

## Video Article

# Solubilization and Bio-conjugation of Quantum Dots and Bacterial Toxicity Assays by Growth Curve and Plate Count

Soonhyang Park, Hicham Chibli, Jay Nadeau  
Department of Biomedical Engineering, McGill University, Montreal, QC Canada

Correspondence to: Jay Nadeau at [Jay.nadeau@mcgill.ca](mailto:Jay.nadeau@mcgill.ca)

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## Abstract

Quantum dots (QDs) are fluorescent semiconductor nanoparticles with size-dependent emission spectra that can be excited by a broad choice of wavelengths. QDs have attracted a lot of interest for imaging, diagnostics, and therapy due to their bright, stable fluorescence<sup>1,2,3,4,5</sup>. QDs can be conjugated to a variety of bio-active molecules for binding to bacteria and mammalian cells<sup>6</sup>.

QDs are also being widely investigated as cytotoxic agents for targeted killing of bacteria. The emergence of multiply-resistant bacterial strains is rapidly becoming a public health crisis, particularly in the case of Gram negative pathogens<sup>7</sup>. Because of the well-known antimicrobial effect of certain nanomaterials, especially Ag, there are hundreds of studies examining the toxicity of nanoparticles to bacteria<sup>8</sup>. Bacterial studies have been performed with other types of semiconductor nanoparticles as well, especially TiO<sub>2</sub><sup>9,10-11</sup>, but also ZnO<sup>12</sup> and others including CuO<sup>13</sup>. Some comparisons of bacterial strains have been performed in these studies, usually comparing a Gram negative strain with a Gram positive. With all of these particles, mechanisms of toxicity are attributed to oxidation: either the photogeneration of reactive oxygen species (ROS) by the particles or the direct release of metal ions that can cause oxidative toxicity. Even with these materials, results of different studies vary greatly. In some studies the Gram positive test strain is reportedly more sensitive than the Gram negative<sup>10</sup>; in others it is the opposite<sup>14</sup>. These studies have been well reviewed<sup>15</sup>.

In all nanoparticle studies, particle composition, size, surface chemistry, sample aging/breakdown, and wavelength, power, and duration of light exposure can all dramatically affect the results. In addition, synthesis byproducts and solvents must be considered<sup>16,17</sup>. High-throughput screening techniques are needed to be able to develop effective new nanomedicine agents.

CdTe QDs have anti-microbial effects alone<sup>18</sup> or in combination with antibiotics. In a previous study, we showed that coupling of antibiotics to CdTe can increase toxicity to bacteria but decrease toxicity to mammalian cells, due to decreased production of reactive oxygen species from the conjugates<sup>19</sup>. Although it is unlikely that cadmium-containing compounds will be approved for use in humans, such preparations could be used for disinfection of surfaces or sterilization of water.

In this protocol, we give a straightforward approach to solubilizing CdTe QDs with mercaptopropionic acid (MPA). The QDs are ready to use within an hour. We then demonstrate coupling to an antimicrobial agent.

The second part of the protocol demonstrates a 96-well bacterial inhibition assay using the conjugated and unconjugated QDs. The optical density is read over many hours, permitting the effects of QD addition and light exposure to be evaluated immediately as well as after a recovery period. We also illustrate a colony count for quantifying bacterial survival.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/3969/>

## Protocol

### 1. QD Solubilization

This is a method appropriate for CdTe. Similar methods can be used with other types of QDs such as InP/ZnS<sup>20</sup> and CdSe/ZnS<sup>21</sup>.

1. Prepare a solution of CdTe QDs in toluene at 15  $\mu$ M (optical density = 2.5 at the first exciton peak).
2. Transfer 200  $\mu$ L of this stock into a glass vial. Do not use plastic!
3. Add 800  $\mu$ L toluene, 1 mL of 200 mM borate buffer (pH 9) and 2  $\mu$ L of 11.5 M mercaptopropionic acid (MPA).
4. Cap the vial and shake it vigorously for 30-60 seconds.
5. The aqueous and organic-solvent phases will separate spontaneously. The aqueous phase will become the color of the QDs. To avoid the toluene layer, remove it and discard it. Then transfer the aqueous phase into a clean vial.
6. Purify the solubilized QDs by washing/filtering four times using a 500  $\mu$ L, 10000 molecular weight cutoff centrifuge filter. The filters should be spun at 3000 x g for 13 minutes. For at least the last two washes, wash with the desired final buffer.

