Ion-Switchable Quantum Dot Förster Resonance Energy Transfer Rates in Ratiometric Potassium Sensors

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Supporting Information

ABSTRACT: The tools for optically imaging cellular potassium concentrations in real-time are currently limited to a small set of molecular indicator dyes. Quantum dot-based nanosensors are more photostable and tunable than organic indicators, but previous designs have fallen short in size, sensitivity, and selectivity. Here, we introduce a small, sensitive, and selective nanosensor for potassium measurements. A dynamic quencher modulates the fluorescence emitted by two different quantum dot species to produce a ratiometric signal. We characterized the potassium-modulated sensor properties and investigated the photonic interactions within the sensors. The quencher’s protonation changes in response to potassium, which modulates its Förster radiative energy transfer rate and the corresponding interaction radii with each quantum dot species. The nanosensors respond to changes in potassium concentrations typical of the cellular environment and thus provide a promising tool for imaging potassium fluxes during biological events.

KEYWORDS: nanosensor, quantum dot, optode, FRET, ion sensing

Real-time ion imaging in cellular systems requires analytical tools with high selectivity and sensitivity. Designing fluorescent reporters that selectively measure sodium or potassium has been particularly difficult, despite significant interest in capturing the spatiotemporal patterns in transmembrane ion currents that regulate cell behaviors for both excitable and nonexcitable cells. In contrast to extensively optimized small-molecule and genetically encoded calcium indicators, dyes such as sodium-binding benzofuran isophthalate (SBFI) and potassium-binding benzo-furan isophthalate (PBFI) are limited by low sensitivity, poor selectivity, and low photoluminescence quantum yield (PLQY). A few previous reports using sodium indicators have successfully imaged the spatiotemporal features of sodium fluxes during action potential firing in neurons, but widespread adoption has been limited by the aforementioned problems.

Optode-based nanosensors address many of the challenges associated with measuring ion concentrations in biological systems. In the optode platform, hydrophobic optode components are contained within a plasticizer nanoemulsion and stabilized by a biocompatible surfactant. The hydrophobic sensing components for a potassium nanosensor consist of a colorimetric pH indicator, a potassium-binding crown ether, and an anion, termed the chromoionophore, ionophore, and additive, respectively. The ionophore extracts a potassium ion into the hydrophobic core, which displaces a proton and deprotonates the chromoionophore. The chromoionophore’s absorbance spectrum depends on protonation, which, in the nanosensor construct, becomes an ion-specific optical change.

Most optode nanosensors use Nile red derivatives for fluorescence measurements because of the dye’s stability and ratiometric readout. The rapid photobleaching of this dye restricts imaging conditions to short durations or low excitation intensities. Imaging rapid ion fluxes requires short exposure times and, consequently, high-intensity illumination to achieve a reasonable signal-to-noise ratio. Replacing organic dyes with semiconductor quantum dots (QDs) has been shown to improve photostability of optodes, but the optical

Received: August 28, 2015
Accepted: April 10, 2016
Published: April 18, 2016

DOI: 10.1021/acsnano.5b05396
ACS Nano 2016, 10, 4020–4030
Figure 1. Nanosensor working principle. Top figure shows the ionophore-mediated $\text{H}^+/\text{K}^+$ ion exchange and changes the chromoionophore’s protonation state. Bottom figure shows that the chromoionophore’s absorbance spectrum is distinct between its protonated (blue dashed line) and deprotonated (red dashed line) state. Quantum dots (bottom, solid lines) are unaffected by $\text{H}^+/\text{K}^+$ exchange.

Figure 2. Size distribution of all particles measured from TEM images of the nanosensors (a). Representative TEM images taken at 4400× (b), 11000× (c), and 350000× (d). Scale bars are 1 μm (b), 500 nm (c), and 50 nm (d). Arrows in (b) point to QD aggregates and arrows in (c) point to individual QDs.
interactions leading to a ratiometric response of these ion nanosensors remain poorly understood.

Here, we design a ratiometric potassium-selective nanosensor based on two QD species (Figure 1 schematic). A non-fluorescent chromoionophore responds dynamically to potassium by changing its absorbance spectrum and the strength of its interactions with either QD species (Figure 1 graph). By characterizing the analytical and physical properties, we confirm that the nanosensor functions via Förster resonant energy transfer (FRET) interactions between the QDs and the nonfluorescent chromoionophore. Using time-resolved photoluminescence spectroscopy we show that the FRET mechanism is generalizable across QD–chromoionophore combinations, which is in agreement with their measured potassium-sensing properties. Furthermore, in vitro experiments with live cells indicate that the nanosensors maintain their potassium sensitivity in physiological environments.

