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Non-cytotoxic Mn-doped ZnSe/ZnS quantum dots for biomedical applications

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ABSTRACT

Quantum dots (QDs) are of high interest in the biomedical field. The most widely used and commercially available CdSe/ZnS QDs have a highly toxic Cd component. High-efficiency luminescent Cd-free Mn-doped ZnSe/ZnS QDs are a reasonable alternative to CdSe/ZnS QDs; however the actual cytotoxicity of ZnSe:Mn/ZnS QDs is relatively unknown. In this study, we apply the ApoTox-Glo™ Triplex assay to test for cell cytotoxicity, viability, and induced apoptosis, by treating macrophage cells with different concentrations of peptide-coated ZnSe:Mn/ZnS QDs at four different incubation times: 6, 12, 24, and 48 hours.

At the concentrations used, which varied between 0.03 μM to 0.25 μM, the macrophage cells showed very little cytotoxic effect. However, cell viability began to decrease with increasing QD concentration beginning with the 12 hour incubation time, with fairly consistent results for 24 and 48 hour incubation times as well. Also, the macrophage cells expressed a measurable degree of induced apoptosis, which scaled with concentration.

While cytotoxicity did not seem to be an issue with macrophage cells treated with the peptide-coated Mn-doped ZnSe/ZnS QDs, the drop in cell viability and the increase in induced apoptosis suggest an antiproliferation effect within the macrophage cell culture.

Keywords: Quantum dots, cadmium-free, zinc selenide, peptide, luminescence, macrophage, cytotoxicity, viability, assay.

1. INTRODUCTION

Luminescent semiconductor nanocrystals, also known as quantum dots (QDs), have been on the frontier of scientific research for the past decade. The number of inherent advantages of QDs over traditional fluorophores, which includes high photoluminescence intensity, high quantum efficiency, high stability, and narrow emission spectra with broad absorption spectra, contribute to their growing use as optical emitters that are compatible with biological processes in the human body [Michalet 2005], [Medintz 2005], [Alivisatos 2005], [Giepmans 2006], [Klostranec 2006]. Further advantages include size-tuneable properties, resistance to photobleaching, broad absorption spectra with 10-50 times larger molar extinction

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coefficients, and high efficiency for applications involving Förster resonance energy transfer (FRET), due to multiple binding sites of energy acceptors [Kim 2012]. A particularly interesting prospect has been the use of QDs for *in vivo* applications, such as imaging, biomarkers, disease treatment, and drug development. Their high stability, large Stokes shifts, and high absorption coefficients make them attractive for complex *in vivo* conditions [Gao 2003], [Vashist 2006].

Furthermore, the narrow emission and broad absorption spectra characteristic of QDs make them more suitable for multicolor detection [Alivisatos 2005], whereas traditional fluorophores can often be hindered by cross talk due to small Stokes shifts, making them unsuitable for monitoring and simultaneous detection of different biomolecules.

These unique properties of QDs make them ideal candidates for the advancements in the biomedical field. The high potential of QDs has generated a lot of worldwide attention in this field of research, and as a result, numerous methods have been developed to utilize QDs to their fullest extent. QDs are widely known for their contribution to biosensing and tracking, cellular imaging, tissue staining, drug delivery, and other *in vivo* and *in vitro* research and diagnostic applications [Yaghini 2009], [Mazumder 2009], [Agasti 2010], [Choi 2010], [Shao 2011], [Fontes 2012], [Kairdolf 2013].

Unfortunately, there is a primary toxicity issue with Cd-containing QDs [Chang 2006], [Su 2009]. Heavy-metal Cd ions are known to be highly toxic to animal and human cells. It remains a challenge to lower cytotoxicity and maintain optimal properties of QDs. Studies show that toxicity varies based upon QD dosage. In the case of maltodextrin-coated CdS QDs, the borderline between toxic and non-toxic is shown to be less than 3.28 nM, but not greater than 4.92 nM [Rodríguez-Fragoso 2012]. Due to the inherent toxicity of QDs containing heavy metals, efforts have been made to change the components of the QDs to make them more biocompatible. A potential solution is to avoid the use of heavy metals, such as lead, mercury, or cadmium, and replace them with transition metals like zinc. Mn:ZnSe QDs have been shown, through confocal microscopy, to pass through the PK15 porcine kidney epithelial cell membranes and localize into the cytoplasm and, through the use of the colorimetric MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay, not to display significant cytotoxicity [Liu 2011]. Replacing the Cd component with Zn in ZnSe doped with Mn ions has also been proven to be sufficient in reducing QD-cytotoxicity in comparison to CdTe QDs [Niu 2012]. In addition, capping CdTe with SiO_2 has been shown to reduce QD toxicity [Niu 2012]. Other options, including CdTe and CdSe QDs capped with L-glutathione (GSH) or thioglycolic acid (TGA), have allowed the QDs to be more tolerable to the BON human pancreatic carcinoid cells and to the Kyse-70 human oesophageal squamous carcinoma cells, with IC_{50} values reaching a micromolar range [Ermilov 2013]. It has been shown *in vivo* using mice that the organic coating of CdSe/ZnS QDs determines their biocompatibility and is a crucial feature for their potential in diagnostic imaging applications [Bakalova 2011].