- After the final wash, suspend the washed QDs in 50 mM borate buffer at the desired pH and store at 4 °C. They will be stable for 1-2 weeks.
- Measure absorbance and emission spectra to estimate concentration based upon published formulas<sup>22</sup>.

**Representative results:** Figure 1 shows an image of CdTe QDs under UV lamp illumination, and emission spectra before and after water solubilization, showing negligible change from the cap exchange. The size values are the core diameter measured by electron microscopy.

## 2. QD Conjugation to Antibiotic

This part of the protocol is applicable to any negatively-charged water-solubilized nanoparticle, including most commercial QDs, metal particles, and more<sup>19</sup>.

- Any molecule will work that either self-assembles to the negatively-charged solubilized QDs, or that has a reactive group that can be conjugated, such as a primary amine. In this example, we are using polymyxin B (PMB), which is positively charged and self-assembles without need for conjugation reagents. Dissolve the PMB in H<sub>2</sub>O at 60 μM. (If the antibiotic chosen is not soluble in H<sub>2</sub>O, dissolve in DMSO or ethanol at a concentration of 0.1-1 mM). This will be a 10x solution (in H<sub>2</sub>O) or a 100x solution (in solvent).
- Calculate how much QD solution you will need for the bacterial toxicity experiments. For a 96-well plate with 0.3 mL per well in quadruplicates, 0.5 mL of 200 nM conjugate is needed. Prepare two tubes containing 100 μL of 1 μM quantum dots in 50 mM borate buffer.
- If coupling to a primary amine, prepare a 19 mg/mL (100 mM) solution of 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) in H<sub>2</sub>O. EDC in solution is not stable; use immediately and throw the rest away.
- Add 50 μL of the 10x antibiotic solution (in H<sub>2</sub>O) or 5 μL of the 100x solution (in solvent) to the conjugate tube. For PMB:CdTe conjugation at molar ratio of 30:1, add 50 μL of 60 μM PMB. Add H<sub>2</sub>O or solvent to the QD only control tube.
- If needed, add 1 μL of the EDC stock solution to the conjugate tube.
- Bring up the conjugate volume to 0.5 mL with the appropriate buffer (borate buffer or PBS; no amine-containing buffers such as Tris, as they will inhibit EDC).
- Shield tubes from light with aluminum foil, and place on a nutator or rocker for 1 hr. If aggregation occurs, repeat the conjugation with lower concentrations of antibiotic. Titrate the concentration upwards until the particles do not aggregate.
- Wash the conjugates by passing through an appropriate filter. 10000 molecular weight cutoff works for most antibiotics, but not for proteins or antibodies.
- Depending upon the molecule, different methods can be used to estimate the number of antibiotic molecules per QD: absorbance or fluorescence spectroscopy, gel electrophoresis, or Fourier transform infrared spectroscopy (FTIR).

**Representative results.** In this example, PMB conjugation is characterized by changes in QD emission spectrum. Figure 2 shows the spectra of CdTe QDs with PMB addition.

## 3. Preparation of Bacteria for 96-well Screen; Determination of Antibiotic IC<sub>50</sub>

This is applicable to almost any bacterial strain grown in the appropriate medium<sup>18</sup>. The exact length of time the recordings should continue depends upon the bacterial growth rate. In our example, we use *Escherichia coli* grown in lysogeny broth (LB) medium.

