Indium Phosphide : Cadmium Free Quantum Dots for Cancer Imaging and Therapy

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ABSTRACT

Fluorescent semiconductor nanoparticles, or quantum dots (QDs), have attracted a lot of attention over the past decade due to their unique optical and chemical properties. Indium phosphide (InP) QDs have emerged as a replacement for the widely used cadmium based QDs but their cytotoxity has not been well examined. Several questions *vis-à- vis* the InP QDs need to be addressed. Are they less toxic than CdSe/ZnS or CdTe? Are they as photoluminescent? Could they be used for imaging and photdynamic therapy (PDT)? Spin-trap electron paramagnetic resonance (EPR) spectroscopy and sulforhodamine B (SRB) viability assay indicates very low cytotoxicity in all cell lines tested, with toxicity proportional to ROS generation. Confocal microscopy showed none specific uptake of InP/ZnS concentrated in the perinuclear region. These data indicate that InP QDs are a viable alternative to cadmium-containing particles for biological applications.

Keywords: *quantum dots, InP/ZnS, toxicity, reactive oxygen species, electron paramagnetic resonance, imaging.*

1 INTRODUCTION

Quantum dots QDs exhibit a broad excitation spectrum, size-tunable emission ranging from ultraviolet (UV) to infrared (IR) and an ability to resist photobleaching. These proprieties make them an attractive alternative to organic dyes [1]. Furthermore, QDs can be conjugated to a variety of bioactive molecules for investigating specific and nonspecific binding [1]. QDs may also be specifically targeted *in vitro* and *in vivo* using antibodies [2], peptides [3], and ligands for cell-surface receptors such as dopamine, folate, and the epidermal growth factor [4].

The most widely used QDs are made of cadmium. Cadmium exposure is associated with tumors in the lung, prostate, liver, kidney, pancreas, urinary bladder and the breast [5]. Indium phosphide (InP) QDs have emerged as a presumably less hazardous alternative to cadmium based QDs. However, reports on colloidal synthesis of InP QDs are few, and usually do not report good size homogeneity. Recent studies performed by Nann's group have demonstrated the surface capping ability of zinc carboxylates during the preparation of InP QDs [6]. The time for shell growth has been shortened from many hours down to 20 min. Furthermore, InP particles have been shown to be nontoxic in animal inhalation experiments [7]. A good predictor of QD cytotoxicity in cell lines is the generation of free radicals, or reactive oxygen species (ROS). We previously reported the genaration of such species by CdSe [8]. There are several mechanisms by which semiconductor QDs generate ROS. Free radicals may be generated from photoexcited nanoparticles by either the *reductive pathway* (involving the electron transferring to an acceptor, A) or the *oxidative pathway* (involving the hole transferring to a donor, D):

$$A + e^-_{CB} \rightarrow A^{\bullet -}$$
$$D + h^+_{VB} \rightarrow D^{\bullet +} \quad (1)$$

If the radicals formed interact with water or oxygen, ROS can result. However, the radicals might also recombine rapidly, such as in the "electron shuttling" seen with quinones [9], for example by the process

$$A + e^-_{CB} \rightarrow A^{\bullet -}$$
$$A^{\bullet -} + h^+_{VB} \rightarrow A \quad (2)$$

In this case, no ROS is produced and the presence of the radicals, which may have femtosecond lifetimes, is difficult to detect.

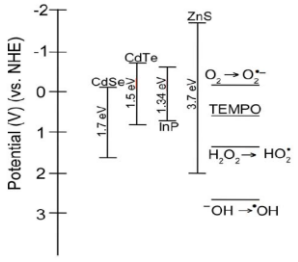

Figure 1: Approximate energy levels (vs. NHE) in aqueous solution for CdSe, CdTe, InP and ZnS bulk semiconductors. The band gaps widen as the materials are made into QDs. In this study, ZnS is a capping shell, which could act as a potential barrier for photogenerated electrons and holes.

