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Quantum dot-FRET systems for imaging of neuronal action potentials

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Abstract— **Fluorescent semiconductor quantum dots (QDs) can act as energy donors or acceptors with a wide variety of environmentally-sensitive molecules. Conjugation of a single QD to a select number of the selected molecule can optimize the range of sensitivity for a given application, and the relatively large size of the QDs allows them to be tracked individually in cells. Using QDs as FRET acceptors, we have created first-generation sensors for membrane potential which shows good signal to noise and time resolution, but prohibitive toxicity. The challenges of delivery, calibration, and toxicity and plans for improvement of the sensors are presented, in the context of the eventual aim of monitoring membrane potential in a cultured motor neuron model of amyotrophic lateral sclerosis.**

I. INTRODUCTION

The field of semiconductor nanoparticle research has grown more than 10-fold over the past five years, particularly focused upon spontaneously photoluminescent particles known as quantum dots (QDs). However, QDs are very different from organic fluorophores, and a lack of quantitative understanding of their response to physiological solutions has thus far limited their biological applications. Nevertheless, recent work by our laboratories and others has shown that QD-biomolecule interactions can be quantified and used to create molecular-scale probes for redox potential [1], pH [2], and specific metabolic processes [3].

Our laboratory is currently targeting several important cellular processes which are difficult or impossible to measure using organic dyes or fluorescent proteins. The sensing mechanism in all of our constructs is alterations in QD fluorescence due to energy or electron-transfer processes. Because of their broad absorbance and narrow emission spectra, QDs are ideal fluorescence resonance energy transfer (FRET) donors. Their has been demonstrated in a variety of biological applications, particularly specific ligand recognition using QDs coupled to antibodies [4] or molecular adapters [5]. On the other hand, very few studies on the use of QDs as FRET acceptors have been published, because the efficiency of these processes is usually low [6, 7]. The potential of environmentally-sensitive probes using QDs as acceptors is great, and thus a full investigation of the properties of these processes is warranted.

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The particular application of interest is voltage sensing particularly detection of single action potentials in neurons, (i.e. a resolution of at least 100 mV/10 nm in 10 ms). The best results obtainable today make use of voltage-sensitive dyes, in particular a class of organic dyes called the aminonaphthyl-ethenyl-pyridinium (ANEP) dyes, such as di-4-ANEPPS and di-8-ANEPPS [8] and a related class of dialkylaminophenylpolyenylpyridinium dyes called the RH dyes (after their inventor, Rina Hildesheim) [9]. These dyes are hydrophobic, being essentially nonfluorescent in aqueous solution, beginning to fluoresce after embedding within a cell's plasma membrane. They then demonstrate (at best) a ~10% change in fluorescence intensity per 100 mV potential difference with millisecond time resolution. While the time sensitivity is ideal, the emission shift is too small to be visualized and must be imaged with a fast CCD camera and deconvolved after the experiment. In addition, the ANEP dyes are significantly phototoxic and usually lead to cell death within 1-2 hours [10]. While some groups have obtained action-potential data using these dyes, the technique is not widespread because of the specialized equipment needed and the low signal to noise in the best data.

Alternatives to voltage-sensitive dyes have been sought in the form of modified fluorescent proteins. The most successful approach to such a probe involves a fusion of the Shaker potassium channel to GFP, called FlaSh. While this fusion protein establishes a proof of principle, its emission changes with voltage are unfortunately very small (~1 %) [11]. The greatest drawback of this type of approach is that the GFP is located outside of the membrane, so it is not directly exposed to the electric field. The lesson from this work is that the attachment of GFP to an extra- or intracellular site of a voltage-sensitive transmembrane protein is insufficient to create a strong voltage-sensitive probe, because the basic structure of the GFP remains the same during the potential-induced conformational change of the protein.

The overall goal is to create tools that will permit optical imaging in cultured motor neurons. This system, which models motor neuron disease either by culturing neurons from mutant mouse models of the disease or by transfecting wild-type cells with superoxide dismutase, contains many unanswered questions which are difficult to address using current technology. In particular, the role of glutamate receptors and calcium permeability in the observed pathology remains highly controversial [12].