- In order to choose the appropriate QD conjugate concentrations, it is important to know the IC<sub>50</sub> of the antibiotic to be conjugated and if the QDs alone are toxic. This should be begun 2 days before the conjugation experiment. In the evening, seed 10 mL of bacterial growth medium from a fresh colony, using correct sterile and biosafety technique.
- The next day, 1-2 hours before the experiment, fill each of the wells of a clear-bottom 96-well plate with a nearly-maximal amount of growth medium. Using a multichannel pipette, seed each well with 1-50 μL of bacterial culture (the concentration will depend upon how fast the particular strain grows and will need to be calibrated by your laboratory).
- Put the plate onto the plate reader and set it to read every 10 minutes for 2 hours at a determined wavelength (usually 600 nm). Monitor the plate so that the bacteria do not grow too dense too quickly.
- When the cells all reach OD=0.1-0.15, remove the plate from the plate reader and stop the recording.
- Add drug alone and QDs alone at different concentrations in at least triplicates. Use one side of the plate as a "dark" control and one half to be illuminated. A suggested layout is given in Figure 3. The concentrations should span a wide enough range to include one concentration that inhibits the bacteria very little, and one that kills them all.
- Shield the "dark" side of the plate with aluminum foil. Expose the other side to a lamp at the desired wavelength. We use a custom 96-well, 440-nm lamp made from 2.4 mW LEDs (Figure 4) and irradiate for 30-60 min. The black plates with clear bottoms help shield the unexposed bacteria from stray light. An empty column may also be used between the exposed and un-exposed sides.
- After light exposure, put the plate into the plate reader and record OD 600 every 10 minutes for 5-12 hours depending upon growth rate. Keep the temperature < 32 °C if possible to avoid drying of the cultures.
- Plot the growth curves at a selected time point vs. log [concentration] and determine the IC<sub>50</sub> of the antibiotic using the Hill equation:

$$y = y_{\min} + \frac{y_{\max} - y_{\min}}{1 + 10^{[\text{Log}(\text{IC}_{50}) - x]H}}$$

where  $H$  is the Hill coefficient,  $y_{\max}$  is the highest point of growth (ideally on a plateau), and  $y_{\min}$  is the zero-point, also ideally on a plateau. It is unlikely that QDs alone will show much toxicity to the cells at the concentrations used, so a value will not be determined.

**Representative results.** At the end of the recording period, clear wells will indicate complete cell death, and a gradient of cell density should appear along increasing concentrations of the drug. The bacteria should show S-shaped growth curves (Figure 5 A); the location of the maximum plateau will vary greatly from strain to strain and also depends upon temperature. A given time point can be chosen as representative and the values plotted vs. Log[antibiotic] to give the IC<sub>50</sub> (Figure 5 B). To evaluate QD toxicity, survival vs. Log[QD] may also be plotted, but achieving significant bacterial killing with QDs alone is rare (Figure 5 C).

## 4. Preparation of Bacteria for 96-well Screen with Antibiotic/QDs

1. The day before the experiment, seed 10 mL of culture from a fresh colony into the appropriate rich medium, using correct sterile and biosafety technique. The QD-antibiotic conjugate should be prepared on this day, unless it is highly unstable.
2. 1-2 hours before the experiment, fill each of the wells of a clear-bottom 96-well plate with a nearly-maximal amount of growth medium. Using a multichannel pipette, seed each well with 1-50  $\mu\text{L}$  of bacterial culture.
3. Put the plate onto the plate reader and set it to read every 10 minutes for 2 hours at the same wavelength as in Part 3.
4. When the cells all reach  $\text{OD}=0.1-0.15$ , remove the plate from the plate reader and stop the recording.
5. Add QD conjugates at different concentrations in at least quadruplicate. Use one side of the plate as a "dark" control and one half to be illuminated. A suggested layout is given in **Figure 6**. A bacteria-only control strip should **always** be included in case problems with the culture, temperature, etc. affect growth conditions. A single strip of drug only and QD only should also be included to ensure that they match the control plate done previously. The rest of the wells are dedicated to conjugates in order to allow for good statistics.
6. Shield the "dark" side of the plate with aluminum foil. Expose the other side to a lamp at the desired wavelength.
7. After light exposure, put the plate into the plate reader and record OD 600 every 10 minutes for 5-12 hours. Keep the temperature  $< 32^\circ\text{C}$  if possible to avoid drying of the cultures.