RESULTS

To obtain ratiometric QD-based potassium sensors, QD490 and QD640 were selected because their fluorescence spectra (Figure 1, solid lines on spectra) overlap well with chromoionophore II (CH) absorbance (Figure 1, dashed lines on spectra) and are completely non-overlapping with each other. The sonication–evaporation process is modified from previous work\(^\text{26}\) to more efficiently encapsulate QDs and optode components into bis(2-ethylhexyl) sebacate (also referred to as dioctyl sebacate, DOS) nanoemulsions stabilized by the phospholipid 1,2-diestearoyl-sn-glycero-3-phosphoethanolamine conjugated with polyethylene glycol (DSPE-mPEG(550)). Filtering the crude product with a syringe filter isolates a nanosensor population with diameters of 77 ± 21 nm as measured by dynamic light scattering (Figure S1) and a ζ-potential of −29 ± 7.6 mV.

Transmission electron microscopy (TEM) images show nanosensors on TEM grids with some globular and some irregular shapes, likely due to the oily nature of DOS. Particles are most commonly 50–100 nm in diameter (Figure 2a). Many, but not all, nanosensors contain at least one QD (Figure 2b,c). Interestingly, the QDs are not evenly distributed throughout the plasticizer matrix. Rather, many QDs appear to aggregate within the nanoparticles with a few individual QDs remaining unaggregated. Even at a magnification of 350 000 times, it is not possible to discriminate between QD490 and QD640 (Figure 2d).

Figure 3. Fluorescence calibration curves for individual QDs (a) and ratio (b) in response to sodium (□) or potassium (●). Error bars indicate one standard deviation.

The nanosensors respond sensitively to potassium in accordance with established optode behavior (eqs 1–3). Nanosensors are intrinsically pH-sensitive, but the chromoionophore, ETH S239, has a pKₐ of 10 and is protonated in standard physiological conditions.\(^\text{27}\) This high pKₐ means that all nanosensor responses in physiological conditions are a consequence of selective ion exchange. The ratiometric response (eq 4) to potassium fits the Hill equation (eq 5) with a half-maximal effective concentration (EC₅₀) of 24 ± 4 mM (Figure 3a,b and Figure S2a), lower and upper limits of detection at 1 and 590 mM, respectively, and a linear range from 2 to 120 mM. The nanosensor sensitivity, defined as the slope of the response at the EC₅₀ measured −37% per log[K⁺], and the ratiometric signal decreases by 10% between 4 and 8 mM. Nanosensors are stable for room temperature storage for 7 days, as the EC₅₀ only shifted to 36 ± 11 mM on day 7 (Figure S3).

Nanosensors respond selectively for potassium over sodium, showing an EC₅₀ for sodium >1 M (Figure 3a,b and Figure S2b), which produces a selectivity coefficient of 1.7 (eq 5, zero background ion). In 150 mM background sodium, the nanosensors have an EC₅₀ of 34 ± 7 mM for potassium (Figure S4), a slope of −29% per log[K⁺], and a ratiometric signal decrease of 9% from 4 to 8 mM. A selectivity coefficient is incalculable in 150 mM background potassium because the nanosensors do not respond to sodium concentration changes in 150 mM potassium (eq 5) or even in 50 mM background potassium (Figure S5a,b). Together, these data show that nanosensors are stable, sensitive, and selective for potassium.

Nanosensor fluorescence responses to potassium (Figure 3a,b and Figure S2a) are consistent with absorbance changes reflecting the chromoionophore’s protonation state (Figure S2c). As potassium concentration increases, CH becomes deprotonated and its shorter absorbance peak increases while its longer absorbance peak decreases (Figure S2c). As the CH absorbance overlapping with QD490 increases, QD490 fluorescence decreases and vice versa for QD640. To show that CH transduces the H+/K⁺ exchange into an optical response, we made potassium nanosensors with (Figure S6a) and without CH (Figure S6b). The result shows that CH is necessary to produce potassium-dependent responses in nanosensors.

Studying deconstructed nanosensor formulations (Table 3) delineates the photonic interactions between all three optical components, −CH and two QDs. Steady-state absorbance

ACS Nano

DOI: 10.1021/acsnano.5b05396
ACS Nano 2016, 10, 4020–4030
spectra show that 0 and 2 M potassium, respectively, fully protonates and deprotonates CH (Figure 4d−f, dashed lines). In the absence of CH, the steady-state fluorescence intensities from both QDs increase between 0 and 2 M KCl (Figure 4a−c). Formulations with CH show CH-mediated fluorescence changes; QD490 decreases intensity, while QD640 increases intensity between 0 and 2 M KCl (Figure 4d−f).

To investigate the physical mechanism of the observed fluorescence changes, we measured FRET rates (eq 8) between the components of optode using time-resolved spectroscopy (setup shown in Figure S7, and streak camera images shown in Figure S8a−i). Nanosensors produce potassium-dependent fluorescence lifetimes with one QD (Figure 5a,b) or both QD species (Figure 5c,d) in the optode formulation. Some of the decay curves for QD490 appear to have more multiexponential features, potentially due to a sufficient signal from unpassivated trap states or charging of dots in blinking phenomenon. To focus on FRET interactions with a simple model for both QDs and avoid complicating fast dynamics, monoexponential functions are fit to data between 10 and 70 ns after excitation. Consistent with observations of FRET in multi-QD systems,28−30 we find that fluorescence lifetime increases from 19.2 ± 0.2 to 26.7 ± 0.4 ns for QD640 and decreases from 20.2 ± 0.2 to 18.5 ± 0.3 ns for QD490 in a two-QD optode as compared to single-QD composites (Figure S9a,b and Table 1). We find that potassium concentration changes have no measurable effect on QD fluorescence lifetimes in the absence of CH (Figure S9a−c), indicating that the FRET rate between the QD490−QD640 pair is independent of potassium concentration. This mean constant QD490−QD640 FRET rate of $(4.7 \pm 1.5) \times 10^6$ s$^{-1}$ was carried through transfer rate calculations for formulations with CH.