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II. Experimental Details

CdSe/ZnS QDs are synthesized as described [3]. Delivery of the probes to the cell membrane is as follows: first, the QDs are washed to remove any traces of TOPO; this is accomplished by several rounds of heating in pyridine followed by drying by rotovap. All traces of pyridine must be removed before addition to cells; this is performed by diluting the QD-pyridine solution 1:1000 in chloroform, drying it to a sludge, and repeating the process five times. The resulting solution is extremely concentrated (~10 □M QDs). 1-5 □L of this solution is added to 500 □L of 2 w/v% Pluronic (Dupont) in DMSO or ethanol (excellent results can be obtained with either solvent). After full dissolution of the QDs, the solution is applied at a 1000-fold dilution to cells in serum-free medium and incubated at 37 ºC for 15-30 min with shaking (because the QDs are dense, the shaking is important to keep them from sinking to the bottom of the dish). ANEPPS and RH421 are applied to cells in a similar fashion: the concentrated dye stock (10 mg/mL in methanol) is diluted to 10 □g/mL in 2% Pluronic in H_2O and added to cells in a 1:100 dilution. Rocking is not necessary, but 1-2 hours are allowed for full labeling. When both QDs and dye are used, the cells are labeled first with dye, then QDs are added.

We use an off-the-shelf fluorimeter for spectral recording from a microscope/CCD system that enables us to quantify the emission spectra of cells in an extremely precise manner. The technology ("Nuance" from Cambridge Research Instruments) uses liquid crystal tunable filters and allows microspectral imaging throughout the visible and near infrared ranges. Multispectral imaging provides images of a scene at multiple wavelengths, rather than simply red, green, and blue, and can generate precise optical spectra at every pixel. The time resolution for switching between wavelengths using this system is milliseconds.

Changing of cell membrane potential is accomplished by glutamate depolarization (for neurons) or by patch clamp. Recordings from single cells are performed with a HEKA EPC-10 Double patch clamp amplifier using the acquisition software PatchMaster. Cells are visualized on an Olympus IX-71inverted microscope enclosed in a Cu mesh Faraday cage and floated on an air table (Kinetic Systems). The bathing solution consists of (in mM) 150 NaCl, 5 KCl, 4 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, pH 7.4, and the pipette solution 140 CsCl, 1 EGTA, 10 HEPES, pH 7.4. Pipettes are pulled on a Sutter P-97 puller and have a resistance of 4-6 MΩ when filled with recording solution. Data are acquired at 10 kHz and filtered at 2.9 kHz; simultaneous spectral data are obtained from the Nuance imaging system.

Cell lines are available from ATCC and are maintained by weekly passage in DMEM supplemented with 10 % fetal bovine serum. Primary neurons were obtained from Prof. Heather Durham (McGill).

III. Results

A. QD as FRET acceptors from voltage-sensitive dyes

In our published work [13], we found that the voltage sensitivity of existing membrane potential dyes can be greatly magnified when these dyes are used as energy donors to membrane-embedded QDs. Very few studies with QDs as energy donors have been done, because the efficiency of these processes is usually low[6, 7]. Although the wide spectra of the voltage-sensitive dyes and the wide excitation of QDs means that exciting the dye without exciting the QDs is not possible, it is nonetheless possible to deconvolve the spectra to prove that the QDs spectrum is enhanced while the ANEPPS spectrum is quenched, and thus the QDs are truly acting as FRET acceptors.

We have recently confirmed that the ability of QDs to act as acceptors is necessary for voltage sensitivity. When blue-emitting QDs are used as donors to ANEPPS and RH dyes, very efficient FRET can be obtained, with clear donor vs. acceptor spectra (not shown; QDemission wavelengths tested: 465 nm and 480 nm). However, there are **no spectral changes** with membrane potential change. Thus, the use of QDs as the acceptors is necessary to observe this effect.

With QD emission peaks in the yellow or red, where they may act as acceptors, very large changes (> 100% peak enhancement, with ~10 nm peak shift) are seen in the emission spectrum upon establishment of membrane potential. Interestingly, the pattern of change seen resembles the monotonic decrease with voltage as seen in the QDs alone, rather than the parabolic shift exhibited by ANEPPS. This change is readily visualized under fluorescence microscopy; the time scale of these changes is well within what is required for resolution of action potentials: all changes were 100% complete within the smallest time step of our spectrofluorimeter (10 ms).

