NOVEL METHODS FOR LABELING MICROORGANISMS

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Abstract: Fluorescent imaging has undergone important changes in recent years, and semi-conductor nanocrystals known as quantum dots (QDs) have been proposed as a 'next generation' fluorescent probe in biological applications. Here we show labeling of bacteria with QDs, and analyze the potential for QDs as a diagnostic tool for microbial populations. We also discuss the stability of QDs in solution, and various methods of achieving solubility of QDs in water. Finally, we examine the toxicity of QDs and the implications for using QDs in future studies.

Introduction

Fluorescent imaging of biological cells has become an invaluable tool in scientific research. Traditionally the fluorescence used in imaging has been accomplished through the use of organic fluorophores, and has thus been limited by certain chemical properties of these molecules. These include a narrow absorbance spectrum, broad emission spectrum, and sensitivity to photo bleaching. For several years now there has been tremendous interest in the use of semi-conductor nanocrystals – commonly referred to as quantum dots (QDs) – as a means of fluorescence. The use of inorganic QDs in imaging allows the research scientist to avoid many of the limitations of organic fluorophores. QDs have a broad absorbance spectrum and narrow symmetric emission spectrum, which can be finely tuned. This means that several distinct populations of QDs, all emitting at a characteristic wavelength, can be used in the same experiment using a single light source. As well, QDs show strong resistance to photo-bleaching with respect to organic dyes. All of these properties make QDs the ideal fluorescent probe for microbial populations such as bacteria.

New possibilities for this technique continue to be found, with an enormous potential in both basic research and in clinical applications. While many reports have used QDs as fluorescent probe [1-3], others studied their possible use in selectively causing cytotoxicity for therapeutic benefit [4]. Here we show labeling of Gram-positive *Staphylococcus* species bacteria, and analyze the potential for QDs in labeling microbial populations, as well as the current technical limitations with this approach. In addition, various different methods of solubilizing QDs in aqueous solution are discussed.

Materials and Methods

CdSe/ZnS 'core-shell' nanocrystals were synthesized as described elsewhere. Briefly, CdSe/ZnS QDs were synthesized as follows: 0.024 g CdO was added to a reaction flask containing 0.44 g stearic acid and heated to 180 °C under inert conditions, forming a colourless solution. The solution was allowed to cool, and afterwards 5 g TOPO and 2 g octadecylamine was added to the flask. The flask was then evacuated and filled with inert gas several times, and the solution was heated to 200 °C-300 °C (exact temperature depends on the desired size). 0.2 g Se was then dissolved in 2-4 mL TOPO under inert conditions, and added to the reaction flask. Finally, 0.4 mL of $Zn(Me)_2$ was added to 0.07 mL $(TMSi)_2$ under an inert atmosphere, and added to the reaction flask. The reaction time could range from minutes to hours depending on the desired size of the nanocrystals. The solution was allowed to cool, dissolved in $CHCl_3$ and precipitated with MeOH. The precipitate was collected by centrifugation and washed several times with MeOH. These TOPO passivated nanocrystals were then dispersed in the desired solvent, including toluene, $CHCl_3$ and hexane.

Solubilization in aqueous solution was accomplished by a ligand-exchange reaction with mercaptopropionic acid (MPA). Solubilized QDs were conjugated to the primary amine group of dopamine with N-(3-Dimethylaminopropyl)-N'-
ethylcarbodiimide hydrochloride (EDC) under an inert atmosphere, using a glove bag and gaseous nitrogen. When used, beta-mercaptoethanol (BME) was added at a concentration of 5.7 mM.

Staphylococcus epidermidis was purchased from MedOx Diagnostics (ATCC 14990). Bacterial suspensions were grown in Luria Bertani (LB) medium and incubated with QD-Dopamine conjugates under overhead fluorescent light for 2.5 hours. Bacteria were then spun down and resuspended in PBS in order to reduce the background fluorescence of the LB.

Bacteria were examined and imaged with an Olympus IX-71 microscope and a Nuance multispectral imaging system. The objective lens was a Nikon PlanFluor 1005 (N.A. = 1.30). UV illumination was through a 'DAPI' filter cube set (excitation = 350/50 nm, dichroic=400 nm, emission = 420 LP).

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Results

Our experiments are aimed at allowing the uptake of QDs into bacteria by their conjugation to biological molecules. Here we report successful labeling of Gram-positive *Staphylococcus* bacteria with QDs that were first conjugated to the biomolecule dopamine. Importantly, labeling was helped by the anti-oxidant beta-mercaptoethanol (BME). We propose that BME is able to help keep the QD conjugates in solution by preventing the oxidation of the conjugates, which can lead to aggregation and precipitation out of solution. We have observed that while dopamine initially quenches the QD fluorescence in the presence of BME, this is reversed upon excitation with a UV light source. We hypothesize that photo-excited QDs are able to cause transient membrane damage to the bacterial cell wall, allowing their attachment or entry. We show the labeling of *Staphylococcus epidermidis* bacteria as Figure 1 below:

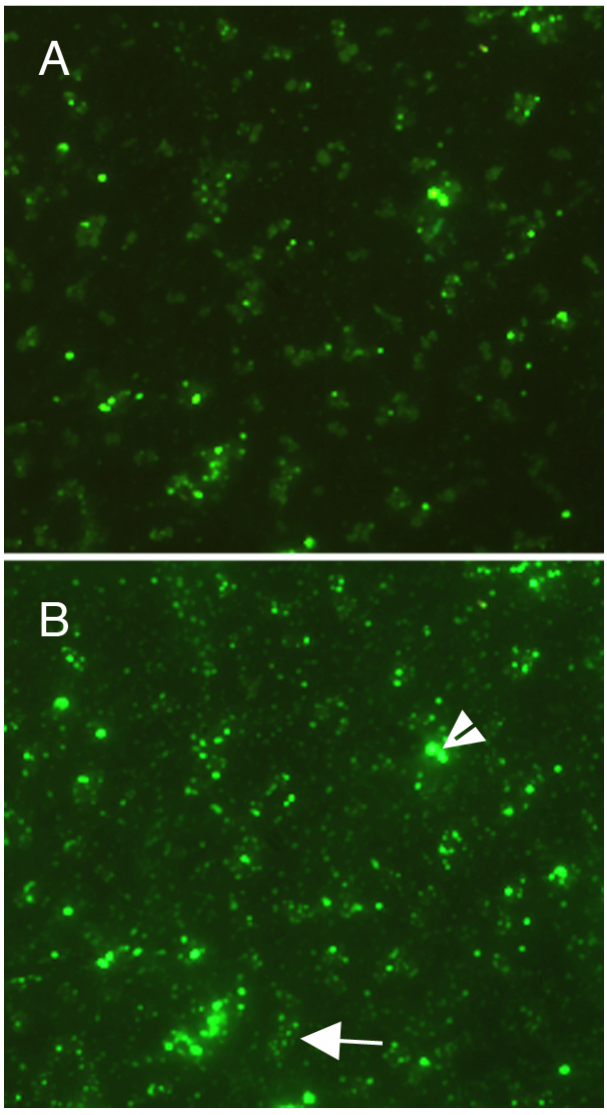

Figure 1: Labeling of bacteria with core-shell QDs. **A** *S. epidermidis* bacteria exposed to QD-dopamine conjugates in the presence of BME and imaged under a DAPI filter. Bright green fluorescence is from the QDs, while light gray fluorescence is bacterial autofluorescence. **B** Same field of view as A, however with 60 seconds of exposure to the UV light source. Notice that QDs appear brighter with greater contrast against background. Arrow points to area showing successful labeling; arrowhead points to aggregate that does not appear to be associated with the bacteria.

It is important to note that the QDs seen in our labeling experiments were always seen as aggregates of various sizes. Solubility of QDs in aqueous solution remains imperfect, and there are reports that photo-oxidation can drastically reduce the stability of QDs in water [5]. The choice of biomolecule conjugated to the QD also affects the solubility; for instance we observed that dopamine conjugates exhibit less aggregation than adenine conjugates, and we can attribute this at least partially to the fact that dopamine has greater solubility than adenine in aqueous solution.

Bare core CdSe QDs were always more soluble with thiol caps than core-shell CdSe/ZnS QDs. We have observed excellent external and internal labeling of bacteria using bare core QDs [2, 6]. However, these QDs are impractical for most applications. Their fluorescence in aqueous solution is weak, and thus the investigator must rely on photooxidation of the nanoparticles in order to obtain a good signal. The fluorescence emission spectrum of bare core QDs after photooxidation, however, is significantly broadened, often showing FWHM of 100 nm or more. This phenomenon is called 'spectral conversion' and has been reported to a lesser extent in capped QDs as well [7]

Most importantly, bare core CdSe QDs are phototoxic. Bacteria showing uptake of these particles demonstrate classic morphological changes indicating genotoxicity, such as greatly elongated cells without septum formation. We show an example of this with *Bacillus subtilis* bacteria below as Figure 2:

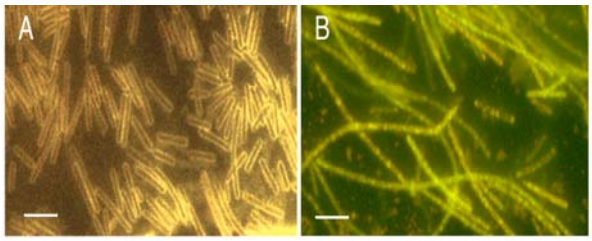

Figure 2: Elongation in *Bacillus* cells after core QD uptake (scale bar = 5 microns). **A** *B. subtilis* labeled externally with QD-lectin conjugate showing normal size and morphology. **B** After QD internalization, greatly elongated cells are seen, indicating DNA damage. Note the variation in fluorescence color from cell to cell.