As expected, deconstructed formulations containing CH show that its protonation state strongly influences fluorescence lifetimes and energy transfer from either QD to CH (Figure S10). Between 0 and 2 M potassium, which corresponds to conditions in which CH is fully protonated and deprotonated, respectively, QD490 to CH transfer increases while QD640 to CH transfer decreases (Figure 5 and Table 2). Moreover, QD fluorescence lifetimes change predictably over the potassium calibration panel, with QD490 lifetime gradually decreasing and QD640 lifetime gradually increasing with increasing potassium concentration (Figure S11a−c). Steady-state fluorescence intensity measurements also reflect these CH protonation-dependent results (Figure 4d−f and Figure S6a), and formulations made without CH show no steady-state response to a potassium calibration panel (Figure S6b). These results show that changes in the net CH protonation state are responsible for potassium-dependent changes in QD fluorescence through changes in the FRET rate.

To further investigate the energy transfer processes within the nanosensors, the FRET radii were calculated for the protonated and deprotonated CH cases. For these calculations, we used experimentally determined PLQY of 0.64 and 0.42 for...
QD640 and QD490, respectively. The resulting FRET radii for protonated CH\textsuperscript{−}QD490 and CH\textsuperscript{−}QD640 are 2.77 and 6.24 nm, respectively, and those radii change to 4.39 and 2.82 nm, respectively, for deprotonated CH. We define \(d_0\) as the optimal separation distance between a QD and CH to achieve the largest difference in FRET efficiency between the protonated and deprotonated states. For QD490−CH, \(d_0\) is 3.49 nm, and for QD640−CH, \(d_0\) is 4.19 nm (Figures S12a,b and S13). Using the optimal radii in the calculation of relative transfer rates (eq 13), we find that the QD490−CH FRET rate can maximally increase by a factor of 16 and the QD640−CH transfer rate can maximally decrease by a factor of 118 (eq 14) upon protonation/deprotonation. By comparison, time-resolved photoluminescence measurements show 7.7 ± 4.2-fold and 9.6 ± 2.0-fold changes in transfer rates for QD490−CH and QD640−CH, respectively (Figure 5a,b), indicating that the existing spacing between the QD donors and CH acceptor is not optimal. The optimal distance for the QD490−QD640 FRET radius was determined to be 3.52 nm, and the mean center-to-center distance calculated from time-resolved spectroscopy of QD490−QD640 is 5.87 nm.

Since the platform is modular and no covalent conjugations are necessary to change the spectral properties of the emission dyes, the same optode cocktail can be used with many unique QD combinations. For example, nanosensors with QD540 and QD640 respond with two dynamic channels and an EC\textsubscript{50} of 55 ± 4 mM (Figure S14a,b). Alternatively, pairing QD560 and QD640 produces a nanosensor with one static signal and one dynamic signal, and the ratiometric signal responds with an EC\textsubscript{50} of 27 ± 7 mM and a slope of 39% per log[\textit{K}\textsuperscript{+}] (Figure S15a,b). As long as one QD acts as a nanoantenna and at least one QD transfers to the chromoionophore’s dynamic portion of its spectrum, the nanosensors’ spectral properties are highly tunable.

Nanosensors imaged in controlled conditions exhibited full reversibility (Figure S16a,b and Video S1) and an EC\textsubscript{50} of 24 ± 5 mM for the ratiometric measurement (Figure S17a,b). Using

Table 1. Fluorescence Decay Time Constants for Deconstructed Nanosensors

<table>
<thead>
<tr>
<th>QD</th>
<th>(\tau) (ns)</th>
<th>0 M KCl</th>
<th>2 M KCl</th>
</tr>
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<tbody>
<tr>
<td>QD490</td>
<td>20.2 ± 0.2</td>
<td>19.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>QD490 in 2QDs</td>
<td>18.5 ± 0.3</td>
<td>17.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>QD490 in (CH+2QDs)</td>
<td>15.9 ± 0.3</td>
<td>12.3 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>QD490 in (CH+QD490)</td>
<td>17.5 ± 0.2</td>
<td>12.3 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>QD640</td>
<td>19.2 ± 0.2</td>
<td>20.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>QD640 in 2QDs</td>
<td>26.7 ± 0.4</td>
<td>26.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>QD640 in (CH+2QDs)</td>
<td>11.5 ± 0.3</td>
<td>23.0 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>QD640 in (CH+QD490)</td>
<td>7.9 ± 0.4</td>
<td>17.9 ± 0.3</td>
<td></td>
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</tbody>
</table>

“Each QD’s lifetime in the formulation conditions.