Pure Zn has much lower toxicity than Cd. However, some materials, which may not be toxic in their bulk form, might in fact exhibit toxicity at the nanoscale. It is therefore necessary to assess the potential cytotoxicity of the ZnSe:Mn/ZnS QDs. Macrophage (MΦ) cells, also called microglia in the context of the central nervous system (CNS), were chosen in this study for evaluation of QD cytotoxicity based on the fact that the resident macrophages of the brain and spinal cord come into direct contact with foreign molecules, including QDs, and phagocytose them, therefore acting as the first and main form of active immune defense in the CNS.

Not only do MΦ cells rapidly take up QDs, but the QDs can undergo a loss of fluorescence, which is due to their instability in the cells. The speed of the uptake and the cytotoxicity have been shown to be affected by the organic coating of the QDs [Clift 2008]. Specifically, the addition of PEG coating resulted in a decreased intracellular uptake, which allowed for minimal toxicity in biological applications; however, this does not preclude possible toxicity of the QDs if they were endocytosed [Chang 2006].

During environmental stress, levels of reactive oxygen species (ROS) become significantly elevated, which contributes to cell damage due to oxidative stress. It has been shown that CdTe QDs without any type of biocompatible coatings do yield damage to the plasma membrane in cells, of which ROS are primary mediators [Lovric 2005]. In addition, despite having components that are not toxic to cells, Cd-free QDs such as InP/ZnS have also been shown to generate ROS when optically excited, due to electron-hole pairs interacting with water and oxygen [Chibli 2011].

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The ZnSe:Mn/ZnS QDs are of interest for *in vivo* biosensing applications and therefore must display very little cytotoxicity, if any. In this paper, we report the low cytotoxic properties of peptide-coated Mn-doped ZnSe/ZnS QDs at relatively low concentrations, namely ≤ 0.25 μM (1.2 mg/mL).

2. ZNSE:MN/ZNS QUANTUM DOT SYNTHESIS

For the ZnSe:Mn core synthesis, the precursors used were zinc stearate ($ZnSt_2$), purchased from Acros Organics, selenourea [$SeC(NH_2)_2$], purchased from Aldrich, and manganese stearate ($MnSt_2$), synthesized in-house using anhydrous manganese(II) chloride ($MnCl_2$), purchased from Acros Organics, and tetramethylammonium hydroxide pentahydrate (TMAH), purchased from Sigma-Aldrich. The precursors used to make the ZnS shell of the QDs were zinc stearate ($ZnSt_2$) and 99% thiourea [$SC(NH_2)_2$], both purchased from Acros Organics. The solvents used for the synthesis were octadecene (ODE) and anhydrous ethanol, both purchased from Aldrich. The surfactants used were 80% - 90% oleylamine and 97% stearic acid (StA), both purchased from Acros Organics. The Mn-doped ZnSe/ZnS QDs were synthesized under air-free conditions in accordance to a synthesis procedure previously developed by our group [Akins 2013]. The synthesis presented forthwith has been modified from [Pradhan 2007] and [Acharya 2010], in that a ZnS shell was added to the nanoparticles using a thiourea precursor for sulfur.

Initially, $MnSt_2$ stock powder was synthesized according to supporting information in [Pradhan 2007]. First 5.690 g of SA were mixed with 37.89 mL of methanol in a reaction flask. The reaction flask was purged with Ar, and the solution was put under stirring and heated to 60 °C until the SA dissolved. The solution was then cooled to room temperature. Next, 3.625 g of TMAH were dissolved into 12.63 mL of methanol and added to the reaction flask. 1.259 g of the Mn precursor $MnCl_2$ were dissolved into 12.63 mL of methanol and added to the primary reaction flask. The resulting mixture was allowed to react at room temperature and under stirring, until a white $MnSt_2$ precipitate formed. The precipitate was centrifuged in methanol at 4,000 rpm three times and then re-dispersed into 18 mL of methanol. The solution was dried under vacuum and stored in a 20 mL vial.

For a typical ZnSe:Mn core synthesis, 63 mg of $ZnSt_2$ were dissolved into 5 mL of ODE and heated to 260 °C in a reaction flask under Ar. In a separate reaction flask under Ar, 123 mg of selenourea (the Se precursor) were mixed with 10 mL of oleylamine 80%-90% and heated to 200 °C in order to dissolve the selenourea into the oleylamine. After the selenourea solution cooled down to 130 °C, it was injected into the flask containing $ZnSt_2$ at 260 °C, after which the temperature was brought to 240 °C. The Mn dopant precursor was prepared by mixing 6.3 mg of $MnSt_2$ into 3 ml of ODE (additional heating was required to fully solubilize the Mn precursor). The Mn precursor was then injected into the reaction flask at 240 °C and allowed to react for one hour. For the shell, 1.159 g of $ZnSt_2$ and 0.515 g of StA were dissolved into 18.4 mL of ODE by heating the mixture under Ar to 150 °C. The temperature of the reaction was increased to 260 °C (the injection temperature) and 0.96 mL of $ZnSt_2$/SA/ODE was injected and allowed to react for 10 minutes and subsequently an additional 0.96 mL of the same mixture was added again. The reaction flask was cooled to 240 °C for 15 minutes and then heated back to the injection temperature for an injection of 1.33 mL of the $ZnSt_2$/SA/ODE mixture before setting the temperature to 240 °C. The injections were repeated in this manner at progressively increasing volumes of 1.69 mL, 2.25 mL, and 2.81 mL at the 260 °C injection temperature, always allowing for a 15 minute anneal step at 240 °C after every injection.