The redox potential of an InP electron is higher than that of molecular oxygen, making the formation of singlet oxygen possible (see figure 1). InP holes are highly oxidizing but might be prevented from interacting by ZnS shell. Given the redox potentials of the InP QD's excitonic electron and hole, the direct formation of hydroxyl radicals is not possible (see figure 1). However, this radical's production could occur through an indirect mechanism, such as the photolysis of peroxide. In the presence of an electron-donating molecule such as dopamine (DA), however, the hole is expected to oxidize the DA, forming a semiquinone radical that can generate singlet oxygen:

$$DA + h^{+}_{VB} \rightarrow D^{\bullet +} \quad \text{(semiquinone)}$$
$$D^{\bullet +} + O_2 \rightarrow O_2^{\bullet -} + \text{DA-quinone} \qquad (3)$$

The formation of singlet oxygen during autooxidation of dopamine was reported earlier [10] and most probably involves semiquinone radicals, as fully chemically oxidized dopamine does not produce singlet oxygen. At the same time, scavenging of holes by dopamine represses charge recombination, allowing for the increase yield of superoxide, and consequent formation of singlet oxygen [11] :

$$O_2 + e^{-}_{CB} \rightarrow O_2^{\bullet -}$$
$$O_2^{\bullet -} + H^{+} \leftrightarrow HO_2, \quad pK = 4.8$$
$$2HO_2 \text{ (or } 2O_2^{-}) \rightarrow H_2O_2 \text{ (or } O_2) + {}^1O_2 \qquad (4)$$

Thus, attachment of dopamine to QD (via conjugation of amino groups) can result in superoxide/singlet oxygen formation both in reduction and oxidation processes and the dopamine conjugated quantum are a potential candidate for photodynamic therapy.

In this paper we test the ability of red and yellow with 1 or 2 shell layers (1 and 2 SL) InP/ZnS and InP/ZnS-DA to generate ROS. We compare this ROS generation to that from CdTe QDs. We also evaluate InP/ZnS as a fluoerscent dye. With spin-trap electron paramagnetic resonance (EPR) spectroscopy we determine the nature of the ROS generated. We demonstrate highly fluorescent labeling of cell lines after nonspecific endocytosis of QD-carboxylate. These data indicate that straightforward methods of synthesis and solubilization result in InP QDs which are a viable alternative to cadmium-containing particles for biological applications

2 RESULTS

2.1 Synthesis and characterisation

Both 1SL and 2SL yellow InP/ZnS had a maximum emission at 570 nm**.** The red InP/ZnS 2 SL had an emission at 600 nm. The diameter of InP/ZnS 1SL is 2.7 nm that of InP/ZnS 2SL 3.0 nm and for the red InP/ZnS is 3.4 nm as seen by TEM (see figure 2).

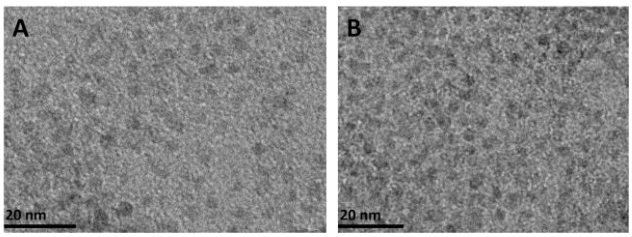

Figure 2: TEM images showing (A) InP/ZnS 1SL and mean diametr 2.7± 0.7nm (B) InP/ZnS 2SL mean diameter 3.0 ± 0.5 nm.

The QDs were transferred into borate buffer at pH 9 *via* a ligand exchange procedure where the hexadecylamine was replaced by mercaptopropionic acid (MPA). The choice of the intermediate solvent was critical for the solubilization procedure. The use of toluene instead of butanol rendered the QDs insoluble. The emission wavelength was unchanged after water solubilization. As expected, the 2SL QDs had a higher relative quantum yield: 9 % for InP-2SL and 4 % for InP-1SL, in accordance with other studies [12]; the red dots had the same quantum as 2SL. InP/ZnS-MPA was stable in borate buffer pH 9 at 4 °C for 5 weeks, after which the solution became cloudy and the QDs aggregated upon centrifugation. CdSe/ZnS and CdTe had higher quantum yields after solubilization (25 %), but aggregated after 1 week at 4^{o}C.

2.2 EPR

The spin trap TEMPO is a stable free radical that can be oxidized by holes, OH radicals or any other oxidative species that have a redox potential ≥ 0.75 V vs. NHE. Thus a decrease in intensity of the EPR spectra of TEMPO following irradiation indicates the presence of photogenerated oxidative species. A decay of the TEMPO signal was observed for all QD samples, but showed significantly faster kinetics for InP/ZnS than for CdTe (see **Error! Reference source not found.**). When conjugated, CdTe did not triger any TEMPO decay while InP/ZnS-DA showed a decay at different rates depending on the number of shell layers and particle size. We found that the 1SL had the slowest rate of decay. The TMP method measures the formation of singlet oxygen or the superoxide anion using the EPR-silent TMP, which reacts with singlet oxygen or superoxide to form a stable, EPR-sensitive radical adduct (nitroxide-type radical). In this case, it is thus the formation of the radical rather than its disappearance that is measured. We report a constant intensity in EPR spectra for all InP/ZnS whether conjugated or not (data not shown). This indicates no formation and/or an undetectable amount of the TMP radical. On the other hand CdTe-DA showed a high level of the TMP radical indicating the formation of singlet oxygen and/or superoxide (data not shown).

