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Na^+-sensing quantum dots for cell-based screening of intracellular Na^+ concentrations ($[Na^+]i$)

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ABSTRACT

We have developed a Na-quantum dot (QD) nanosensor for $[Na^+]i$ measurements. Using this Na-QD, we determined the dynamic physiological responses of $[Na^+]i$ in nonexcitable human HEK-293F cells and excitable primary rat cardiac myocytes by pharmacologically manipulating the membrane permeability to Na^+, the Na–K–2Cl cotransporter, and the Na^+/H^+ antiporter. These data suggest that the mechanisms of $[Na^+]i$ homeostasis can now be elucidated with this novel Na-QD nanosensor. This could have a broad impact on Na^+ channel drug discovery.

1. Introduction

Na^+ channels are ubiquitously involved in almost all crucial cellular functions, including ion homeostasis and fluid transport, as well as in the excitability of cardiac and neuronal cells. Patch clamping and radioactive tracers are the conventionally deployed assays used to interrogate the physiopharmacological profiles of these Na^+ channels [1,2]. Recently, Förster/fluorescence resonance energy transfer (FRET)-based membrane voltage sensing probes, in conjunction with genetically inserted ion channels in cells, have been introduced to indirectly examine the Na^+ channels [3]. These assays and the assays of green/yellow/red fluorescent proteins fused with targeted proteins are beset by complex protocols and low quantum efficiency [4,5]. Although organic fluorophores such as SBFI [6] and Na-green [7] provide direct measurements of $[Na^+]i$, these dyes are characteristically plagued by photobleaching, cytotoxicity, low sensitivity and autofluorescence induced by UV excitation. A robust fluorescence assay is needed for the investigations of the ionic cellular signal transduction regulatory mechanisms of $[Na^+]i$. Such assay should also be amendable for cell-based high-throughput screening (HTS) platforms to enable the interrogations of large compound libraries for translational drug discovery.

Quantum dots (QD) are nanoscale crystals made of semiconductor compounds that can be excited with visible wavelength and emitted light at a specific wavelength according to its size. QD-based biosensor has been reported to be ~100 times brighter compared to the organic-based fluorophores and fluorescent proteins [8]. QD, served as the FRET donor, has the potential to greatly improve the sensitivity of the biosensor without invoking UV excitation that could also be harmful to the cells. However, there are some technical issues in using QD as a FRET donor for sodium sensing. First, the Na^+ sensor should be specific to Na^+. Second, the Na^+ sensor needs to be stable and amendable to chemical modifications so that it could be conjugated to the QD to form Na-QD. Third, the Na-QD needs to have minimal cell toxicity for biological applications. Fourth, the translocations of the Na-QD should not affect the integrity of the cellular signal transduction pathways of the live cells.

Crown ethers have been well-established to form complexes with metal ions, including Na^+ [9]. ZnS, in addition to being a protective layer of CdSe, also provides a surface for stable chemical modifications because of the high affinity of the thiol groups to metals [8]. CdSe/ZnS has been shown to have minimal cellular toxicity compared to the other types of QDs [8]. It is one of the most common QDs being exploited for biological applications. Based on these principles, we have successful developed a CdSe/ZnS QD-based chloride sensor, Cl-QD™, for cell-based applications recently [10]. By integrating the aforementioned technologies, herein we describe a synthesis protocol and validation of a novel Na^+-sensitive luminous quantum dot (QD) nanosensor, Na-QD, to measure $[Na^+]$. To synthesize the Na-QD sensor, monolayers of bis-thioctic ester derivatives [11] were used to conjugate the Na^+ sensor, 12-crown-4, to the CdSe/ZnS QD. We have applied Na-QD to determine the $[Na^+]i$ dynamics in HEK-293F cells and primary rat cardiac myocytes. The

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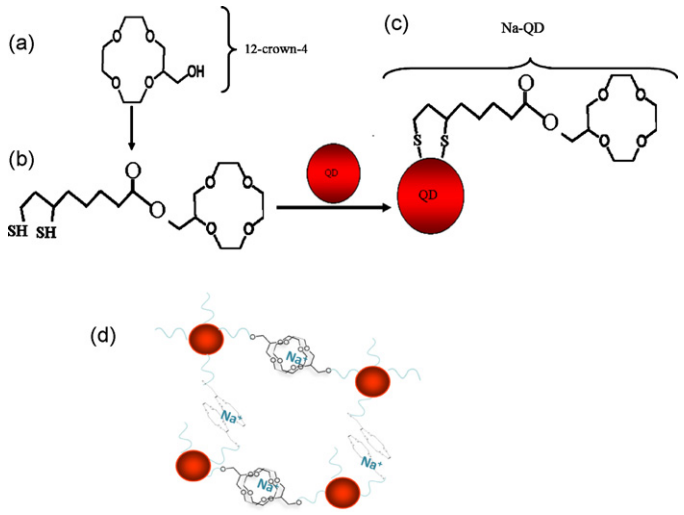

Fig. 1. Synthesis of Na^+-sensing QD by using 12-crown-4 as a Na^+ sensor. (a) Structure of 12-crown-4-ether, (b) 6,8-dimercapto-octanoic acid 1,4,7,10 tetraoxa-cyclododec-2-methyl-ester, (c) Na-QD sensor, (d) schematic of Na-QD undergoing FRET mechanism.

potential broad utilities of Na-QD as applied to basic physiology and pharmacology investigations, as well as drug screening discovery of Na^+ channels, can now be performed using this novel nanosensor.