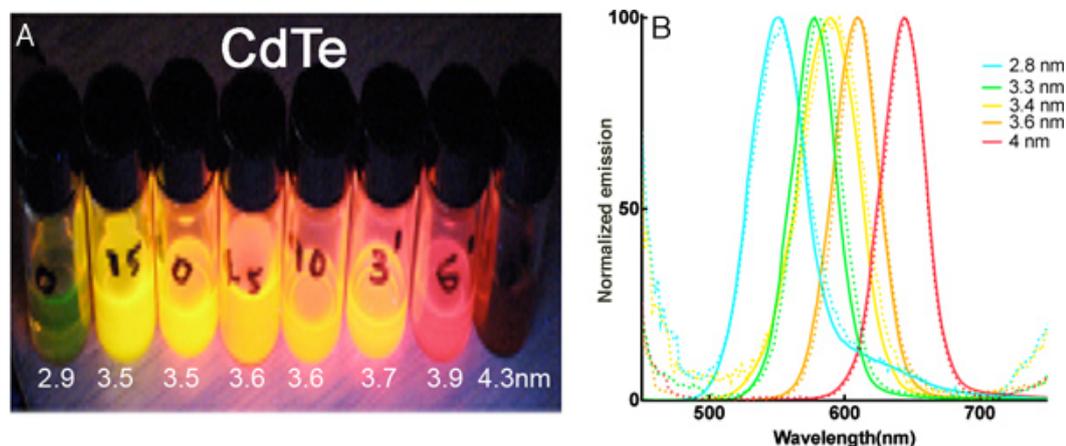
**Representative results.** The combination of QDs and antibiotic may be less toxic than antibiotic alone; equally toxic; or more toxic. This can be quantified using the growth curves and  $\text{IC}_{50}$  measurements. **Figure 7** shows an example of conjugates that are equally toxic as antibiotic alone, and an example of conjugates that are more toxic.

## 5. Plate Count

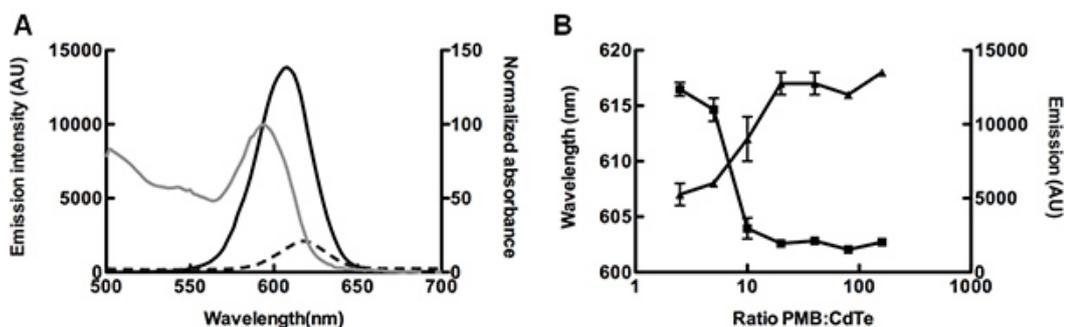
1. Choose one or more wells of the treated 96-well plate to be used for serial dilutions and colony counting.
2. Make a serial dilution of each of the selected bacterial samples with PBS or saline solution (0.9 % NaCl). Transfer 20  $\mu\text{L}$  of the bacterial solution and dilute it with 180  $\mu\text{L}$  saline solution, change the pipette tip, mix and transfer 20  $\mu\text{L}$  of diluted bacterial solution to 180  $\mu\text{L}$  saline solution. Repeat 6 times.
3. Take 10  $\mu\text{L}$  from each dilution and plate on an appropriate solid media plate. All 8 concentrations of one sample may be plated on a 10 cm round Petri dish, though rectangular dishes are preferred as it is easier to line things up.
4. Let it dry on the bench for 15 min. then incubate at appropriate temperature for your bacteria for 16 hours.
5. Count colonies and calculate colony forming units (CFU) according to  $(\# \text{ of colonies} \times \text{dilution factor})/\text{volume plated} = \text{CFU/mL}$ .