For the ZnS shell synthesis, 86 mg of thiourea were mixed with 3 mL of anhydrous ethanol and dissolved with additional heating. The reaction solution was brought to 70 °C. The entire thiourea solution was injected into the reaction flask at 70 °C. In order to evaporate the ethanol, the solution was put under vacuum and the temperature was slowly increased, first to 80 °C and then to 120 °C, while always allowing for the bubbling to stop before increasing the temperature. Finally, the temperature of the reaction mixture was increased to 260 °C and 3.65 mL of the $ZnSt_2$/SA/ODE solution were injected. The reaction temperature was then dropped to 240 °C for 30 minutes before being increased to 260 °C for a final injection of 4.75 mL of the $ZnSt_2$/SA/ODE mixture, followed by 30 minutes of annealing at 240 °C.

The synthesized QDs were then washed and centrifuged with acetone and finally stored in chloroform.

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3. BIOFUNCTIONALIZATION OF ZNSE:MN/ZNS QUANTUM DOTS

For biofunctionalization, the cysteine-containing peptides were purchased from GenScript, 99% benzylamine was purchased from Acros Organics, and dimethylformamide was purchased from EMD.

The synthesized QDs were biofunctionalized with cysteine-containing peptides [Shi 2007], and these peptides were custom made to contain the substrate motif Val-Val-Leu-Ser-Leu-Arg-Ser [Turk 2001], as well as a C-terminal cysteine residue. Certain modifications were made from the procedure reported in [Shi 2007], which includes replacing pyridine with benzylamine 99%, because pyridine was etching the ZnSe:Mn/ZnS QDs, causing the QDs to lose a significant portion of their fluorescence intensity. Also, because the benzylamine is already basic, with a pH of 11.3, no tetramethyl ammonium hydroxide was added to increase the pH. The reason to increase the pH is to negatively charge the thiol group in order to facilitate the binding of the peptides to the QDs.

The biofunctionalization procedure was as follows. 1 mL of 1 μM ZnSe:Mn/ZnS QDs in chloroform was centrifuged at 4,000 rpm after the addition of acetone. After pouring off the supernatant, the QDs were resuspended in 2 ml of 9:1 (v:v) benzylamine/dimethylformamide (DMF) solution. 1 mg of the MMP-9 peptide substrate was dissolved into 0.200 mL of DMF. The peptide/DMF solution was added to the QD/benzylamine/DMF solution, and the resulting mixture was vortexed for 30 minutes. The QD/peptide conjugates were centrifuged and redistributed in 2 mL of DMF. The mixture was centrifuged again and re-dispersed in 2 mL of sterile DI water. The QD/peptide conjugate solution was subjected to 2 cycles of spin dialysis by using 4 Microcon (ultracell YM-30) centrifugal filter devices. Afterwards, the QD/peptide conjugates were dispersed into 2 mL of sterile DI water and stored in a refigerator at 4 °C.

4. MACROPHAGE CELL CULTURE

For cell culture, RAW 264.7 macrophage cells, established from an ascites (accumulation of fluid in the peritoneal cavity) of a tumor induced in a male mouse infected with Abelson murine leukemia virus, were purchased from ATCC. DMEM cell medium, containing 10% fetal bovine serum and 1% penicillin/streptomycin, was purchased from Life Technologies.

The cell cultures were prepared using 4 different 384-well plates, purchased from Thermo Scientific, seeded with mouse RAW 264.7 MΦ cells, at a concentration of 5,000 cells per well in DMEM cell medium. Each of the four wells corresponded to 6, 12, 24, and 48 hour incubation treatments, with triplicate wells set up for the 6 hour treatment and hextuplicate wells for the 12, 24, and 48 hour treatments.

5. APOTOX-GLO™ TRIPLEX ASSAY

In order to characterize the cytotoxic potential of peptide-coated Mn-doped ZnSe/ZnS core/shell QDs on mammalian cells, the ApoTox-Glo™ Triplex assay, purchased from Promega, was used. The assay's reagents include substrates that release fluorescent molecules when cleaved by protease enzymes for live and dead cells. The viability substrate, glycyl-phenylalanyl-aminofluorocoumarin (GF-AFC), enters live cells and is cleaved by protease enzyme to release the florescent AFC. The intensity of AFC fluorescence is the indicator of cell viability. The cytotoxicity substrate, bis-alanylalanyl-phenylalanyl-rhodamine 110 (bis-AAF-R11O), does not enter live cells and is cleaved by dead cell proteases, releasing a fluorescent substance known as R110. The intensity of the R11O fluorescence is the indicator of cellular cytotoxicity. To measure apoptosis, the Caspase-Glo 3/7 reagent, a caspase substrate will result in a luminescent signal upon cleavage. The digitonin, ionomycin, and staurosporine reagents were purchased from Sigma-Aldrich. The fluorescence and luminescence signals were measured with a Tecan Microplate Reader, collecting the light from the bottom of the well plate.