2. Experimental protocols

2.1. Synthesis of sodium-sensitive luminous QD, Na-QD

Conventional organic chemistry synthesis techniques were applied throughout the protocol unless stated otherwise. Water-soluble CdSe/ZnS emitted at 620 nm (QD_{620}) was used as the FRET donor [12]. To synthesize the Na-QD nanosensor, we used monolayers of bis-thioctic ester derivatives to conjugate the 12-crown-4 (Fig. 1a) to the QD on the basis of a 2:1 dimerization scheme analogous to the synthesis of a K^+-sensitive QD sensor [13]. In brief, (12-crown-4)-2-methanol and thioctic acid were mixed with CH_2Cl_2 at 0 °C under N_2. 4-Dimethylaminopyridine and dicyclohexylcarbodiimide in cold CH_2Cl_2 were added under N_2 and allowed to warm to room temperature. After 6 h of reaction, the resultant filtrate was washed. The solutions of CdSe/ZnS QD_{620} and 12-crown-4 resultant mixture in chloroform (Fig. 1b) were refluxed for 24 h. With the solvent removed, the residue was resuspended in acetonitrile and centrifuged at 12,500 rpm for 5 min. The centrifugation step was repeated where necessary to enable precipitation of the Na^+-sensitive QD nanosensor, Na-QD (Fig. 1c).

2.2. Characterization of the emission spectra and quantum yield of Na-QD

A Na^+ concentration-dependent emission spectrum of Na-QD was generated by using a spectrofluorometer (PTI, Birmingham, NJ). Na-QD solution was placed in a quartz microcell. An interference filter of 440 nm was placed in front of the xenon light, which was focused perpendicularly to a cuvette. The emitted light, filtered with a tunable dual-grating monochromator, was collected at a 90-degree scattering configuration with a high bandwidth 2″ diameter photon-counting photomultiplier tube (R647, Hamamatsu). The photon counts were processed with a high-gain impedance amplifier and 200 MHz bandwidth discriminator (EG&G). The photon counts were processed by using a 100 MHz multichannel scaler (ACE MCS) counter board. Fluorescence of Na-QD for each Na^+ concentration was scanned at emission wavelengths that ranged from 550 nm to 650 nm.

To determine the quantum yield of Na-QD, a relative optical method was used [14]. The absorption and emission spectra of fluorescein with known quantum yield of 0.79 as a reference standard were established. The absorption spectrum was determined with the spectrofluorometer operated in a 180-degree transmission configuration. The incident light was scanned from 440 nm to 660 nm using a tunable dual-grating monochromator. The emission spectrum was determined using a 90-degree configuration as described with the emission wavelengths ranged from 440 nm to 660 nm. Subsequently, the absorption and emission spectra of Na-QD were determined using the same experimental conditions. The measurements of the absorption and emission spectra of fluorescein and Na-QD, both at the concentrations of 1 μM, were each repeated for 2 samples.

2.3. Cultures of HEK-293F cells

In brief, HEK-293F cells (Invitrogen, Carlsbad, CA) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM HEPES, 10% fetal bovine serum, 2 mM L-glutamine, and BSD (blasticidin S deaminase) at 10 μg/mL (1% penicillin/streptomycin sulfate). Cells were maintained at 37 °C and 5% CO_2 in T25 flasks. The cells were then cultured in collagen-coated chambers for the fluorescence microscopy studies as followed.

2.4. Cultures of primary rat cardiac myocytes

Primary rat cardiac myocytes (ScienCell, San Diego, CA) arrived frozen in 1:1 Medium 199 and DMEM/F12 containing 5% fetal calf serum. To passage these cells, we thawed them in a 37 °C water bath and then resuspended them in fresh culture media. On the following day, any residual dimethyl sulfoxide and floating cells were removed from the culture. This process was repeated every other day. For healthy and viable cultures, spindle-shaped cells were doubled every 2–3 days under these conditions. When the culture reached 50% confluence, the medium was exchanged every day. When the culture reached ~90% confluence, it was passaged by using a conventional trypsin/EDTA protocol. In brief, cells were harvested, transferred, resuspended in fresh medium, and plated in a T25 flask according to the recommended cell density. The cells were then cultured in collagen-coated chambers for the fluorescence microscopy studies as followed.

2.5. Collagen-coated coverglass chambers

HEK-293F cells and rat cardiac myocytes in a T25 flask were transferred to coverglass chambers for the fluorescence microscopy studies. In brief, approximately 100 μL of type 1 collagen solution (0.3%, ICN Pharmaceuticals) was coated on each well of the four-well coverglass chambers (Lab-Tek II, Nalge Nunc International). The chambers were air-dried in a laminar flow hood under UV light for 2–3 h. They were maintained at room temperature until use. An aliquot of the cell suspension was added to each collagen-coated culture chamber. Cells attached to the collagen matrix in 1 day in 5% CO_2–air, 100% humidified, in a 37 °C incubator.

