with 500 Da MWCO membranes. $^1$HNMR (500 MHz, CD$_3$OD, $\delta$): 3.39 (t, $J = 6.5$ Hz, 8H), 3.277 (br t, 16H), 3.08 (m, 2H), 2.94 (br t, ~16 H), 2.80 (t, $J = 6.5$ Hz, 24H), 2.62–2.5 (m, ~16 H), 2.40 (t, $J = 6.5$ Hz, 24 H), 2.16 (t, $J = 7$ Hz, 4H), 1.92–1.19 (m, ~20H).

**Cap–exchange of CdSe/CdZnS with DHLA–PAMAM.** One mL of the stock CdSe/CdZnS solution was precipitated by methanol and resuspended in 3 mL chloroform. 0.2 g of DHLA–PAMAM was dissolved in 2 mL of methanol, mixed with the chloroform solution of NCs, and stirred vigorously for 5 h. The solution was then transferred to a 40 mL vial, to which 30 mL of H$_2$O was added and stirred briefly. The resulting mixture was allowed to sit overnight, after which the dendrimer solubilized NCs phase transferred into the aqueous layer. Although most of the excess dendrimer remained in the organic phase, excess ligands were further removed through dialysis of the NC containing aqueous layer, using Millipore centrifuge tubes equipped with 50,000 Da molecular weight cut–off (MWCO) filters. The isolated aqueous compatible NCs were diluted in distilled water to a final volume of 10 mL. The purified dendrimer–solubilized NCs were found to have a quantum yield of $\Phi = 26\%$ in water and a FWHM = 32 nm.

**Conjugation of SNARF–5F 5 (and –6) to NCs.** SNARF–5F 5 (and –6) carboxylic acid (2.5 mg, 0.53 μmol) was activated for amide coupling with EDC (0.50 mg, 2.6 μmol) and NHS (0.31 mg, 2.7 μmol) in pH 6 MES buffer. After vigorous stirring for 20 min, the NHS–activated dye was added to 3.3 mL of the DHLA–PAMAM solubilized NCs and stirred overnight. The unreacted dye was removed through dialysis with 50,000 Da MWCO filters.

**Spectroscopic Characterization.** UV–vis spectra of SNARF coupled CdSe/CdZnS were obtained on a Hewlett–Packard 8453 UV–vis Spectrophotometer. The samples were diluted in appropriate standard pH phosphate buffers for pH 6–8 and a borate buffer for pH 9. Potassium phosphate buffered solution with 4% BSA were used for BSA calibration studies.

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Size measurements were obtained by using ProteinSolutions DynaPro Titan Dynamic Light Scatterer equipped with Dynamics version 6.7.7.9 instrument control software. Samples were filtered through a 0.2 μm syringe filter and microcentrifuged to remove large particulates (such as dust) before measurements were taken at 25 °C. Steady-state fluorescence measurements were obtained in a 1 cm pathlength cuvette from a custom-built Photon Technology Instruments fluorometer installed with a Hamamatsu R928 photomultiplier tube and a 150 W Xe excitation lamp. Quantum yield (Φ) measurements were calculated using rhodamine 590 as the reference using eq. (1), where \( A \) is the measured absorbance at the excitation wavelength, \( I \) is the integrated emission intensity, and \( η \) is the refractive index of the solvent.

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Φ_{sample} = Φ_{ref} \left( \frac{A_{ref}}{A_{sample}} \right) \left( \frac{I_{sample}}{I_{ref}} \right) \left( \frac{η_{sample}}{η_{ref}} \right)
\]  

(1)

The quantum yield of the reference, \( Φ_{ref} \), was taken to be 0.94 for rhodamine 590 in methanol.\(^4\) A 400 nm emitting Ti: Sapphire laser equipped with a gated intensified CCD camera was used to obtain time-resolved fluorescence spectra. Data were collected at room temperature using a 1 cm optical path fluorescence cuvette. Origin 6.0 was used to fit the lifetime decay curve as a biexponential decay.