For the testing, the QD bioconjugates were incubated with the cells at 0.03, 0.06, 0.13, and 0.25 μM concentrations for each treatment. Each final individual well consisted of 20 μL of solution with 10 μL of cells in DMEM cell medium and 10 μL of QDs in water. Background measurements were taken and subsequently subtracted from every average reading. As a control, measurements involving untreated cells were included. Positive controls included treatments from 2 compounds known to be toxic, digitonin and ionomycin (markers for cytotoxicity and necrosis, respectively), as well as staurosporine, a compound known to induce apoptosis. The concentrations for the positive controls were 15 μg/mL for digitonin (15 minute incubation time) used for the 6 hour treatment, 30 μg/mL for digitonin (25 minute incubation time) used for the 12, 24, and

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48 hour treatments, 100 μM for ionomycin (6 hour incubation time) used for all four treatment times, and 10 μM for staurosporine (6 hour incubation time) used for all treatment times. The concentrations and incubation times were determined empirically for the 12, 24, and 48 hour treatments.

Cytotoxicity/viability reagents were added to the wells immediately after the incubation period, and following a brief mixing in an orbital shaker for 30 seconds, the plates were incubated for 30 minutes before measurements were taken. Viability measurements were taken using 400 nm excitation wavelength and 505 nm emission wavelength for all 4 treatment times (6, 12, 24, and 48 hours). Cytotoxicity measurements were taken using 485 nm excitation wavelength and 520 nm emission wavelength. The caspase reagent was added last to designated wells and was briefly mixed with an orbital shaker for 30 seconds and incubated for 30 minutes, before measurements were taken with the plate reader.

6. RESULTS AND DISCUSSION

In order to test for any sort of possible interference from the QDs, control tests were done with QDs in DMEM with and without the cells. For the tests, QDs were diluted to concentrations of 0.06 μM, 0.13 μM, 0.25 μM, and 0.50 μM. A 364 well plate was seeded with 10 μL/well of DMEM solutions and cells in DMEM solutions at a concentration of 5,000 cell per well. 10 μL of the QD solutions were added to wells, making overall concentrations of 0.03 μM, 0.06 μM, 0.13 μM, and 0.25 μM, prepared for hextuplicate wells. In addition, hextuplicate background wells (containing only 10 μL of DMEM and 10 μL of water) as well as another hextuplicate set of wells containing untreated cells with no QDs (*i.e.*, containing only 10 μL of cells in DMEM and 10 μL of water), were prepared. Measurements were taken with the microplate reader, using 400-nm excitation and 505-nm emission wavelengths for viability, 485-nm excitation and 520-nm emission wavelengths for cytotoxicity, and measuring luminescence for apoptosis. None of the wells containing QDs showed any signal beyond that of the background wells or wells containing untreated cells for all three measurements. The 400-nm emission is expected to excite the ZnSe:Mn/ZnS QDs, but the characteristic 595-nm emission from these QDs is well outside the range of the 505-nm emission wavelength used in viability testing. The 485-nm excitation wavelength used in cytotoxicity testing is not expected to excite the QDs at all.

For the tests discussed in Sections 6.A-6.D, cytotoxicity/viability reagents were added to the wells immediately after the incubation period, and following a brief mixing in an orbital shaker for 30 seconds, the plates were incubated for 30 minutes before measurements were taken. Viability measurements were taken using 400-nm excitation and 505-nm emission wavelengths for all 4 treatment times (6, 12, 24, and 48 hours). Cytotoxicity measurements were taken using 485-nm excitation and 520-nm emission wavelengths. The caspase reagent was added last to designated wells and was briefly mixed with an orbital shaker for 30 seconds and incubated for 30 minutes, before luminescence measurements were taken with the plate reader.

In Figs. 1-4, RFU stands for relative fluorescence units, RLU denotes relative luminescence units, UTC means untreated cells, and N.S. means no significant change.