Table 2. Transfer Rates for Optical Components within Fully Formulated Potassium-Selective Nanosensors

<table>
<thead>
<tr>
<th>QD</th>
<th>(\text{transfer rates (}1 \times 10^6\text{ s}^{-1})</th>
<th>0 M</th>
<th>2 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>QD490−QD640</td>
<td>4.5 ± 1.5</td>
<td>4.9 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>QD490−CH</td>
<td>3.2 ± 1.7</td>
<td>24.4 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>QD640−CH</td>
<td>74.4 ± 6.6</td>
<td>7.7 ± 1.4</td>
<td></td>
</tr>
</tbody>
</table>

“Conditions of 0 M KCl and 2 M KCl permit CH to be fully protonated or fully deprotonated.

Figure 5. Intensity-normalized time-resolved fluorescence of QDs showing potassium-dependent changes in QD lifetime for QD490 (a) and QD640 (b) when formulated with either QD alone. Intensity-normalized plots for QD490 (c) and QD640 (d) when formulated with both QDs.
dialysis tubing introduces a diffusional barrier for potassium, thus causing the nanosensor response times to appear to be slow. The temporal response in this experiment is limited by the diffusion of potassium into the inner bore of the dialysis tubing. To assess the utility of our nanosensors in physiological environments, we incubated them with HEK-293 cells. We find that nanosensors are distributed almost exclusively on the cell periphery (Figure 6a–c), and they respond to in situ calibrations with an EC₅₀ of 40 mM (Figure 6d). While there is significant variability in photoluminescence intensity of individual QD spectra (Figure S18), the ratiometric signal shows a clear concentration-dependent response to potassium in phosphate buffered saline (PBS).

A similar experiment using a microinjector to simulate a localized potassium source by delivering a quick, gentle puff of 150 mM potassium solution produced a rapidly reversible nanosensor response. The timing-matched image sets, acquired separately for each QD spectral channel, show that photoluminescence intensity decreases for QD490 and increases for QD640 in response to an increase potassium concentration (Figure S19). This yields a brief decrease of the ratio of fluorescence intensities of QD490/QD640 (Figure 6f). The short, gentle puff of the pipet solution avoided introducing motion artifacts into the region of interest (Figure 6e, yellow oval), and the pipet had no positive pressure before or after the puff. This approach dilutes the pipet solution to an unknown potassium concentration less than the original 150 mM, but these measurements still demonstrate that nanosensors can detect a brief, small, and local increase in potassium concentration. The fluorescence ratio drifts because CH absorbs light in the illuminating wavelengths but does not emit detectable fluorescence in either channel. However, this absorbance makes the CH subject to some degree of photobleaching. This problem may be addressed in future designs by using chromoionophores lacking absorbance in the illuminating wavelengths.

**DISCUSSION**

We designed a bright, stable, and sensitive ratiometric nanosensor to address the challenges associated with imaging potassium in real-time. Of the existing small-molecule indicators, only PBFI can quantitatively image potassium concentrations. It requires 340 and 380 nm excitation, exhibits low quantum yields of 0.04−0.08, and absorbs with an extinction coefficient of 45 000 M⁻¹ cm⁻¹. The remaining indicators such as Asante Potassium Green (analogues exist for sodium, as well) are significantly brighter, but none are ratiometric. Given that ratiometric indicators more accurately...
quantify ion concentrations than single wavelength indicators,33 a new family of bright and ratiometric ion indicators would be valuable research tools.

The QD490–QD640 nanosensor formulation responds sensitively to potassium and can measure potassium throughout the entire physiological range (Figure 2a,b). Combinations of QD540–QD640 and QD560–QD640 demonstrated similarly sensitive and physiologically relevant ratiometric responses (Figures S14a,b and S15a,b). For this study, FRET rates and radii were calculated only for QD490–QD640 sensors, but the same approach translates to other QD combinations; it presents a rational route toward optimizing a nanosensors formulation, an approach that had not been described in QD-based, ion-selective nanosensors until now.

The optimal predicted FRET radii between QDs represent very close spacing that would not occur if QDs were evenly distributed throughout a nanosensor. Computed FRET radii suggest that even 8–10 QDs within a 70 nm particle should not have measurable FRET interactions. However, TEM images show that QDs aggregate within nanosensors (Figure 2b,c), which is consistent with spectroscopic measurements of FRET between QDs.

When considering the FRET spacing between either QD and CH, it is important to keep in mind that, in a 70 nm diameter particle, the current formulation contains ~370 CH molecules. An even spacing model would produce CH molecules with an average spacing of 9.7 nm.34 For every QD, there would then be multiple CH FRET acceptors within 5 nm, explaining the strong FRET effects observed here. This arrangement means that donor–acceptor distances are reasonably within the observed FRET radii ranging from 2.77 to 6.24 for QD640 and 2.82 to 4.39 for QD490.