Such bacteria grow, measured by optical density increase, but cannot form colonies. Previous reports [8] have shown Cd release in irradiated core-shell QD

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samples to be in the toxic range for a wide variety of bacteria (in the 100 ppm range). Our lab currently uses ion chromatography-mass spectrometry (ICP-MS) to quantify Cd release from our QD conjugates. ZnS overcoating reduces the amount of Cd released at least a thousandfold, to ~100 ppb after 2 hours of UV irradiation. Correspondingly, bacteria incubated with these particles have shown no signs of genotoxicity, measured both by colony formation and quantitative colorimetric testing for hydroxyguanine. However, it is still unknown whether this decreased toxicity primarily reflects decreased particle uptake.

While uptake of QDs into mammalian cells is thought to occur through receptor-mediated endocytosis, the mechanism in bacteria has not yet been elucidated. We hypothesize that the formation of oxygen radicals is important in uptake, causing transient damage to the bacterial wall and allowing QDs to enter. The mechanism we propose is shown below as Figure 3:

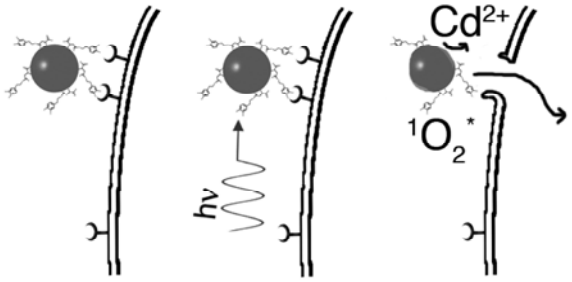

Figure 3: Proposed mechanism by which QDs can enter bacterial cells. QDs conjugated to a biomolecule (shown here is adenine) may bind to the bacterial surface. Photoexcitation of the QD conjugate can allow the formation of reactive oxygen species, and also liberates Cd^{2+} ions from the QD into the surrounding environment. Oxygen radicals can cause transient damage to the bacterial wall and membrane, allowing QD entry into the cell. Cd^{2+} ions may also participate in membrane damage, and could help explain why labeling is more easily accomplished with core QDs.

Discussion

While it is possible to synthesize QDs directly in aqueous solutions, this technique is used infrequently as the fluorescence of the QDs synthesized in this manner often have a relatively low quantum yield. QDs are normally synthesized in non-polar solvents, and in a separate reaction made soluble in water. There are several strategies for this. These include using simple mercaptohydrocarbonic acids, coating with silica [9], or coating with ogliomeric phosphines [10]. Our research has focused on the use of simple mercaptohydrocabonic acids, such as mercaptopropionic acid (MPA) and mercaptosuccinic acid (MSA). This approach is advantageous as it is very quick, and biological molecules that have a primary amine group can be easily conjugated to the QD via EDC using a well-defined biochemical reaction. However, our work is in keeping with other reports that show relatively low stability for QDs solublized with MPA or MSA. For this reason, we have begun to explore the use of phosphine oxide polymers as a means of solubilizing QDs in aqueous solution. We expect that increased solubility will lead to enhanced labeling of biological cells, especially in microorganisms such as bacteria.

There are several different methods whereby the advantageous fluorescent properties of QDs can be used in biological experiments. Conjugation of QDs to biotin has allowed incorporation with immune complexes that can give fluorescent labeling of the cell surface in a very specific manner; this approach has been used in order to detect single bacterial pathogens [11], as well as in immunochromatography assays [12]. However there are few studies that report uptake of QDs into the cell to allow imaging of the intracellular space, particularly in organisms such as bacteria that do not endocytose. We aim to accomplish this by means of conjugation to biological molecules. Our data suggest that the choice of biological molecule will dictate the outcome of labeling biological cells. Both the solubility of the biomolecule, as well as its redox potential relative to the QD it is conjugated to seem to be important.

While there have been several reports on the toxicity of both core and core-shell QDs [13-15], there is not yet a firm answer as to whether QDs have high levels of toxicity in their use as fluorescent probes. Our goal is to use QDs in order to provide fluorescent imaging in a manner that is non-toxic. While we have qualitative data that suggests that QDs can be toxic to bacterial species, our lab is interested in quantifying this toxicity using techniques such as ICP-MS. Understanding the mechanism by which QDs are toxic to cells could reveal insights into how to overcome this toxicity, and also to potentially use QDs as species-specific anti-microbial agents.

Conclusion

There are several attractive properties of QDs that can be exploited in biological applications. Their optical properties give them the potential to be a 'next generation' fluorescent probe, where emission is narrow and can be finely tuned while absorbance remains broad. Resistance to photo-bleaching is also an advantage, especially in assays that require fluorescent counts over long periods of time. Here we have shown labeling of bacteria with core-shell QDs, and discussed differences between the labeling previously shown with bare core QDs. Means of solubilizing QDs in aqueous solution has also been analyzed. Finally, the toxicity of QDs remains somewhat controversial, and we propose ways in which toxicity may be quantified. This information will allow our lab and others to better assess the use of QDs as a means of fluorescence in biological cells.

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