2.6. Cell viability study

The effect of Na-QD on cell viability was evaluated using a MultiTox-Fluor Multiplex (MTMF) Cytotoxicity Assay (Promega, Madison, WI). In brief, ~100,000 HEK-293F cells were cultured in each well of a 96-well plate. Eight wells were used per condition. In brief, cells were loaded with Na-QD over night using a cationic liposome protocol as previously described [10] and cells without Na-QD

were used as controls. Cells were allowed to restabilize in culture and experiments were performed two days later. The solution containing the substrate for live-cell protease was added according to the manufacturer's instructions. After 30 min of incubation, live-cell fluorescence was measured at $400_{Ex}/505_{Em}$ using a microplate reader (Cytofluor 2350, Millipore). Viability of cells are normalized to the cell numbers in each well and expressed as percentages.

2.7. Inverted fluorescent microscope system for dynamic $[Na^+]i$ measurements

The system has been described elsewhere [10]. In brief, a 425 nm He–Cd laser (Series 56, Omnichrome, Melles Griot, Carlsbad, CA) as an excitation light source was reflected 90° upwards by using a long-pass 45° incident angle dichroic mirror placed under the microscope objective (Nikon Diaphot, Japan). Fluorescence emitted from the excited Na-QDs trapped in the cells and that of the laser beam were visually focused at the same plane by using a 50× oil immersion objective. Na-QD fluorescent signals, transmitted through the dichroic mirror, were directed to an interference filter of 620 nm and detected with a photon-counting photomultiplier tube (HA1040, Hamamatsu, Japan). The photon counts were processed at a sampling rate of 100 Hz in real time with a photon counter board interfaced to a PC. Programs were written in MATLAB with the Real Time Toolbox.

2.8. Physiopharmacological experimental protocols

Na-QD was applied to investigate the dynamic cellular responses of $[Na^+]i$ in nonexcitable HEK-293F cells and in excitable primary rat cardiac myocytes. Na-QDs were loaded into the cells for a minimum of 3 h by using a cationic liposome (Invitrogen, Carlsbad, CA), as previously described [10]. A volume ratio of Na-QD to liposome of 1:3 was used in the studies. After the cell samples were washed with culture media to remove the extraneous Na-QD and liposome, cells were restabilized in culture media for a minimum of 4 days prior to commencing the experiments. On the day of the experiment, cells were again washed with the media to eliminate any cellular debris and exogenous Na-QD. Dynamic cellular $[Na^+]i$ responses were measured by using a He–Cd laser inverted microscope fluorescent system.

Two studies were performed on the basis of established cellular mechanisms to demonstrate the dynamic Na-QD responses measured in the nonexcitable HEK-293F cells: (i) $[Na^+]i$ was increased with SQI-Pr (TEFLabs, Austin, TX, USA), a highly Na^+ selective sodium ionophore to increase the membrane permeability of Na^+; and (ii) $[Na^+]i$ was decreased with bumetanide (Bumex, Hoffmann-La Roche, Nutley, NJ), a potent and specific inhibitor of Na–K–2Cl cotransporter to block the influx of extracellular Na^+ into the cells. A minimum of five experiments were performed per study. Following establishment of the baseline, the agents of interest, namely either SQI-Pr at 1 μM and 10 μM or bumetanide at 2.5 μg/mL, 25 μg/mL, and 250 μg/mL were added topically to each sample 5–10 min apart in a cumulative manner and the $[Na^+]i$ responses were measured accordingly.

We also tested two common cardiac agents to determine whether Na-QD can be applied to measure $[Na^+]i$ in subsided beating rat cardiac myocytes. Two studies were performed in which $[Na^+]i$ was decreased: (i) with lidocaine (Abbott Laboratories, North Chicago, IL, USA), a local anesthetic and common anti-arrhythmia agent that inhibits the Na^+/H^+ antiporter; and (ii) with bumetanide, a diuretic that specifically inhibits the Na–K–2Cl cotransporter to decrease the influx of extracellular Na^+ into cells. Two experiments were performed per study. Similarly, following establishment of the baseline, the agents of interest, either bumetanide at 0.25 μg/mL, 2.5 μg/L and 25 μg/L or lidocaine at 0.01%, 0.1% and 1% were added cumulatively to the cells 5–10 min apart and the $[Na^+]i$ responses were measured accordingly.

In all HEK-293F cells and rat cardiac myocytes experiments, baseline fluorescence photon counts were greater than 1000 a.u. (counts)/channel when sampled at a frequency of 100 Hz (10 ms/channel). These agents were all prepared in phosphate-buffered saline.

3. Results and discussion

3.1. Na-QD nanosensor

A Na-QD sensor was constructed with a 12-crown-4 structure conjugated within the FRET distance of the QD to enable quenching of the energy transferred from the QD FRET donor to the Na/12-crown-4 complex acceptor (Fig. 1a). 12-crown-4, the smallest and simplest unadorned structure of crown ether, has long been sought as one of the potential structures for sensing Na^+. Considering the radii of Na^+ (~1.02 A) and K^+ (~1.38 A), it is intuitively reasonable to base on "hole size" relationship [9] to choose 12-crown-4 as the Na^+ sensor. With similar size of the cation of the interior cavity ('hole'), binding will be optimal. There are two speculative FRET mechanisms of binding Na^+ to the crown—ethers that could cause quenching. In principle, bindings of increased Na^+ to 12-crown-4 enhance FRET transfer from the QD to the sensor which in turn cause a dose dependent quenching of the fluorescence intensity upon binding of the Na^+ [11,13]. Another possibility is the sandwich complexation of Na^+ conformation enables the assembly of the Na-QD in proximity and results in intermolecular interactions that alters the fluorescence transfer [9,11,13]. These FRET mechanisms of the Na-QD is schematically summarized in Fig. 1d.