**Figure 8** shows an example CFU plate.

## 6. Representative Results



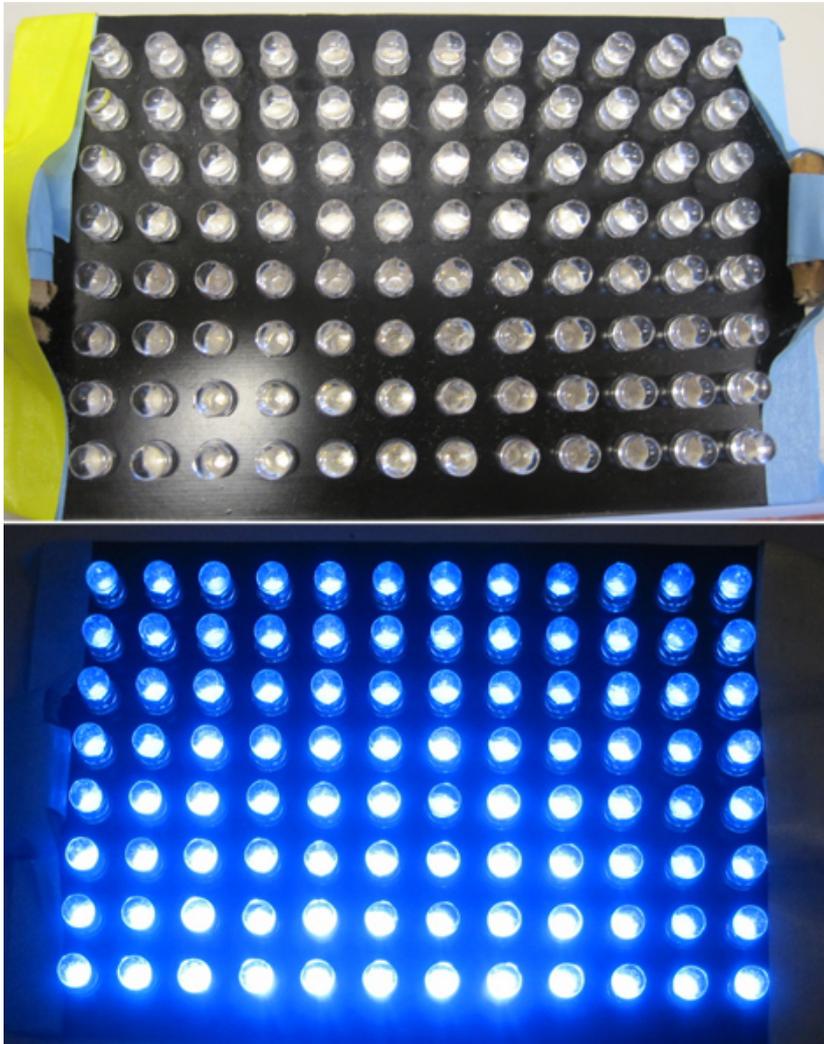
**Figure 1.** CdTe QDs. (A) Eight preparations of CdTe QDs illuminated with a UV wand (365 nm). (B) Absorbance and emission spectra of five selected sizes before and after water-solubilization. The dashed lines are spectra in toluene, the solid lines are in water.