Two photon-excited emission spectra were taken in the following manner. NC-SNARF solutions were prepared in potassium phosphate buffers with 4%–bovine serum albumin. Buffered sample solutions were placed in 0.1 × 1 mm\(^2\) inner diameter glass microslides attached to a standard microscope slide. All spectral measurements were taken on a custom-built multiphoton laser scanning microscope (MPLSM) with the emission output fibre-coupled to a spectrometer. Multiphoton excitation was performed by a Spectra-Physics Broadband MaiTai diode pumped Ti:Sapphire laser using 800–920 nm light at sample powers ranging from 10–60 mW. All measurements were made with the MPLSM Bio–Rad MRC600 scan–head parked versus scanning on a Zeiss Axioskop20 microscopy using a Zeiss X20/0.5NA.water objective. Fluorescence emission was coupled into a 100–micron core fibre bundle after passing through a 720 nm short-pass filter. Spectra of the fibre-coupled emission were collected on a Shamrock 303 spectrograph with Newton DU–420 CCD detector system (Andor Technology) using a 100 micron slit opening and 1–sec detector integration. Emission spectra were analyzed in MATLAB.

Two–photon laser scanning microscopy imaging was performed on a Olympus Fluoview 300 Laser Scanning Microscope modified with a Spectra–Physics MaiTai laser. All images were taken at either 800 or 850 nm with excitation powers of 42 or 35 mW, respectively. Non–descanned fluorescence emission was detected by Hamamatsu HC125–02 PMTs. Nanocrystal and dye fluorescence was directed to separate PMTs using a 565–nm shortpass dichroic filter. The nanocrystal and dye channels were further selected using 660/50 nm and 535/40 nm AR–coated broadband filters respectively. Image stacks (512 × 512 pixels and 5–μm z–step) were collected throughout the 100 micron pathlength of the microslide for each calibration sample using an Olympus ×20/0.95NA/water objective. Image analysis was performed using NIH ImageJ. Briefly, average intensity projections for each stack were created and converted to 32–bit. Then the dye channel was divided by the nanocrystal channel. The pixel average and standard deviation of the ratiometric image were used to create a calibration curve.

Analysis. According to Förster theory, the efficiency ($E$) of energy transfer, or the fraction of photons absorbed by a donor that is transferred to a set of $m$ acceptor(s) at a radius $r$, is described by Eq. (2):

$$E = \frac{mk_{D\rightarrow A}}{mk_{D\rightarrow A} + \tau_D^{-1}} = \frac{mR_0^6}{mR_0^6 + r^6}$$

where $\tau_D$ is the donor excited–state decay lifetime, and $k_{D\rightarrow A}$ is there energy transfer rate. $R_0$ is the characteristic distance at which $k_{D\rightarrow A} = \tau_D^{-1}$ such that $E = 50\%$. The characteristic distance (in cm) is given by $R_0 = (8.79 \times 10^{-25} \kappa^2 n^{-4} J \Phi_D)^{1/6}$ for donor quantum yield $\Phi_D$ and spectral overlap integral $J$, with orientation factor $\kappa^2$ ($\kappa^2 = 2/3$ for random orientations) and refractive index $n$ ($= 1.33$). For a given sample, $E$ can be obtained from the quench of the NC donor emission intensity with respect to a control prepared with no dye, $m$ is determined from the absorption spectrum with an appropriate estimate of the NC molar extinction coefficient, and $R_0$ can be calculated from the spectral overlap and measured NC quantum yield. These parameters allow the typical separation $r$ to be estimated and compared with the radius measured by other means, e.g., DLS.

The efficiency can also be obtained using the emission lifetimes of the donor in the absence and the presence of an acceptor (eq. 3):

$$E = 1 - \frac{\tau_{DA}}{\tau_D}$$
Fig. S1 Plots of the ratio of SNARF emission to NC emission with respect to pH under (a) one–photon excitation at 365 nm and (b) two–photon excitation of the NC–SNARF pH sensor at 800 nm. The data shown represent the average of three measurements. Note that the ratio y-axis values cannot be directly compared because of differing bandwidths and quantum yields for the two PMT channels in the image mode detection; this will introduce a linear scaling of the y axis.
Fig. S2 Lifetime decay traces of (a) NC alone at pH 6, (b) NC–SNARF at pH 6, and (c) NC–SNARF at pH 9. Biexponential fit shown by the red trace; lifetimes are shown in the panels.
Fig. S3 (a) The emission of the nanocrystal in PBS (— blue line) and in BSA (— green line). While the emission in BSA appears to be diminished by ~25%, scattering may account for the differences in emission (b) The emission of SNARF–5F in PBS (— blue line) and in BSA (— green line). SNARF–5F is significantly quenched in a 4% BSA solution.