It is reasonable to suggest that a QD titration series would arrive at an optimized formulation. However, increasing the QD content is likely to affect both the mobility of optode components and the properties of the nanoparticle that determine partitioning behaviors that govern optode responses (eqs 1–3). Thus, an optimization will realistically include between up to 11 parameters: surfactant type, surfactant concentration, ionophore type, ionophore concentration, chromoionophore type, chromoionophore concentration, additive type, additive concentration, plasticizer type, plasticizer concentration, QD490 and QD640 concentration. Such work may be addressed as the central focus for future investigations.

Basal potassium and sodium are 4 and 140 mM, respectively, and this high background sodium interferes with potassium measurements. The observed 50-fold selectivity for potassium over sodium makes these QD-based potassium sensors compare favorably to previously reported sensor designs.35–37 Although small-molecule indicators have progressed significantly,38–40 QD resistance to photobleaching and narrow emission spectra make them appealing reporter components. More importantly, this dual-QD system produces a quantifiable ratiometric signal that responds to potassium calibrations with an identical EC50 of 24 mM in a well-plate calibration and in close proximity to the cell membrane.

The in vitro experiments indicate that HEK-293 cells do not internalize the nanosensors (Figure 6d and Figure S18). Any nanosensors sequestered in endosomes would not have responded to the in situ calibration and diminished the overall sensitivity. The passive external attachment mechanism is unknown at this point, but the high concentration of nanosensors during the incubation period and strong ζ-potential41 may explain the phenomenon. In the future, nanosensor formulations will be targeted using peptides or other groups to specifically label cells of interest as other groups have done.42,43 Altogether, these results show that nanosensors are capable of sensitively responding to rapid changes in potassium concentration in physiologically relevant conditions.

CONCLUSIONS

We aimed to design and characterize nanosensors capable of measuring real-time potassium concentration measurements in the physiological environment. The results show that optodes incorporating QDs have the sensitivity and selectivity to detect potassium in conditions of low and high interference from sodium. Measuring steady-state and time-resolved optical properties provided conclusive evidence that FRET between all three optically active elements is necessary for a dynamic ratiometric signal. Comparing the FRET rates from fluorescence lifetime measurements and overlap integral calculations showed that optimizing the FRET donor–acceptor distance will produce even more sensitive nanosensors. Finally, controlled in situ calibrations demonstrated that the nanosensors sensitively respond to potassium over concentration ranges corresponding to physiological conditions. Future work will fine-tune formulations to achieve greater sensitivity by maximizing the number of particles that contain two or more quantum dots and apply these nanosensors to image potassium efflux from cells during signaling events.

METHODS

Materials. Bis(2-ethylhexyl) sebacate, chromoionophore II (9-dimethylamino-5-[4-(16-buty1-2,14-dioxo-3,15-dioxaeicosyl)phenylimino][benzo[a]phenoxazine, ETHE 2439), potassium ionophore III (KIr3, 2-dodecyl-2-methyl-1,3-propanedithiol bis[N-[3-nitro(benzol-1’5-crown-5)-4’-yl] carbamate]), sodium tetrakis[3,5-bis-(trifluoromethyl)phenyl]borate (NaBARF), (2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), sodium chloride (NaCl), dichloromethane (DCM), and tetrahydrofuran (THF) were purchased from Sigma-Aldrich. Quantum dots were purchased from NN-Laboratories (emission peak 640 nm, C2640) and Cytodiagnostics (emission peak 490 nm, FN-490). Tris base was purchased from Fisher BioReagents. Spectra/Por in vivo microdialysis hollow fibers (inner diameter 200 μm; outer diameter 280 μm, MWCO 13 kDa) were purchased from Spectrum Laboratories, Inc. 1,2-Distearoyl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-550] (ammonium salt) (DSPE-mPEG550) was purchased from Avanti Polar Lipids, Inc.

Nanosensor Fabrication. Quantum dots were flocculated from chloroform by mixing as-received stocks in anhydrous methanol at a volume ratio of 4:1 MeOH/CHCl3 and centrifuging for 5 min at 5000g. After repeating the wash and centrifugation steps three times, we re suspended the quantum dots in THF and determined their concentration based on absorbance. The sensing components KI3 (4 μmol, 2 mg), CH (1.36 μmol, 1 mg), and NaBARF (13.5 μmol, 12 mg) were reconstituted separately with THF and combined in a 2 mL glass vial so that the total THF volume was 300 μL. DOS (428 μmol, 200 μL) was added to the sensing components and vortexed briefly to homogenize the mixture. Just prior to nanosensor fabrication, 5 μg of DSPE-mPEG550 was dried in a glass scintillation vial and reconstituted in 400 μL of DCM. Next, 50 μL of the optode stock, 189 μg of QD490, and 182 μg of QD640 were added to the mixture. Four milliliters of aqueous buffer (10 mM HEPES buffer; 6 mM Tris base) was added, and the mixture was sonicated for 1 min with a Branson digital sonifier (S-450D) at 10% intensity with a 1/8 in. diameter tip. After sonication, the organic solvents were removed using a rotavap (Buhle) for 10 min at room temperature. The resulting emulsion was filtered with a 100 nm syringe filter.
Particle Size and ζ-Potential Measurements. Hydrodynamic diameters were measured by dynamic light scattering using a 90 Plus particle size analyzer by Brookhaven Instruments Corporation. All particle size measurements were taken in triplicate by diluting the sample solution in 10 mM HEPES buffer, pH 7.2, so that the detector count rate measured 50–450 kcps. The detector measured scattered 640 nm light at a fixed angle of 90° and calculated the effective diameter based on the intensity of scattered light. ζ-Potentials were measured using the same 90 Plus instrument in PALS5 mode. Sample concentrations were adjusted until the detector count rate measured 100–450 kcps, and then 25 cycles of 10 runs per cycle were collected. Three batches of nanosensors each from two separate stock optode preparations were measured.