3.2. Characterizations of Na-QD nanosensor

The emission spectra of Na-QD were determined (PTI spectrophotometer) when $[Na^+]$ was increased from 0 mM to 130 mM. The excitation wavelength was set at 450 nm and the emission wavelengths were scanned from 550 nm to 650 nm. Fig. 2a shows the normalized Na-QD fluorescent signals against the increased concentrations of the $[Na^+]$ determined at physiological pH 7.4. The quenching of the fluorescent signals with increased $[Na^+]$ is consistent with the mechanisms of energy transfer from the QD to the Na/12-crown-4 complex. A binding constant of 2.54 has been determined from the linear regression ($R^2 = 0.99$) between the normalized fluorescent intensity of the Na-QD and the $[Na^+]$ (Fig. 2b). Note that (i) the luminosity of the Na-QD is inversely proportional to the Na^+ concentration; and (ii) the peak emission wavelength of the Na-QD, 620 nm, was not red-shifted in all the measurements of different concentrations of Na^+ compared with the original QD. These data imply that the conjugation of the sodium receptors, 12-crown-4, to the QD does not alter the predominant intrinsic energy characteristics of the QD.

We evaluated the selectivity of Na-QD toward Na^+ over K. When the total concentrations of $[Na^+]$ and $[K^+]$ are maintained at a constant 130 mM, the binding constant (β) derived from the emission spectra is 2.51. There is minimal difference between these emission spectra and those shown in Fig. 2a. When the data for $[Na^+]$ at 65 mM are compared with and without $[K^+]$ at 65 mM, there is also no discernable difference in fluorescence spectral responses (Fig. 2c). These data demonstrate that Na-QD has a high selectivity for Na^+ over K^+ for the measurement of $[Na^+]$ at physiological pH.

We also evaluated the effect of Ca^{2+} from 0.001 mM to 1 mM on Na-QD. Basal $[Ca^{2+}]$ and the sequestered $[Ca^{2+}]$ in the intracellular organelles of cells are ~0.001 mM and 1 mM, respectively. Upon bindings of the cell surface receptors with calcium mobilizing ago-