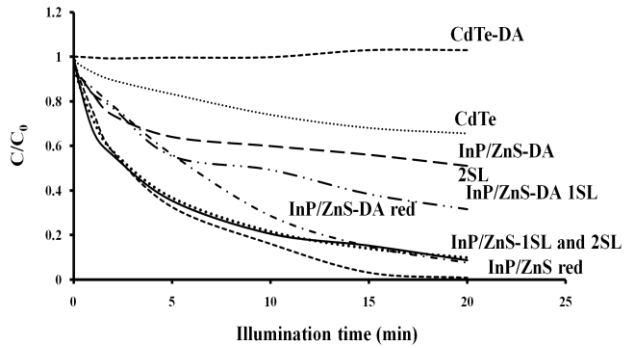

Figure 3: EPR spectroscopy using TEMPO radicals as spin traps showing the decay of TEMPO radical relative concentrations with time illumination

2.3 ROS Indicator Assays

XTT must be reduced, rather than oxidized, to form an absorbance peak at 470 nm. Therefore, direct oxidation by the QDs will not yield a false positive [13]. The XTT assay is sensitive to perhydroxyl and superoxide anions; disappearance of the signal in the presence of superoxide dismutase (SOD) indicates the formation of superoxide. We observed signals from single and double shell InP/ZnS, with those from single shell QDs showing significantly higher counts. In the presence of SOD, signals were reduced to <10 % of their original signal. A change in the amount of SOD from 25 to 50 units/mL did not show a notable difference in this reduced signal. This indicates that the superoxide anion, rather than perhydroxyl is predominantly responsible for the reduction of XTT (see Figure 4). The hydroxyl radical indicator sodium terephthalate showed emission peaks at 430 nm. We have previously observed hydroxyl radical from CdTe upon light irradiation[14], so we used these QDs as a positive control. Both single and double shell InP/ZnS QDs showed positive signals that increased with irradiation time; single shell QDs showed higher signals than 2SL, comparable to those from CdTe (see Figure 4). Sodium terephthalate in the presence of QDs with SOD (25 units/mL) showed an undetectable signal.

2.4 Cell labelling

The fluorescence intensity of 1SL InP/ZnS inside cells decreased dramatically after 2 min of exposure to typical Hg lamp epifluorescence illumination (Quantum Dot filter: excitation 380-460 nm, approximately 2 mW). Because this did not demonstrate a significant improvement over most dyes, InP/ZnS 2SL were chosen for imaging. Cells labelled with 2SL QDs could be visualized under the same excitation conditions for 15-20 minutes before they began to photobleach. The cells could be fixed and stored at 4 ^{o}C for several days and the fluorescence of internalized QDs was retained. For nonspecific uptake, a minimum concentration of 50 nM QDs was needed to get a strong signal. Most of the QDs were localized in the peri-nuclear region, indicating nonspecific uptake, a minimum concentration of 50 nM QDs was needed to get a strong signal. Most of the QDs were localized in the peri-nuclear region, indicating endosomal uptake, with all the cell lines tested demonstrating some degree of uptake. B16 murine melanoma cells showed significantly greater uptake than the other cell lines tested (see Figure 5).

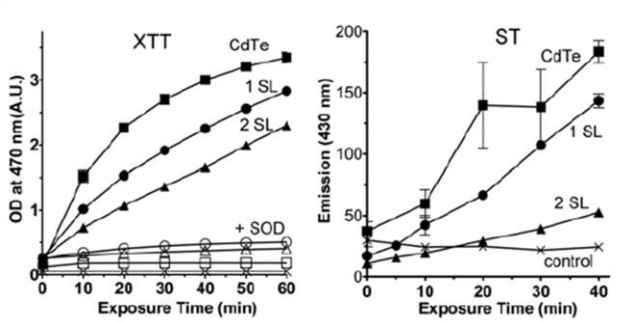

Figure 4**:** To the left Superoxide anion detection with the XTT assay showing CdTe, 1 SL InP/ZnS, and 2 SL InP/ZnS with and without SOD (the open symbols with SOD correspond to the same QDs as the filled symbols). To the right the hydroxyl radical sensor, sodium terephthalate (ST), showing a marked reduction in signal with 2SL InP vs. 1SL InP.