Electron Microscopy. Diluted samples of the PUNQs were air-dried onto 300-mesh carbon-film-coated copper grids (Electron Microscope Sciences). The grid was washed three times with distilled water, placed onto a 5 µL drop of 1.5% phosphotungstic acid stain for 5 min, and dried with filter paper to remove excess liquid. Images were acquired at 120 kV accelerating voltage on an FEI Tecnai multipurpose TEM. Images were analyzed using ImageJ to measure nanoparticle diameters.

Fluorescent Response to Potassium. Nanosensors were calibrated for their fluorescent response to potassium in 10 mM HEPES buffer, pH 7.2. Acid and base standards of 10 mM HCl and NaOH were used as end points for fully protonated and deprotonated HEPES buffer, pH 7.2, so that the detector count rate measured 50–450 kcps. The detector measured scattered 640 nm light at a fixed angle of 90° and calculated the effective diameter based on the intensity of scattered light. ζ-Potentials were measured using the same 90 Plus instrument in PALS5 mode. Sample concentrations were adjusted until the detector count rate measured 100–450 kcps, and then 25 cycles of 10 runs per cycle were collected. Three batches of nanosensors each from two separate stock optode preparations were measured.

Table 3. Formulations for Deconstructed Nanosensors

<table>
<thead>
<tr>
<th>DOS</th>
<th>CH</th>
<th>KI3</th>
<th>NaBARF</th>
<th>QD490</th>
<th>QD640</th>
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<tr>
<td>CH</td>
<td>10</td>
<td>0.05</td>
<td>0.4</td>
<td>1.2</td>
<td>0</td>
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<tr>
<td>QD490/QD640</td>
<td>10</td>
<td>0</td>
<td>0.4</td>
<td>1.2</td>
<td>0.1</td>
</tr>
<tr>
<td>QD490–CH</td>
<td>10</td>
<td>0.05</td>
<td>0.4</td>
<td>1.2</td>
<td>0</td>
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<tr>
<td>QD640–CH</td>
<td>10</td>
<td>0.05</td>
<td>0.4</td>
<td>1.2</td>
<td>0</td>
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<tr>
<td>QD490/QD640–CH</td>
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<td>0.05</td>
<td>0.4</td>
<td>1.2</td>
<td>0.1</td>
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<tr>
<td>QD490</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>1.2</td>
<td>0</td>
</tr>
<tr>
<td>QD640</td>
<td>10</td>
<td>0</td>
<td>0.4</td>
<td>1.2</td>
<td>0</td>
</tr>
</tbody>
</table>

For each calibration point, the nanosensor solution was mixed with standard solutions so that the final KCl concentrations were 10–150 mM KCl and solutions of 10 mM NaOH or 10 µM HCl were used to, respectively, fully deprotonate or protonate the chromoionophore in order to determine the fraction protonated during measurements in KCl. The fluorescence intensities for QD490 (ex = 405, em = 490) and QD640 (ex = 405, em = 650) were measured with a Spectravmax M3 plate reader (Molecular Devices) in bottom read mode through clear-bottom 96-well plates. The fluorescence ratio for QD640/QD490 was calculated as

$$R = \frac{QD640}{QD490}$$

The EC_{50} for the nanosensors was calculated from the ratiometric values according to the dose–response (Hill) equation:

$$R = R_0 - \left( \frac{R_1 - R_0}{1 + 10^{(EC_{50} - \log R_f)/x}} \right)$$

where R_0 and R_1 are the ratio values in conditions that fully protonate and deprotonate CH, respectively. The Hill slope is p, and x is a potassium concentration at which a ratio was measured.

Nanosensor selectivity was determined by measuring the fluorescent response to NaCl and fitting the calibration data to the Nicolskii–Eisenman model with a fixed interfering ion,18,45 which defines the selectivity coefficient for an optical sensor as

$$\log K^{opt} = \log EC_{50} - \log EC_{50}$$

where EC_{50} and EC_{50} are the EC_{50} constants for the interfering ion (sodium) and primary (potassium), respectively.

Nanosensor Shelf Stability. In order to characterize the shelf life for the nanosensors, their fluorescent response to potassium was evaluated over the course of 7 days. In triplicate, nanosensors were fabricated and stored at room temperature with no protection from ambient light. At days 0, 1, 2, 3, 6, and 7, aliquots were removed to produce a calibration curve in response to potassium and sodium. The calibration conditions were identical to those described in the previous section, and the results were fit to a dose–response model to calculate the EC_{50}.