B. Voltage-related spectral changes in primary neurons

Quantum dots capped with small hydrophobic molecules (pyridine or small amines) and dissolved in a solution of Pluronic will assemble spontaneously into cell membranes, including those of primary neurons (Fig. 1).

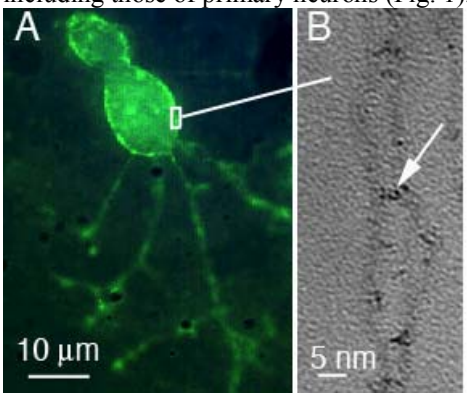

Figure 1. Delivery of hydrophobic QDs to primary rat cortical neurons in culture (3 days *in vitro*). A, Epifluorescence image showing strongest QD fluorescence from the cell edges and patchy labeling in the processes. B,

Transmission electron micrograph of a thin section of a labeled vesicle, showing dark areas within the membrane that are identified as Cd and Se at a ~1:1 ratio by energy-dispersive X-ray spectroscopy (EDS).

An image of a primary neuronal culture labeled with the voltage-sensitve dye RH421 is shown in Fig 2 A, and its corresponding spectrum in Fig. 2 C. When the culture was depolarized with 30 mM glutamate, a spectral change consistent with that observed in vesicles was measured (Fig. 2 B, C). Results obtained with di-4-ANEPPS were qualitatively similar but quantitatively smaller by a factor of 2-3 (data not shown).

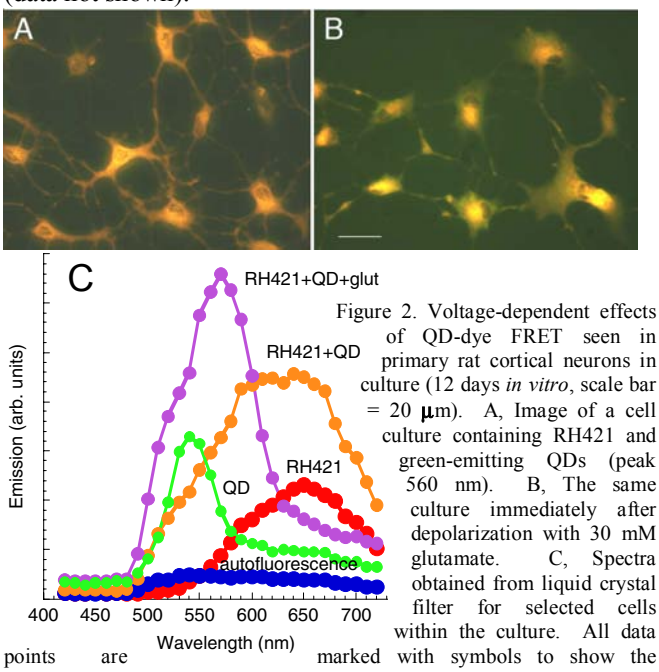

Figure 2. Voltage-dependent effects of QD-dye FRET seen in primary rat cortical neurons in culture (12 days *in vitro*, scale bar = 20 μm). A, Image of a cell culture containing RH421 and green-emitting QDs (peak 560 nm). B, The same culture immediately after depolarization with 30 mM glutamate. C, Spectra obtained from liquid crystal filter for selected cells within the culture. All data points are marked with symbols to show the accuracy of the curves obtained. Without QDs or dye, the cell autofluorescence is small (blue). Addition of RH421 alone or QDs alone leads to classic spectral peaks in the cell membrane. When both are present in a resting cell with 5 mM external KCl (expected membrane potentials ~ -60 mV), a broad peak somewhat larger than the sum of the RH421 and QD peaks alone is seen. When the cell is depolarized with glutamate, a dramatic blue-shift and increase and intensity are observed on the edges of the cells.