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6.A. 6-Hour Exposure Time

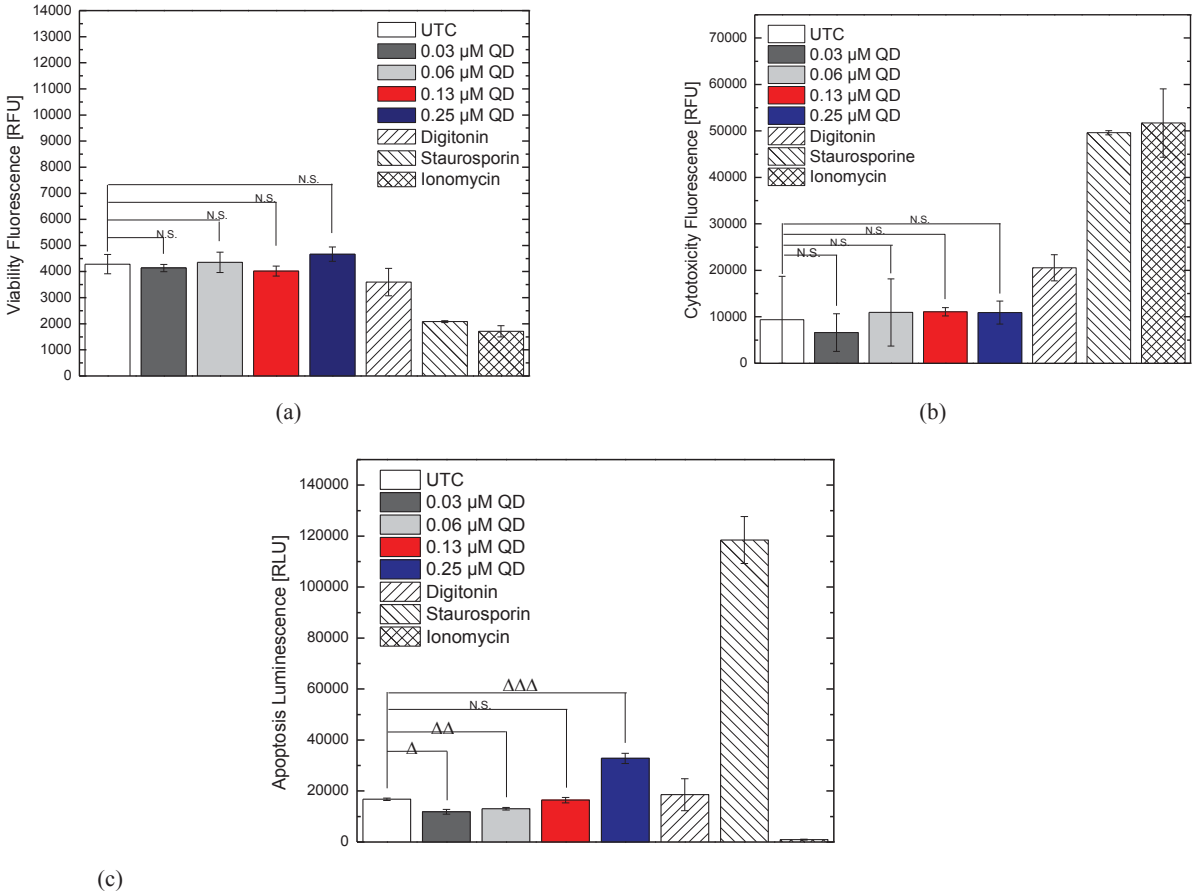

Fig. 1. ApoTox-Glo™ Triplex test results for a) viability, b) cytotoxicity, and c) apoptosis on murine MΦ cells after 6-hour exposure to peptide-coated ZnSe:Mn/ZnS QDs, together with positive controls. The bars indicate the mean value ± standard deviation, with $n = 3$.

As shown in Fig. 1a, a 6-hour exposure to ZnSe:Mn/ZnS QDs at any concentration in the 0.03 – 0.25 μM range resulted in no appreciable difference or statistical significance when comparing to the untreated cells, indicating that there was no effect on cell viability. Likewise, no significant difference was observed between the cytotoxicity from the QDs compared to the untreated cells, even taking into account the relatively large standard deviations on some fraction of the data, as indicated in Fig. 1b.

Fig. 1c shows the luminescence results from the 6-hour incubation test, in which induced apoptosis was quantified. The symbols in Fig. 1c have the following meaning: Δ: $p = 0.0005$ (< 0.01); ΔΔ: $p = 0.0004$ (< 0.01); and ΔΔΔ: $p = 0.00008$ (<< 0.01). Induced apoptosis can be inferred from the luminescent signal from the cells incubated with 0.25 μM QDs, with a 96% increase in luminescence compared to that of the untreated cells.

6.B. 12-Hour Exposure Time

Fig. 2a shows the results of cell viability testing after a 12-hour exposure of murine MΦ cells to ZnSe:Mn/ZnS QDs at various concentrations from 0.03 to 0.25 μM. The symbols in Fig. 2a have the following meaning: *: $p = 0.009$ (< 0.01); **: $p = 0.005$ (< 0.01); ***: $p = 0.001$ (< 0.01); and ****: $p = 0.00000007$ (<< 0.01). It can be seen from Fig. 2a that cell viability starts to become an issue and appears to scale inversely with QD concentration at the 12-hour incubation time. The fluorometric response with regards to cell viability, when compared with the untreated cells, decreases by 8% for 0.03 μM

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QD exposure, 13% for 0.06 μM QD exposure, 19% for 0.125 μM QD exposure, and as much as 50% for 0.25 μM QD exposure.

Fig. 2b shows the cytotoxicity testing results for 12-hour incubation time. The symbol † indicates $p = 0.009$ (< 0.01). Conflicting with the viability data of Fig. 2a, it would seem that the signals from the cell incubated with three of the four QD concentrations showed no statistically significant variability for cytotoxicity, with 0.13 μM QD having a 70% increase over the untreated cells, which was drastically lower than the positive control signals.