Determination of Fluorescence Dynamics within Nanosensors. Nanosensor formulations were fabricated using the emulsification method described above with compositions described in Table 3, in milligrams.

The crude product from each combination was diluted 1:4 for the subsequent measurements. The absorbance spectra from 450 to 740 nm were recorded in 5 nm steps. Fluorescence spectra were recorded with 400 nm excitation and collected from 450 to 740 nm in 5 nm steps. The emission spectra were normalized with respect to the peak emission of QD490 for the formulation with only QD490.

Time-resolved fluorescence spectra were collected on a custom-built free-space optical system (Figure S7). Laser excitation of 400 nm at 1 kHz (Coherent Libra with OPerA) was focused into a quartz cuvette (Starna Cells) at a 45° angle to minimize detection of reflected laser light. Emission was filtered with a 450 nm long-pass filter (Thor Laboratories), and a portion of the subsequent sample fluorescence was focused into the streak camera (Hamamatsu C5680) using optical reimaging systems.

From the streak image, emission data of each QD donor were selected based on the full width at half-maximum of the photoluminescence (PL) spectrum. The resulting spectrally integrated time-resolved PL for each quantum dot was plotted on a semilog scale and approximated using a single exponential. A fitting was performed for each QD emission from 10 to 70 ns after excitation to compute τ, the exciton lifetime:
I(t) = I_0 e^{-t/τ} \tag{7}

where \( I(t) \) is the intensity of fluorescence detected at time \( t \), after a laser pulse and \( I_0 \) is the fluorescence intensity at \( t = 0 \). The excited lifetime, \( τ \), for donors is determined based on the rate of radiative (\( k_0 \)), nonradiative recombination (\( k_{nr} \)), and energy transfer to lower-energy QDs and/or acceptor molecules (\( k_{trans} \)):

\[
τ = k_0^{-1} = \frac{1}{k_i + k_{nr} + k_{trans}} \tag{8}
\]

Time-resolved spectra were recorded with full nanosensors in 0, 1, 4, 8, 20, 50, 100, 150, and 1000 mM KCl in 10 mM HEPES (pH 7.4 with Tris base). Additionally, each formulation from Table 1 was placed in 0 or 2 M KCl, and time-resolved fluorescent spectra were recorded with 400 nm excitation. To further examine the effects of QDs on the fluorescence lifetime of CH acceptors, fluorescent spectra were recorded for 0 M KCl with CH, QD640, QD490/CH, and QD490/CH from Table 1. In the case of QD490/QD640–CH, the CH fluorescence was difficult to resolve from the strong QD signal, so time-resolved spectra were recorded from two formulations made with 0.02 mg of each QD and 0.004 mg of each QD in 0 M KCl.

We set out to determine the three-dimensional radius at which FRET is 50% efficient relative to its maximum, \( R_0 \), for each QD with CH, when CH is protonated or deprotonated. CH was dissolved into THF at a concentration of 54 mM, and CH absorption in both states was measured for the wavelength range of 350–800 nm in 1 nm steps using a UV–vis–NIR spectrophotometer (Cary 5000). The absorption data were used to compute the molar absorptivity, \( ε_λ(λ) \), based on the Beer–Lambert law:

\[
ε_λ(λ) = \frac{-\ln\left(\frac{I}{I_0}\right)}{lc} = \frac{-\ln(1 - \text{Abs}(λ))}{lc} \tag{9}
\]

Photoluminescent spectra for each QD in THF were taken using a fluorometer (Cary Eclipse) for the wavelength range of 350–800 nm in 1 nm steps and normalized according to

\[
f_λ(λ) = \frac{F_0(λ)}{\int F_D(λ)dλ} \tag{10}
\]

The overlap integral, \( J \), was computed using the CH wavelength-dependent and protonation-dependent molar extinction coefficients and the QD photoluminescent spectra:

\[
J = \int f_λ(λ)ε_λ(λ)λ^4 dλ \tag{11}
\]

The FRET radius, \( R_0 \), was thus computed as

\[
R_0^6 = \frac{9\ln(10)\kappa^2Φ_DΦ_J}{128πn^3N_A} \tag{12}
\]

where \( \kappa^2 \) is the dipole orientation factor (assumed to be 2/3 for isotropically oriented donor and acceptor), \( f_0 \) is the overlap integral, \( n \) is the refractive index of DO5 (1.45), \( Φ_D \) is the fluorescence quantum yield of the donor QD in the absence of CH, and \( N_A \) is Avogadro’s number. The predicted transfer rate is calculated according to

\[
K_0(τ) = \frac{1}{τ_0} \left( \frac{R_0}{r} \right)^6 \tag{13}
\]

where \( τ_0 \) is the QD lifetime in the absence of CH and \( r \) is the QD–CH distance. The relative change in transfer rate between the protonated and deprotonated CH case was calculated according to

\[
\frac{K_0^+(r)}{K_0^-(r)} \tag{14}
\]

where \( K_0^+(r) \) and \( K_0^-(r) \) are the transfer rates for the deprotonated and protonated CH cases, respectively.