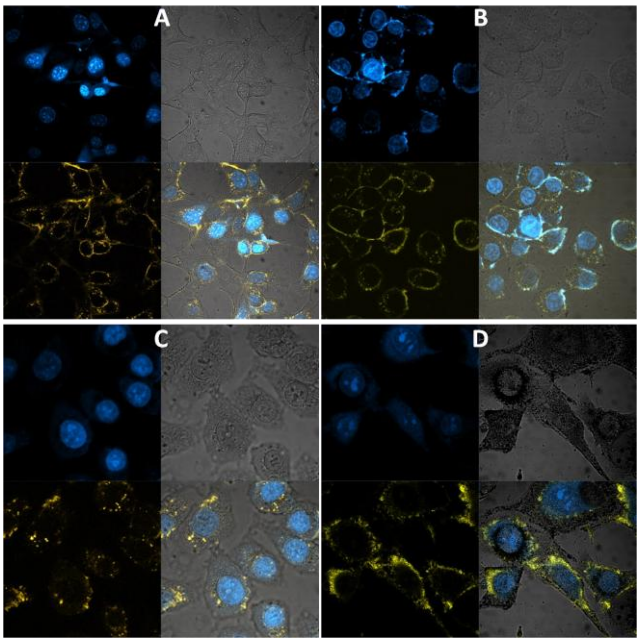

Figure 5: Comparaison between (A) fibroblat NIH 3T3(B) KB (C) MDA and (B) B16 cellular uptake of InP/ZnS 2SL at 100 nM (30 min incubation time). showing DAPI (blue), InP/ZnS (yellow), DIC and merged channels

DISCUSSION AND CONCLUSION

The water soluble InP/ZnS are more stable in solution than CdSe/ZnS and CdTe solubilized by the same alkenethiol. The ZnS shell prevent the QDs from fast photobleaching and increase the quantum yield. The photoluminescence of these nanoparticles are lower than that of Cadmiun-based QDs, which implies that more need to be used to get the same signal intensity. This is possible without any side effect. The toxicity of InP/ZnS is very low. We previuosly demonstrated that no or little cell death occurred up to 200 nM of InP/ZnS 2SL on sevral cell lines compared to 20 % with CdSe/ZnS at 100 nM[8]. Others have also reported 70 % of cell death when they used CdTe [15]. The spin-trap EPR results were in accordance with the low toxicity of the QDs. The absence of TMP formation when InP and InP-DA are involved supported the lack of singlet oxygen, the most damaging species of ROS. We suspect that the TEMPO decay is the result of the photogenerated holes, rather than from the toxic production of the perhydroxyl radical. Firstly, the reduction potential of the valence band of InP does not allow the formation of hydroxyl radicals. Secondly, we observe identical TEMPO decays for both 1 and 2 SL InP/ZnS, yet different signal levels from the Sodium Terephthalate assay. Lastly, the reduction potential of photogenerated holes is +0.94 V vs. NHE, which is greater than +0.75 V vs. NHE for TEMPO, thus making a TEMPO decay from the photogenerated hole seem plausible. A TEMPO decay was still observed with the InP/ZnS samples conjugated to DA; this was not the case for CdTe QDs (Figure 3). We suspect that photoinduced electron transfer occurs from the DA to the QD (as described in equation 1). This would imply that QD holes are filled with electrons from DA. Such a process would be supported by a lack of TEMPO decay, since photogenerated holes are no longer accessible by the TEMPO spin-trap. This mechanism likely occurs with CdTe QDs, as supported by the lack of TEMPO decay with the conjugate. However, all InP QDs conjuagted to DA show TEMPO decays with different kinetics, thus suggesting that photoinduced electron transfer is inefficiently occuring from the DA molecule to the QDs. Ultimately, this results in a decreased toxicity as observed from the lack of singlet oxygen production. EPR using TMP is an indicator of superoxide/singlet oxygen generation. The radical was formed with CdTe-DA and CdSe/ZnS-DA, however none was detected when the InP/ZnS and InP/ZnS-DA.

The results from EPR and repoter assays are highly consistent with the SRB toxicity assay previously done in our lab (nanoscale 2011 paper in press). In all except B16 cells 100 nM of InP/ZnS-2SL was not significally toxic. The greater toxicity in B16 cells can be attributed to their ability to take up more QDs by nonspecific binding (see figure 5). A better selectivity could allow for bright labelling with non-toxic concentartion of QDs. Even more importantly InP/ZnS might eventually be developed for in vivo applications.

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