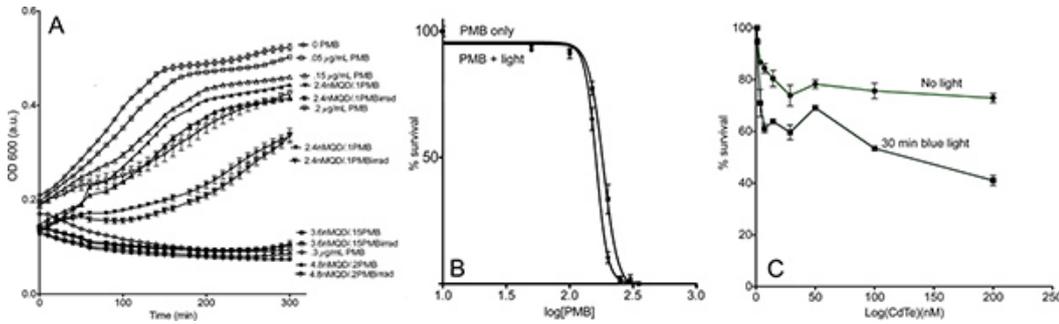
**Figure 2.** Spectral and gel analysis of QD-PMB conjugates. Orange-emitting CdTe QDs were used for this example; the effects on other types of QDs will need to be evaluated for each experiment. (A) Typical absorbance (grey line) and emission spectra (black line) of the QDs before conjugation of PMB and the emission (dashed line) after the addition of 160 molar equivalents of PMB. (B) Relationship between the ratio of PMB and the QD emission intensity (squares) and peak wavelength location (triangles).

	1	2	3	4	5	6	7	8	9	10	11	12
A	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0
B	0.1 $\mu$ M PMB	0.1 $\mu$ M PMB	0.1 $\mu$ M PMB	1 nM QD	1 nM QD	1 nM QD	0.1 $\mu$ M PMB	0.1 $\mu$ M PMB	0.1 $\mu$ M PMB	1 nM QD	1 nM QD	1 nM QD
C	0.2 $\mu$ M PMB	0.2 $\mu$ M PMB	0.2 $\mu$ M PMB	2 nM QD	2 nM QD	2 nM QD	0.2 $\mu$ M PMB	0.2 $\mu$ M PMB	0.2 $\mu$ M PMB	2 nM QD	2 nM QD	2 nM QD
D	0.3 $\mu$ M PMB	0.3 $\mu$ M PMB	0.3 $\mu$ M PMB	3 nM QD	3 nM QD	3 nM QD	0.3 $\mu$ M PMB	0.3 $\mu$ M PMB	0.3 $\mu$ M PMB	3 nM QD	3 nM QD	3 nM QD
E	0.4 $\mu$ M PMB	0.4 $\mu$ M PMB	0.4 $\mu$ M PMB	4 nM QD	4 nM QD	4 nM QD	0.4 $\mu$ M PMB	0.4 $\mu$ M PMB	0.4 $\mu$ M PMB	4 nM QD	4 nM QD	4 nM QD
F	0.5 $\mu$ M PMB	0.5 $\mu$ M PMB	0.5 $\mu$ M PMB	5 nM QD	5 nM QD	5 nM QD	0.5 $\mu$ M PMB	0.5 $\mu$ M PMB	0.5 $\mu$ M PMB	5 nM QD	5 nM QD	5 nM QD
G	0.6 $\mu$ M PMB	0.6 $\mu$ M PMB	0.6 $\mu$ M PMB	6.7 nM QD	6.7 nM QD	6.7 nM QD	0.6 $\mu$ M PMB	0.6 $\mu$ M PMB	0.6 $\mu$ M PMB	6.7 nM QD	6.7 nM QD	6.7 nM QD
H	0.7 $\mu$ M PMB	0.7 $\mu$ M PMB	0.7 $\mu$ M PMB	8.3 nM QD	8.3 nM QD	8.3 nM QD	0.7 $\mu$ M PMB	0.7 $\mu$ M PMB	0.7 $\mu$ M PMB	8.3 nM QD	8.3 nM QD	8.3 nM QD

**Figure 3.** Suggested plate layout for control growth plate. A wide range of PMB and QD concentrations is represented. One half of the plate is irradiated (highlighted blue), and an identical half is protected from light. [Click here to view larger figure.](#)