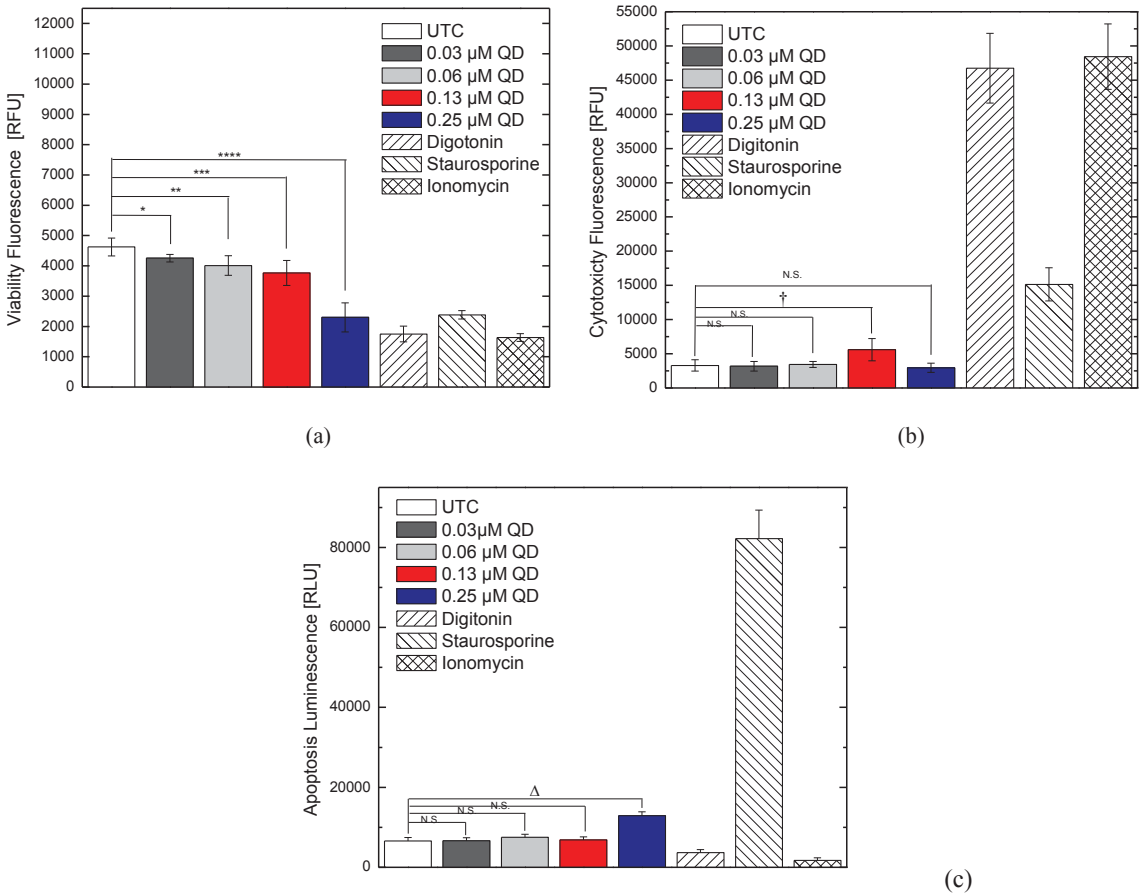

Fig. 2. ApoTox-Glo™ Triplex test results for a) viability, b) cytotoxicity, and c) apoptosis on murine MΦ cells after 12-hour exposure to peptide-coated ZnSe:Mn/ZnS QDs, together with positive controls. The bars indicate the mean value ± standard deviation, with $n = 6$.

As shown in Fig. 2c, a relatively small degree of induced apoptosis was observed for samples incubated with with 0.25 μM concentration of the QD, with the 96% signal increase over that of the untreated cells, while none of the other three lower concentrations showed any statistically significant variation in luminescence. The symbol Δ indicates p =0.00000002 (<< 0.01).

6.C. 24-Hour Exposure Time

Fig. 3a shows the results of cell viability testing after a 24-hour exposure of murine MΦ cells to ZnSe:Mn/ZnS QDs at various concentrations from 0.03 to 0.25 μM. The symbols in Fig. 3a have the following meaning: *: $p = 0.002$ (< 0.01); **: $p = 0.0002$ (<< 0.01); and ***: $p = 0.00000005$ (<< 0.01). The 50% decrease in viability at the highest QD concentration observed in the 12-hour test is seen in Fig. 3a as well, with viability as a whole continuing to scale inversely with the QD concentration. Compared to the untreated cells, the viability has decreased to 94% when incubated with 0.03 μM QDs ($p = 0.1$), 85% when incubated with 0.06 μM QDs, and 77% when incubated with 0.13 μM QDs.

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Fig. 3b shows the cytotoxicity testing results for 24-hour incubation time. The symbols in Fig. 3b have the following meaning: †: $p = 0.03$ (< 0.05); and ††: $p = 0.000003$ (<< 0.01). As shown in Fig. 3b, a strong increase in cytotoxicity by 315% of the untreated control was observed for cells incubated with 0.25 μM QDs. For all other QD concentrations, however, no statistical deviation was observed from the fluorometric intensity of the untreated cells.