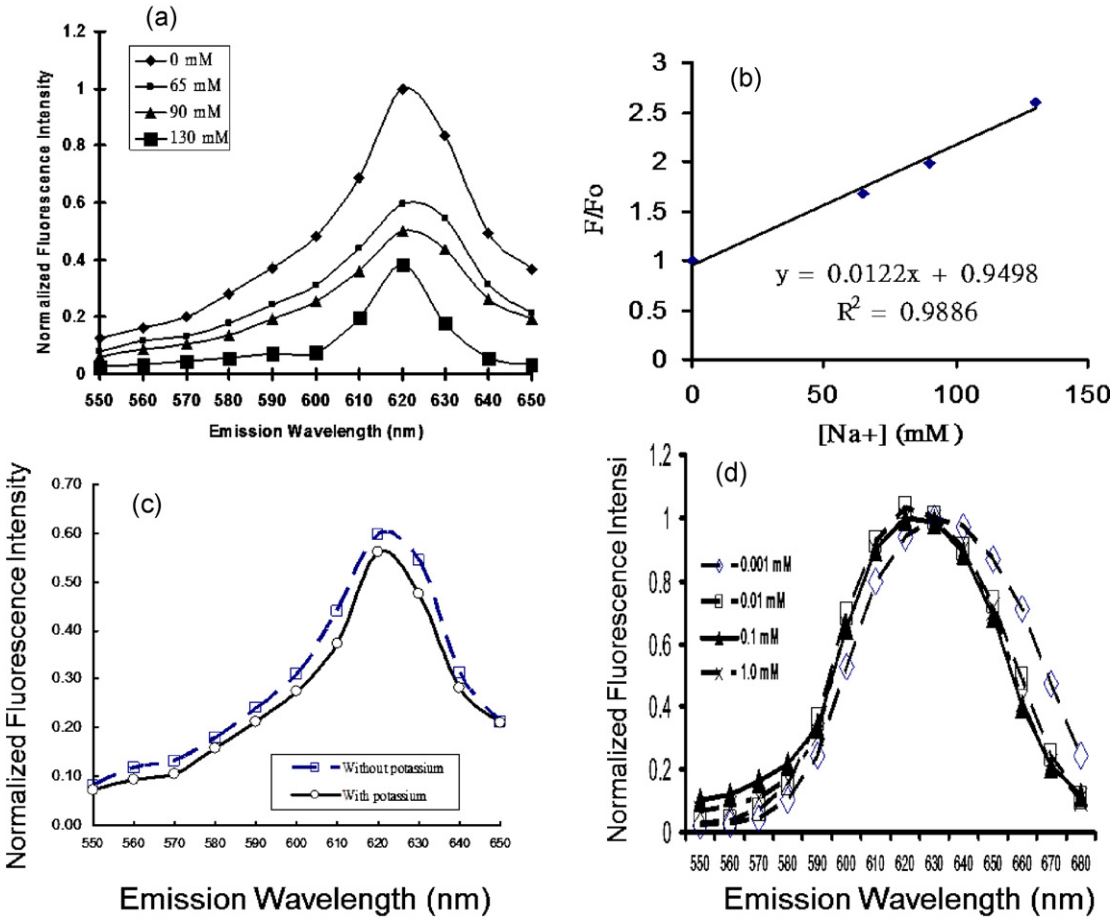

Fig. 2. (a) Emission spectra of Na-QD against increased [Na^+]. Measurements were done at pH 7.4. (b) The Stern-Volmer linear relationship derived from the emission spectra of Na-QD shown in (a). Note that the fluorescence intensity of Na-QD is inversely proportional to [Na^+]. (c) Emission spectra of Na-QD in increased [Na^+] with and without K^+. The final concentrations of [K^+] together with [Na^+] ([K^+] + [Na^+]) were maintained at 130 mM. (d) Effect of 0.001 mM, 0.01 mM, 0.1 mM and 1 mM [Ca^{2+}] on the emission spectra of Na-QD.

nists, Ca^{2+} could release from the intracellular organelle and also cause influx of extracellular calcium via a calcium-induced-calcium mechanism such that [Ca^{2+}]*i* could transiently reach 0.01–0.1 mM. Fig. 2d shows the effect of 0.001 mM, 0.01 mM, 0.1 mM and 1 mM [Ca^{2+}] on Na-QD in physiological buffer solution. All four concentrations of [Ca^{2+}] did not alter the responses at 620 nm emission wavelength as well as the emission spectra of Na-QD. This is expected as Ca^{2+} sensing probe is largely developed based on BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid) structure rather than the 12-crown-4 and 5-crown-5 structures for Na^+ and K^+, respectively [5]. In addition, basal [Na^+]*i* of cells ranges 4 mM to 25 mM while basal [Ca^{2+}]*i* is ~0.001 mM in most cell types. Upon stimulation, [Na^+]*i* could transiently increase to 90 mM in some cell types but [Ca^{2+}]*i* has never been reported to be higher than 0.1 mM. This 3-log concentration difference between [Na^+]*i* and [Ca^{2+}]*i* in cells also ensures the minimal effect of [Ca^{2+}]*i* on Na-QD.

The quantum yield of Na-QD was 0.59 ± 0.01, consistent with the quantum yields of CdTe QDs which have been reported to be 0.27–0.73 [14]. K-QD has been reported to be 0.15 and 0.21 with no biological data has ever been reported in association with this probe [11]. The respective quantum yields of SBFI and sodium green are 0.08 and 0.2 (Data Sheet, Molecular Probes), while PBFI is 0.072 [15]. For practical purposes, the quantum yield of a sensor should be close to 0.4 or greater when the sensor is used for cell applications [16]. The quantum yield of Na-QD is in the range to be a robust Na^+ sensor for cell applications.

3.3. Physiopharmacological experiments

An example of Na-QD trapped inside the HEK-293F cells is shown in Fig. 3a. The 460 nm epi-illuminated picture was taken with a dimmed bright-field light using a 20× objective to show both the cells and the Na-QD trapped in the cells. The super-luminosity of the Na-QD trapped inside the HEK-293 cells is apparent when the picture of the same view was taken without the microscope bright-field light (Fig. 3b). Fig. 3C shows the corresponding bright-field picture of the view to illustrate the healthy morphologies of the cell culture. Consistent with our previous observations [10], the liposome loading protocol of QD-based nanosensor for intracellular measurements preserves the integrity of the cells as indicated by their healthy morphologies and the undiminished super-luminosity of the Na-QD. It is notable that some of the Na-QDs inside the cells were clustered together. This is more apparent when they were observed under high magnification using a 40× oil-immersion objective (Fig. 3d). This is consistent with the hypothesis (Fig. 1d) that sandwich complexation of Na^+ conformation could be one of the mechanisms enabling fluorescence transfer [13] in crown ethers [9].

The MTFM assay applied to the HEK-293F cells indicates that the cell viabilities for cells loaded with Na-QD was 93.3% compared to the control of 94.2% ($p > 0.01$). This is consistent with the healthy morphology shown in Fig. 3a–d.

The viability of Na-QD for [Na^+]*i* measurements depends on the preservation of the integrity of the predominant cellular