Voltage-clamping of individual cells containing QDs has certain advantages over bulk depolarization experiments, as it allows for (a) precise control of voltage and time course; (b) repeated voltage cycles to test the durability of the probes; (c) elimination of possible effects due to direct interaction of QDs and/or ANEPPS with the ionophores; (d) wide variation of the ionic makeup of the internal and external solutions; (e) a study of the toxicity of the particles to living cells.

We have established the ability to combine patch-clamp with multispectral imaging (Fig 3 A). Although the time scale is not yet sufficiently fast to allow resolution of spectral changes with action potential, we were able to test the repeatability of the RH421-QD FRET system to a series of long, slow voltage steps. The results are intriguing but promising: although the emission intensity of the hyperpolarized state is decreased by ~25 % after 10 cycles of 3 min each, the spectra of the depolarized state remain remarkably constant (Fig. 3 B).

These experiments were only successful in epithelial cell lines. Primary neuronal cultures, including hippocampal, motor neuron, and cortical neurons, were too damaged after QD-Pluronic delivery to permit patch-clamp.

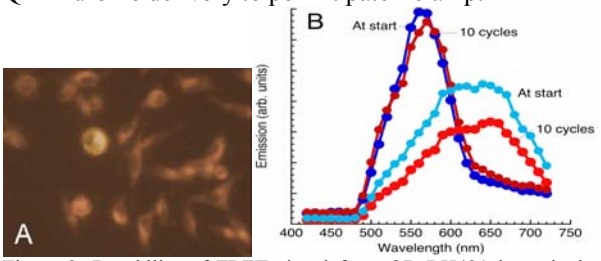

Figure 3. Durability of FRET signal from QD-RH421 in a single patch-clamped cell. A, Mouse A9 cell line in the depolarized state (holding +50 mV) showing spectral difference in clamped cell (the bright spot is the recording electrode; the presence of the electrode also leads to a blurred image and color distortion to the eye). B, Spectral signals at the start from holding potentials at +50 mV (light blue) and –100 mV (dark blue). After 10 cycles of 1 min at each of the two potentials, the signal at –100 mV is decreased (red) whereas the spectrum at +50 mV has remained constant (maroon). Qualitative shapes and shifts have not changed.

C. Constructs to be tested

In order to clarify the photophysics and improve toxicity, we have created but not yet tested a modified ANEPPS that has been directly linked to a QD. In this case, the QD is hydrophilic and located external to the membrane. This construct will have three advantages: (1) it will allow a quantitative measurement of energy transfer efficiency vs. the distance between the ANEPPS fluorophore and the QD; (2) it will restrict the QD:dye stoichiometry to 1:1, allowing for more quantitative data interpretation; (3) it will likely be less toxic to cells, as it will not be located directly within a membrane, and will not be restricted to small QDs. The initial construct is a "zero-length" ethyl linker, easily variable to any length alkyl chain. The distance to the QD core may also be varied by changing the alkanethiol used to solubilize the particle: from 2 carbons (mercaptoacetic acid) to 16 carbons.

We have also designed a hybrid ion-channel/dye system that does not require embedding of hydrophobic structures into cell membranes. This was based upon previous work in which a variation of di-4-ANEPPS that is hydrophilic was shown to demonstrate voltage-sensitive behavior as part of a hybrid with the potassium selective ion channel protein Shaker [14]. The channel was expressed in a cell line, and the modified dye was then reacted with a cysteine residue cloned into a specific site in the protein. The choice of a site near the putative voltage gate of the channel resulted in the greatest effect.

Our hybrid system allows for FRET system between this modified ANEPPS and QDs. The modified ANEPPS was be synthesized via a β-[2-(Dimethylamino)-6-naphthyl]-4-vinyl pyridine precursor (Fig. 4 A). A maleimide group makes the channel thiol-reactive, but insufficiently reactive to label cell membranes nonspecifically [14]. Rather than Shaker, we have chosen to use a voltage-dependent sodium channel (NaChBac) for which the positively-charged voltage gate has been identified [15]. The last arginine will be converted to a cysteine by site-directed mutagenesis. To attach

a QD to the protein, a 6His tag will be employed on the first extracellular loop, chosen for its flexibility (Fig. 4 B).