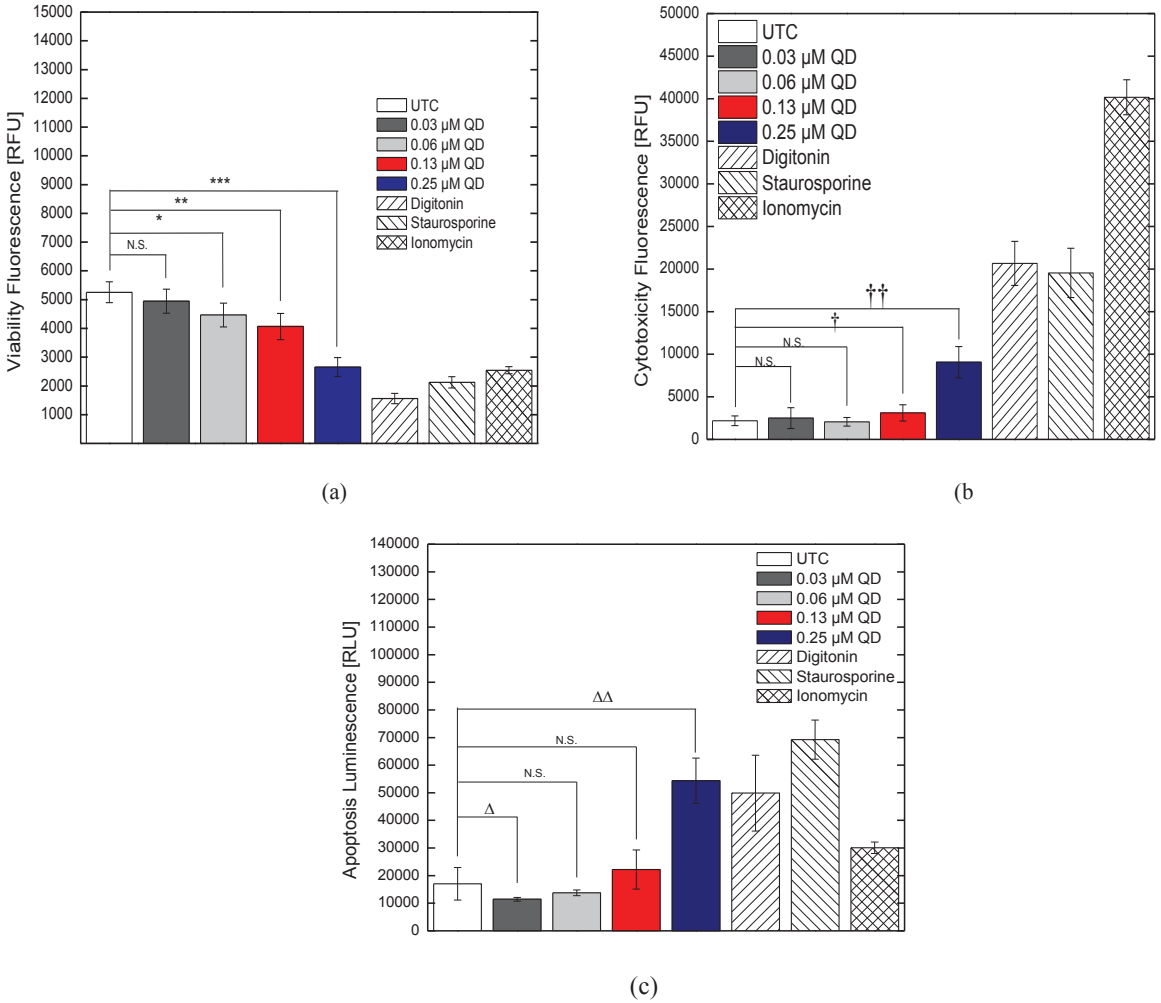

Fig. 3. ApoTox-Glo™ Triplex test results for a) viability, b) cytotoxicity, and c) apoptosis on murine MΦ cells after 24-hour exposure to peptide-coated ZnSe:Mn/ZnS QDs, together with positive controls. The bars indicate the mean value ± standard deviation, with $n = 6$.

In testing for apoptosis, the cells incubated for 24 hours with 013 μM QDs showed an elevation of 30% ($p = 0.1$ (> 0.05) in the luminescence signal over the untreated cells, while incubation with 0.25 μM QDs resulted in a 219% increase the luminescence signal over the untreated cells (Fig. 3c). None of the other QD concentrations resulted in a significant increase in apoptosis. The symbols in Fig. 3c have the following meaning: Δ: $p = 0.02$ (< 0.05); and ΔΔ: $p = 0.000002$ (<< 0.01).

6.D. 48-Hour Exposure Time

Fig. 4a shows the results of cell viability testing after a 48-hour exposure of murine MΦ cells to ZnSe:Mn/ZnS QDs at various concentrations from 0.03 to 0.25 μM. The symbols in Fig. 4a have the following meaning: *: $p = 0.049$ (< 0.05); **:

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p = 0.002 (< 0.01); and ***: p = 0.0002 (<< 0.01). Consistent with both 12-hour and 24-hour incubation periods, an inverse relationship between viability and QD concentration is observed. Exposure to QDs with 0.03 µM concentration resulted in a statistically insignificant decrease of 3% in cell viability, increasing to a 17% drop at 0.06 µM, 41% drop at 0.13 µM, and 62% for 0.25 µM QDs.

As shown in Fig. 4b, 48-hour incubation with ZnSe:Mn/ZnS QDs at all concentrations up to 25 µM has not produced any statistically significant cytotoxic effects.

Luminescence readings at 48 hours (Fig. 4c) displayed apoptosis elevated by 17% for 0.06 µM, 45% for 0.13 µM, and 60% for 0.25 µM QD concentration. The symbols in Fig. 4c have the following meaning: Δ: p = 0.045 (< 0.05); ΔΔ: p = 0.004 (< 0.01); and ΔΔΔ: p = 0.002 (< 0.01).