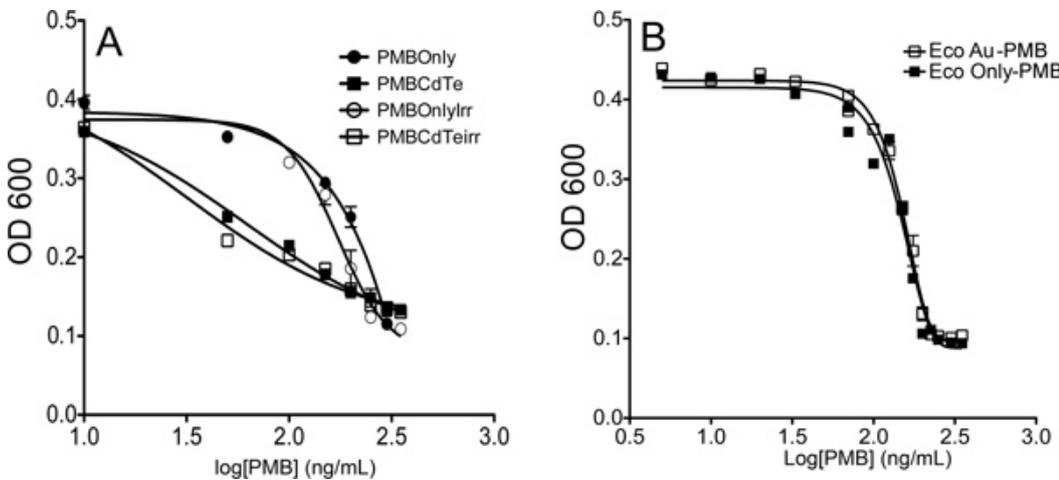
**Figure 4.** Custom 96-LED lamp for uniform plate irradiation, showing appearance off and on. A typical hand-held UV lamp may also be used, but will not cover the entire plate uniformly.



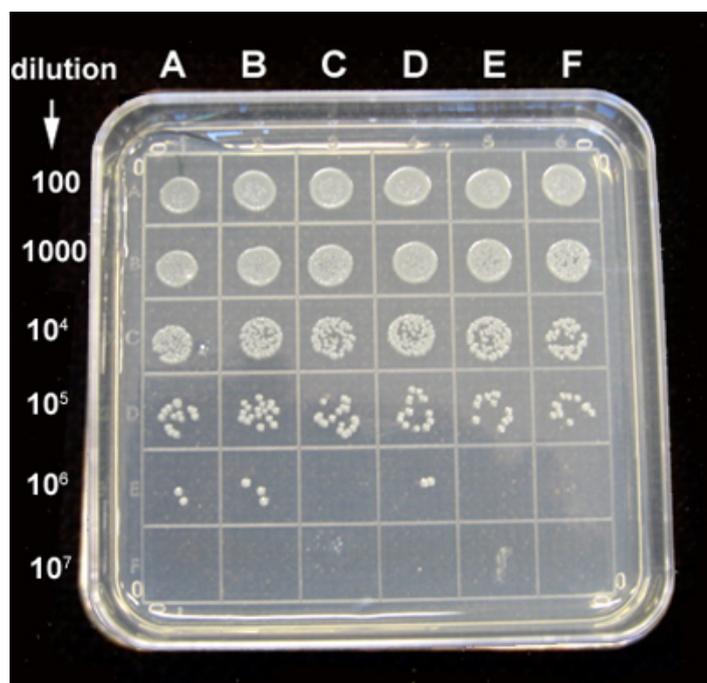
**Figure 5.** Example results for control growth plate. (A) Representative bacterial growth curves with different CdTe drug concentrations, from 0 to complete cell death. The open symbols are PMB only with concentrations given; the solid symbols are CdTe-PMB without irradiation; and the half-filled symbols are CdTe-PMB with irradiation. Irradiation had no effect on the PMB-only samples, so these curves were omitted for clarity. All PMB-CdTe conjugates are 30:1 PMB:QD ratios. (B) Plots of growth curve values at 200 min vs. Log[PMB] and fits to Eq. (1). To control for the effects of light, a curve is done with antibiotic only with 30 min of light exposure. (C) Bacterial survival at 200 min vs. QD concentration, using CdTe QDs. Some toxicity is seen with light exposure, but too little to determine an IC<sub>50</sub> value. [Click here to view larger figure.](#)