**Reversibility with a Confocal Microscope.** Nanosensors were imaged with a laser scanning confocal microscope by immobilizing the nanosensors inside of a piece of sealed dialysis tubing that was fixed to a glass coverslip. The setup, described and demonstrated previously, involved filling a 1.5 cm length of microdialysis tubing (200 μm i.d., 250 μm o.d., 13 kDa MWCO, Spectrum Laboratories) with nanosensors suspended in 10 mM HEPES buffer, sealed on its ends with fast-curing epoxy, and fixed to a 15 mm diameter circular glass coverslip with two drops of fast-curing epoxy. No attempt was made to control or measure the concentration of nanosensors between batches. The glass coverslip was then secured in a flow chamber (Warner Instruments, RC-21BRFS) and mounted for imaging on a laser scanning confocal microscope (Zeiss LSM 700). A 405 nm laser (1% intensity, 5 mW full power) excited both quantum dots simultaneously, and the two emissions were split with a diffraction grating set to split the emission at 580 nm. Images were acquired at 20 s intervals, and the solutions were changed every 3 min—alternating between 10 and 100 mM KCl in 10 mM HEPES, pH 7.2, with Tris base.

Images were analyzed with the Fiji plugin suite of ImageJ (NIH) with the region of interest defined as the interior of the dialysis tube, and the region of interest definitions for both channels were identical.

**Calibration with a Confocal Microscope.** Nanosensors were immobilized in dialysis tubing as described above and mounted in a perfusion chamber for imaging on a confocal microscope. Solutions of 0, 4, 8, 20, 50, 100, 150, and 1124 mM KCl were perfused through the chamber, and three images were acquired at each of the eight concentrations measured. Nanosensors were excited with a 405 nm laser set to 1.1% intensity (5 mW full power). For image analysis, a threshold mask was created using Otsu’s method; the mean channel values for QD490 and QD640 were measured separately, and the ratio of QD490/QD640 fluorescence was calculated and fit to a dose–response curve.

**In Situ Calibration and Response to Exogenous KCl in the Cellular Environment.** In situ calibrations were performed on HEK-293 cells (ATCC) cultured on 25 mm glass coverslips. Exogenous KCl application was performed on HEK-293 cells cultured on glass-bottom dishes (Mat-tek). Growth medium contained 89% DMEM, 10% FBS, and 1% Pen-strep, and cells were cultured for 3 days after plating. One hour prior to imaging, cell culture medium was aspirated out of a glass-bottom dish and replaced with nanosensors in PBS, 2 mM CaCl₂, 1.2 mM MgCl₂, and 10 mM glucose and allowed to incubate for an hour at 37 °C. After 1 h, the cells were rinsed three times with PBS and then moved to an upright microscope (Zeiss AxioObserver) for imaging with a 40x oil immersion objective (NA = 1.3).

For calibrations, 10X PBS (Boston BioProducts) was spiked with additional KCl to bring the final KCl concentration to 4, 8, 16, 20, 50, 100, and 150 mM KCl in 1X PBS. Cells cultured on glass coverslips were mounted in a perfusion chamber, and three images were acquired in each condition using a 405 laser set to 0.6–0.9% power (5 mW full power). A diffraction grating split the emission at 580 nm, and two channels were collected: one for QD490 and one for QD640 fluorescence. In each solution, three images were collected for each channel.

Images were analyzed in Fiji/ImageJ (NIH). First, the images were averaged, and then a binary mask was generated based on Otsu’s method for thresholding on the QD640 channel. An image based on the pixel-wise ratio of QD490/QD640 was also generated, and averaged in each solution condition to produce a calibration curve for the nanosensors when they were attached to cells and in physiological solutions.

To apply exogenous KCl, a pulled glass micropipette (1 mm o.d., 0.5 mm i.d., Sutter) was filled with 150 mM KCl in 10 mM HEPES (pH 7.4 with 6 mM Tris base) and brought close to proximity with a cell without any positive pressure on the pipet. An imaging sequence was initiated and controlled with digital logic in pClamp 10.4 (Molecular Devices). The microscope epifluorescence shutter was opened, and then 300 ms of frames were recorded with 50 ms exposures (Hamamatsu Flash 4.0) before a gentle 200 ms puff of KCl from the pipet tip was delivered. The sequence was first recorded with
a GFP filter set (ex = 470/40; BS = 495; em = 525/50) and then with a Cy3 filter set (ex = 545/25; BS = 570; em = 605/70).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.5b05396.

Extended characterization of nanosensor characterization, time-resolved spectroscopy, and in situ calibrations (PDF)

Video S1 (ZIP)

TEM images (ZIP)

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The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Dr. Zhang (Jane) Wang for help with figure preparation. T.T.R., C.G.S., and H.A.C. are supported by the NIH through Grant No. R01NS081641. T.T.R. is supported by the NIH through Grant No. F32EB015847. C.G.S. is supported by the National Academy of Sciences Ford Foundation Fellowship through Grant No. 2014-PGA084488. W.C. and V.B. are supported by the U.S. Department of Energy through Grant No. DE-FG02-07ER46474.

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