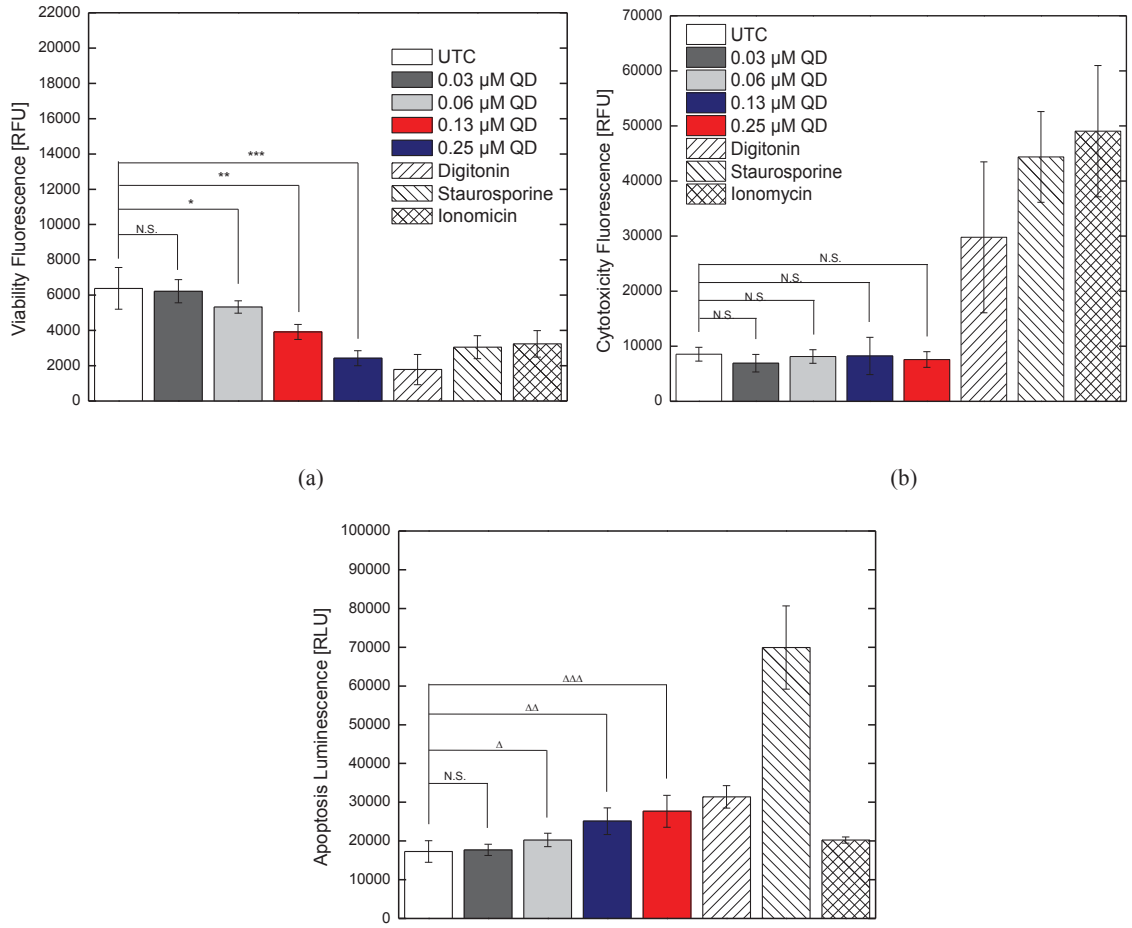

Fig. 4. ApoTox-Glo™ Triplex test results for a) viability, b) cytotoxicity, and c) apoptosis on murine MΦ cells after 48-hour exposure to peptide-coated ZnSe:Mn/ZnS QDs, together with positive controls. The bars indicate the mean value ± standard deviation, with n = 6.

7. CONCLUSIONS

The data obtained from the ApoTox-Glo™ Triplex assay yielded a complete profile for the effects of cell viability, cytotoxicity, and apoptosis from the peptide-coated ZnSe:Mn/ZnS QDs at four different concentrations for four different exposure times. Noteworthy is the higher sensitivity in cell viability that becomes apparent at 12 hours (Fig. 2a) and

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remains fairly consistent for 24 (Fig. 3a) and 48 hours (Fig. 4a). Most interesting is the fact that despite the drops in viability, the QDs show very little cytotoxic effect, particularly for 6, 12, and 48 hour incubation times, with an exception of a 315% increase in cytotoxicity for the most concentrated 0.25 μM QD solution for 24 hours (Fig. 3b). These results are consistent with the report of [Liu 2011], who applied the MTT assay to PK15 cells incubated with Mn^{2+}-doped ZnSe QDs and observed the cell viability above 80% after 48-hour incubation time.

The main conclusion from the tests is that, despite their low cytotoxicity, the peptide-coated ZnSe:Mn/ZnS QDs still seem to be having an unhealthy effect on the cells. Lowered viability without any increase in the cytotoxicity biomarker is indicative of an antiproliferation effect on the cells. In the case of primary necrosis, where all the cells die early, cytotoxicity may not be measurable in later testing times [Promega 2014]. If the cells were to die significantly much earlier than the testing time, the enzyme markers for cytotoxicity could degrade early on, and cytotoxicity would end up being underestimated [Niles 2008]. However, in the case of the MΦ cells being treated with the QDs, there is a measurable caspase activation from induced apoptosis at all four incubation times, as seen from the luminescence measurements in Figs. 1c, 2c, 3c, and 4c, with a statistically significant measurable increase in luminescence for the most concentrated 0.25 μM QD for all 4 incubation times, so primary necrosis can be ruled out.

Overall, it would seem that there is very little cytotoxic effect on the MΦ cells from the presence of the peptide coated ZnSe/ZnS:Mn QDs. However, a fortunate aspect the ApoTox-Glo™ Triplex assay allows for more thorough testing on other effects from the cells. The results outside of the cytotoxicity measurements show that these QDs cause a good deal of decreased viability and induced apoptosis, a result which would have gone unnoticed had only cytotoxicity been tested. Further studies on why cell viability is dramatically affected in this case, despite very little cytotoxicity, are needed. Also, further testing could be done for these particular QDs at higher concentrations than the ones presented herein.

8. ACKNOWLEDGEMENTS

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