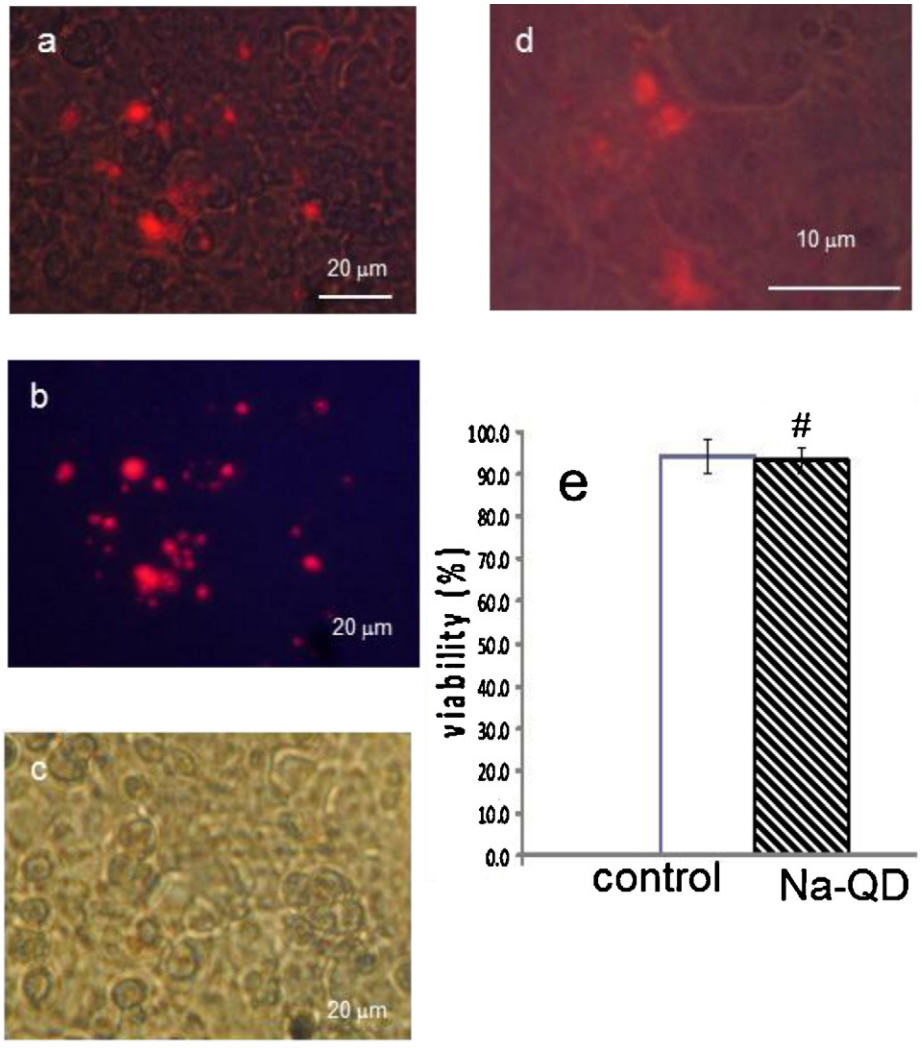

Fig. 3. Pictures of the Na-QD trapped in HEK-293F cells. The cells were epi-illuminated using a Xenon light source coupled with a 440 nm interference filter. The pictures were taken with a 20× objective of the same sample when the microscope overhead bright-field light was (a) dimmed to show both Na-QD trapped inside the cells and the perimeters of the cells, (b) off to show only the luminosity of the Na-QD, (c) in full power to show the healthy morphologies of the cells. (d) Picture of another sample taken with a 40× oil immersion objective when the microscope overhead bright-field light was dimmed to illustrate both Na-QD trapped inside the cells and the perimeters of the cells. The size of HEK-293 cell was determined to be ~13 μm based on the micrometer embedded in the eye-piece and the scale shown in the pictures, consistent with the data published in the brochure by InVitrogen. (e) Comparisons of the cell viabilities of the HEK-293F cells with and without Na-QD loaded into the cells ($n = 8$, $\#p > 0.001$).

signal transduction mechanisms following its loading into the cells. HEK-293F cell is a human embryonic cell line with established uncompromised cellular signal transduction mechanisms. Primary cardiac myocytes with subsided spontaneous beating should, purportedly, preserve all the predominant signal transduction mechanisms. Both cell types exhibit dynamic $[Na^+]i$ baselines. They were used to evaluate the Na-QD.

Fig. 4a shows an example of the dynamic responses of $[Na^+]i$ measured by the Na-QD when the HEK-293F cells were challenged with the sodium ionophore. SQI-Pr has a Na^+ to K^+ selective ratio of 80–100:1. When 1 μM and 10 μM SQI-Pr were added cumulatively to the cells, they predictably decreased the fluorescent intensity of the Na-QD. These data imply that $[Na^+]i$ was increased in a dose-dependent manner. Of note in this example is that the rate of decrease of fluorescent intensity for the first dose of 1 μM SQI-Pr was 0.25-fold per 100 s. It reached a plateau value of 0.7-fold 200 s following its application. The rate of decrease of fluorescent intensity for the second dose of 10 μM SQI-Pr was 0.1-fold per 100 s and reached a plateau value of 0.4-fold, also 200 s following its application. These data are consistent with the conventional kinetics defined by a single diffusive mechanism without invoking other pathways. In five experiments (Fig. 4b), SQI-Pr at 1 μM and 10 μM decreased the fluorescence of Na-QD from the baseline and control to 0.7-fold and 0.5-fold, respectively. Note that 0.1 μM SQI-Pr caused a slight increase in $[Na^+]i$ in some samples (data not shown). These data are consistent with our previous findings that this Na^+-selective ionophore [6] depolarized the cell membrane potential of epithelial cells as a result of the increase in $[Na^+]i$.

In the study in which the Na^+ influx was inhibited via the Na–K–2Cl cotransporter in HEK-293F cells, 100 μL of each dose of bumetanide at 2.5 μg/mL, 25 μg/mL, and 250 μg/mL predictably induced a corresponding increase of fluorescent intensity by 1.2-fold, 1.5-fold, and 3.5-fold (Fig. 4c). Of note is that the corresponding rates of increase of fluorescence were also increased from 0.2-fold per 100 s, 0.5-fold per 100 s, and 1-fold per 100 s. These rates of increase of fluorescence are consistent with the receptor transport mechanisms. In five experiments, bumetanide at 2.5 μg/mL, 25 μg/mL, and 250 μg/mL increased the corresponding mean responses of Na-QD fluorescence by 1.2-fold, 1.8-fold, and 2.2-fold in a dose-dependent manner (Fig. 4d).