	1	2	3	4	5	6	7	8	9	10	11	12
A	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0	E. Coli 0
B	1 nMQD +0.03 μM PMB	1 nMQD	0.1 μM PMB	1 nMQD +0.03 μM PMB	1 nMQD +0.03 μM PMB	1 nMQD +0.03 μM PMB	1 nMQD +0.03 μM PMB	1 nMQD	0.1 μM PMB			
C	2 nMQD +0.06 μM PMB	2 nMQD	0.2 μM PMB	2 nMQD +0.06 μM PMB	2 nMQD +0.06 μM PMB	2 nMQD +0.06 μM PMB	2 nMQD +0.06 μM PMB	2 nMQD	0.2 μM PMB			
D	3 nMQD +0.09 μM PMB	3 nMQD	0.3 μM PMB	3 nMQD +0.09 μM PMB	3 nMQD +0.09 μM PMB	3 nMQD +0.09 μM PMB	3 nMQD +0.09 μM PMB	3 nMQD	0.3 μM PMB			
E	4 nMQD +0.12 μM PMB	4 nMQD	0.4 μM PMB	4 nMQD +0.12 μM PMB	4 nMQD +0.12 μM PMB	4 nMQD +0.12 μM PMB	4 nMQD +0.12 μM PMB	4 nMQD	0.4 μM PMB			
F	5 nMQD +0.15 μM PMB	5 nMQD	0.5 μM PMB	5 nMQD +0.15 μM PMB	5 nMQD +0.15 μM PMB	5 nMQD +0.15 μM PMB	5 nMQD +0.15 μM PMB	5 nMQD	0.5 μM PMB			
G	6.7 nMQD +0.2 μM PMB	6.7 nMQD	0.6 μM PMB	6.7 nMQD +0.2 μM PMB	6.7 nMQD +0.2 μM PMB	6.7 nMQD +0.2 μM PMB	6.7 nMQD +0.2 μM PMB	6.7 nMQD	0.6 μM PMB			
H	8.3 nMQD +0.25 μM PMB	8.3 nMQD	0.7 μM PMB	8.3 nMQD +0.25 μM PMB	8.3 nMQD	0.7 μM PMB						

**Figure 6.** Suggested layout for conjugate test plate. The blue-highlighted half of the plate should be exposed to light, and the unhighlighted half is protected. [Click here to view larger figure.](#)



**Figure 7.** Example results for conjugate test plate. Growth curve values at 200 min were plotted and fit to Eq. (1). (A) CdTe-PMB conjugates show increased toxicity over PMB alone. (B) Gold nanoparticle Au-PMB conjugates show no increased toxicity over PMB alone.



**Figure 8.** Example of a CFU plate. *E. coli* seeded in a 96-well plate was treated with QD-PMB with or without irradiation for 30 min. then incubated at 32 °C for 4 hours. Serial dilutions of each bacterial sample were made with saline solution, and 10  $\mu$ L of 100 X to 10<sup>7</sup> X dilutions were plated on agar plates. The plates were incubated at 37 °C and colonies were counted after 16 hours. The plate shows the dilutions along the rows as indicated; the columns are: (A) 0.06  $\mu$ M PMB + 2 nM CdTe, (B) 0.12  $\mu$ M PMB + 4 nM CdTe (C) 0.2  $\mu$ M PMB + 6.7 nM CdTe, (D) 0.06  $\mu$ M PMB + 2 nM CdTe irradiated, (E) 0.12  $\mu$ M PMB + 4 nM CdTe irradiated, (F) 0.2  $\mu$ M PMB + 6.7 nM CdTe irradiated.

## Discussion

Nanoparticles represent a promising approach to creation of new anti-microbial agents. Growth curve analysis is a way to monitor bacterial cell density that distinguishes actively-growing cells from growth-inhibited cells. When coupled with plate counts, it allows for a thorough analysis of the antibiotic potential of a conjugate. The 96-well format permits relatively high-throughput variations of concentration and other conditions such as light exposure; the latter is crucial for light-activated agents such as quantum dots. Many variations on this basic approach are possible, making it a versatile method for nanotoxicology.

## Disclosures

No conflicts of interest declared.

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