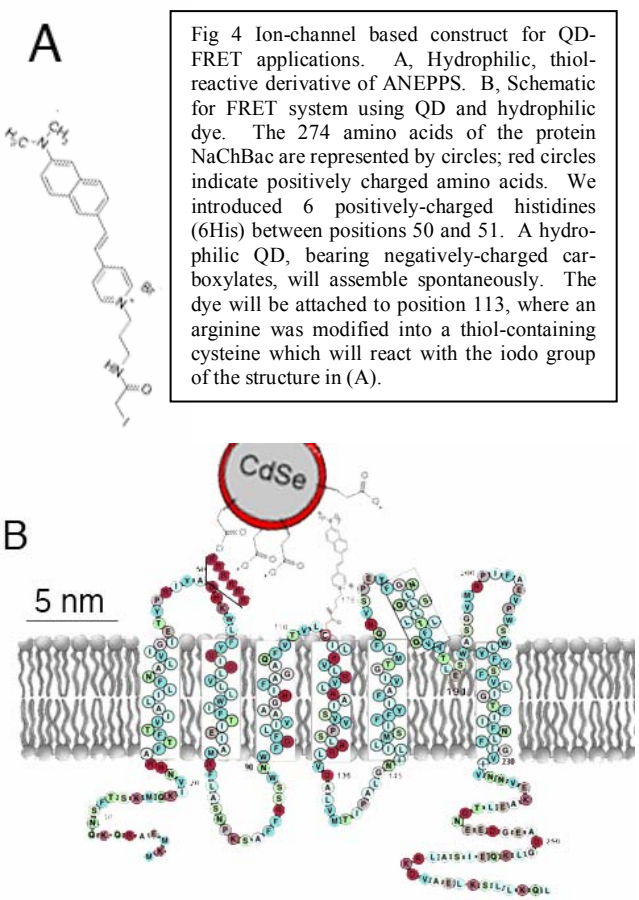

Fig 4 Ion-channel based construct for QD-FRET applications. A, Hydrophilic, thiol-reactive derivative of ANEPPS. B, Schematic for FRET system using QD and hydrophilic dye. The 274 amino acids of the protein NaChBac are represented by circles; red circles indicate positively charged amino acids. We introduced 6 positively-charged histidines (6His) between positions 50 and 51. A hydrophilic QD, bearing negatively-charged carboxylates, will assemble spontaneously. The dye will be attached to position 113, where an arginine was modified into a thiol-containing cysteine which will react with the iodo group of the structure in (A).

IV. Discussion

The utility of QDs as FRET acceptors has not been well explored, and determination of the resulting properties will determine whether this avenue of research is potentially fruitful for the creation of sensors for specific biological processes. Additional basic physical chemistry is needed to fully understand the energy transfer processes in this system, since the spectra obtained are not "clean" since the excitation wavelength excites both donor and acceptor. One useful approach to this is to perform time-resolved emission measurements. we have found emission lifetimes of 10-20 ns in our QD preparations, and the published lifetime for voltage-sensitive dyes are on the order of 0.4 ns. Hence, time-resolved spectroscopy with this range of sensitivity will discriminate between the two species and allow an interpretation of the energy transfer mechanisms.

Measurement of effects of changes of surface potential on cells or vesicles containing QDs and/or voltage-sensitive dyes may also be of great interest. From Fig. 3 A, it is seen that QDs can be located at different positions within a cell membrane, from very shallow near the inside or outside of the cell, to deep within the membrane. Thus, it is expected that changes in the surface potential will affect the spectra of the vesicle membranes differently on a spatial scale of nanometers.

The problem of QD toxicity remains a serious one. While the constructs discussed in Part III C are likely to eliminate toxicity caused by direct embedding (mechanical damage to the membrane) and toxicity due to hydrophobic capping materials such as pyridine, the oxidative phototoxicity of the QDs may well remain important. It will almost certainly be necessary to add antioxidants to cells containing these constructs, especially sensitive neuronal cultures.

V. Conclusions

QDs can show voltage sensitivity as FRET acceptors from voltage-sensitive dyes. However, the toxicity problems of the current constructs have not been resolved.

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