When three doses of 60 μL of lidocaine per application were applied cumulatively to the cardiac myocytes, lidocaine at 0.01%,

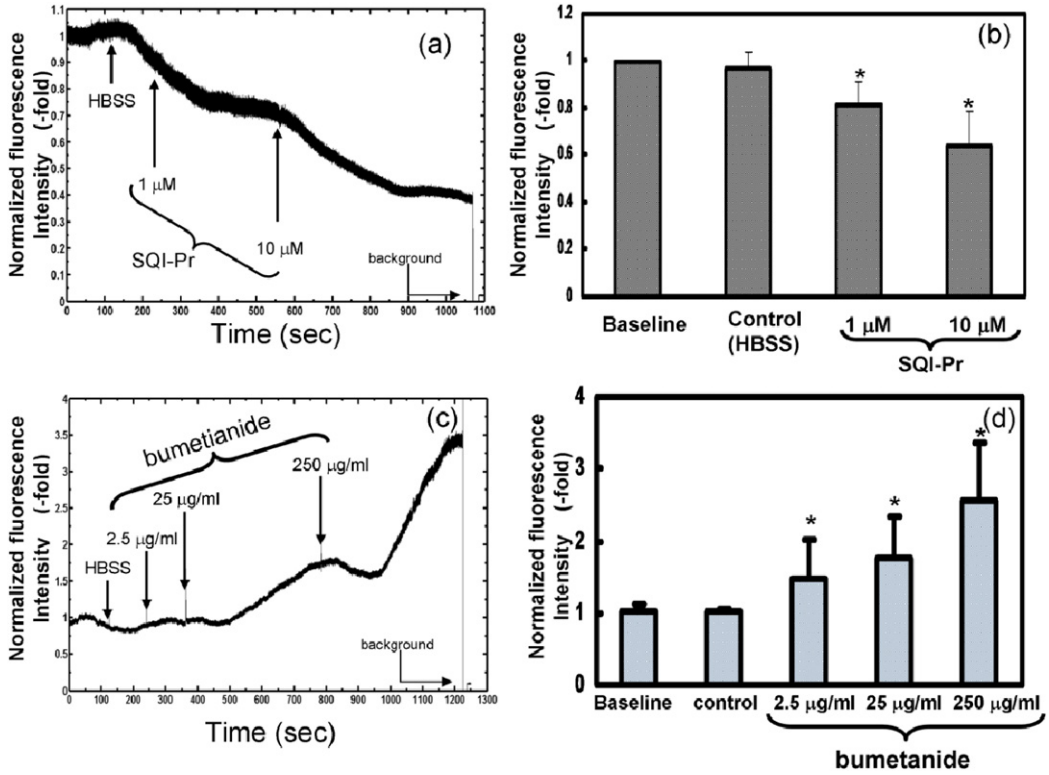

Fig. 4. $[Na^+]i$ responses in HEK-293F cells measured by the Na-QD. (a) Example of dynamic responses of $[Na^+]_i$ by SQI-Pr, a specific sodium ionophore. Two doses of SQI-Pr were added cumulatively to the sample. (b) Mean dose responses of $[Na^+]_i$ to SQI-Pr of five samples. * Mean $p < 0.05$, ** mean $p < 0.01$ and *** mean $p < 0.001$. (c) Example of dynamic responses of $[Na^+]_i$ by bumetanide, a specific sodium ionophore. Three doses of bumetanide were added cumulatively to the sample. (d) Mean dose responses of $[Na^+]_i$ to bumetanide of five samples. * Mean $p < 0.05$, ** mean $p < 0.01$ and *** mean $p < 0.001$.

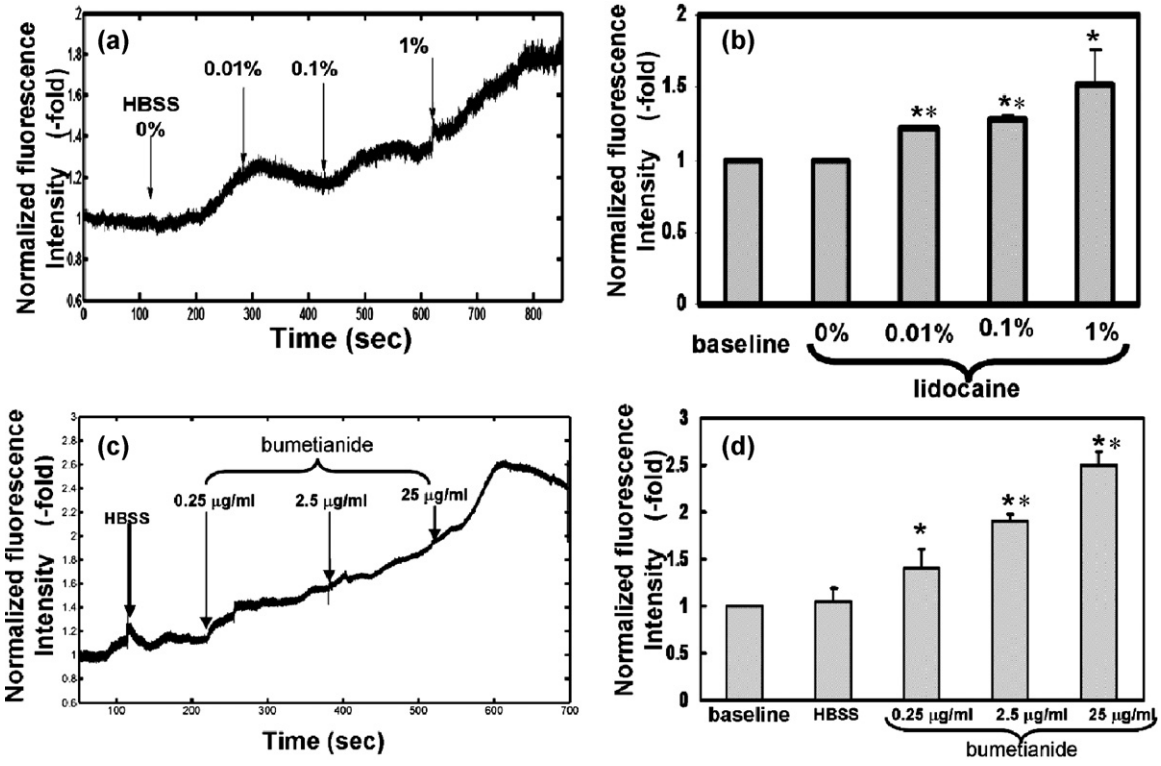

Fig. 5. $[Na^+]i$ responses in primary mouse cardiac myocytes measured by Na-QD. (a) Example of dynamic responses of $[Na^+]_i$ to lidocaine. (b) Mean dose responses of $[Na^+]_i$ to lidocaine in three samples. * mean $p < 0.05$, ** mean $p < 0.01$ and *** mean $p < 0.001$. (c) Example of dynamic responses of $[Na^+]_i$ to bumetanide. (d) Mean dose responses of $[Na^+]_i$ to bumetanide in three samples. * Mean $p < 0.05$, ** mean $p < 0.01$ and *** mean $p < 0.001$.

0.1%, and 1% caused a dose-dependent increase in fluorescence from the baseline by 1.2-, 1.3-, and 1.8-fold, respectively (Fig. 5a). The mean responses of two samples showed a similar consistent trend (Fig. 5b). When 100 μL of each dose of bumetanide at 0.25 μg/mL, 2.5 μg/mL, and 25 μg/mL was applied cumulatively to each cardiac myocyte sample, it caused a corresponding increase in fluorescence intensity of 1.5-fold, 1.8-fold, and 2.5-fold compared with the baseline and control (Fig. 5c). Bumetanide at 0.25 μg/mL, 2.5 μg/mL, and 25 μg/mL predictably induced a dose-dependent increase of fluorescence intensity by 1.5-fold, 2.0-fold, and 2.5-fold, respectively, in two samples (Fig. 5d). Note that the bumetanide is10× more potent when it is acting on cardiac myocytes than it is on HEK-293F cells.

Baseline $[Na^+]i$ of HEK-293 cells and primary cardiac myocytes exhibited some intrinsic dynamic physiological responses. These "fluctuations" were likely not due to the intrinsic quenching of the Na-QD, since the in vitro characterizations of Na-QD experiments summarized in Fig. 2a–d did not exhibit photobleaching. The physiological actions of both Na–K–2Cl cotransporter and the Na^+/H^+ uniporter require active phosphorylation processes. The predictable results in inhibiting these two transporters by the respective bumetanide and lidocaine not only demonstrate the viability of these cells, they also show the applications of this Na-QD sensor for Na^+-channel drug discovery. Direct $[Na^+]i$ measurements related to cardiac Na-channels has only been reported in 1993 using ^{23}NMR to assay $[Na^+]i$ [17]. The data reported herein represent the first dynamic measurements of $[Na^+]i$ in cardiac myocytes.

We have also performed experiments using Na-green to measure the dynamic $[Na^+]i$ responses in cardiac myocytes and other cell types. The baseline fluorescence was 50–150 AU, compared to the baseline fluorescence 1000 AU unit using Na-QD. This accounts for 5- to 15-fold difference in the baseline luminosities. When the common therapeutic agents such as bumetanide and lidocaine at their respective concentrations were tested, Na-green did not provide notable changes in the fluorescent signals (data not shown). This is possibly due to the lack of sensitivity of this probe as indicated by its low quantum yield and luminosity. The second possibility is that a higher probe concentration was usually used in these studies to increase the luminosities (brightness). However, the ester forms of Na-green were toxic to the cells.

4. Conclusions

We have synthesized and characterized a novel Na-QD nanosensor by conjugating 12-crown-4 to water-soluble CdSe/ZnS QDs. We have applied this highly Na^+-selective Na-QD nanosensor to measure the dynamic physiological $[Na^+]i$ responses in HEK-293F cells and primary rat cardiac myocytes when the cells were being pharmacologically manipulated. The characteristics exhibited by this Na-QD nanosensor demonstrate that this highly sensitive and specific $[Na^+]$ probe holds the promise and potential to be developed into a robust cell-based high-throughput screening assay for Na^+ channel drug discovery.

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