Submitted to small NANO MICRO

Quantification of Reactive Oxygen Species Generation by Photoexcitation of PEGylated Quantum Dots

Elnaz Yaghini[1], Katharina F. Pirker[2, 3], Christopher W. M. Kay[3, 4], Alexander M. Seifalian[5], and Alexander J. MacRobert[*1]

[1]Division of Surgery & Interventional Science, Charles Bell House, University College London, London, W1W 7EJ, UK

[2]Department of Chemistry, Division of Biochemistry, University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria

[3]Institute of Structural and Molecular Biology, University College London, Gower Street, London, WC1E 6BT, UK

[4]London Centre for Nanotechnology, University College London, 17-19 Gordon Street, London, WC1H 0AH, UK

[5]Centre for Nanotechnology and Regenerative Medicine, Division of Surgery & Interventional Science, Royal Free Campus, University College London, London, NW3 2QG, UK

[*]Corresponding author: Prof. A. J. MacRobert, a.macrobert@ucl.ac.uk

Division of Surgery & Interventional Science, Charles Bell House, University College London, London, W1W 7EJ, UK

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Keywords:

EPR spectroscopy, quantum dots, singlet oxygen, spin trapping, superoxide anion radicals

Abstract

Photocatalytic generation of reactive oxygen species (ROS) from quantum dots (QDs) has been widely reported yet quantitative studies of ROS formation and their quantum yields are lacking. This study investigates the generation of ROS by water soluble PEGylated CdSe/ZnS QDs with red emission. PEGylation of QDs is commonly used to confer water solubility and minimise uptake by organs of the reticuloendothelial system; therefore studies of ROS formation are of biomedical relevance. Using non-photolytic visible wavelength excitation, the superoxide anion radical is shown to be the primary ROS species generated with a quantum efficiency of 0.35 %. The yield can be significantly enhanced in the presence of the electron donor, nicotinamide adenine dinucleotide (NADH), as demonstrated by oxygen consumption measurements and electron paramagnetic resonance spectroscopy with *in situ* illumination. Direct production of singlet oxygen is not detectable from the QDs alone. A comparison is made with ROS generation by the same QDs complexed with a sulfonated phthalocyanine which can generate singlet oxygen via Förster resonance energy transfer between the QDs and the phthalocyanine.

Introduction

Quantum dots (QDs) are inorganic semiconductor nanoparticles and have many potential applications ranging from electronics to medicine, due in part to their unique photophysical properties. [1-5] Photoexcitation of QDs results in the promotion of an electron from the valence band to the conduction band, yielding an electron-hole pair. [5;6] The excited QD can return to the ground state via two different pathways: radiative recombination of the electron-hole pair or non-radiative relaxation. The luminescence properties of QDs originate from the radiative electron-hole recombination, resulting in the emission of light. Alternatively, the conduction band electron may localize in surface traps within the bandgap, resulting in a long-lived electron-hole pair to form a metastable 'dark' state analogous to the long-lived triplet state of conventional organic photosensitisers. The electron or hole may then interact with electron acceptors or donors respectively, which enables photoexcited QDs to mediate both oxidative and reductive electron transfer processes, thereby providing a mechanism for production of reactive oxygen species (ROS), such as superoxide radical anions ($O_2^{-\bullet}$), and hydroxyl radicals ($OH^\bullet$). Direct generation of singlet oxygen ($^1O_2$) from QDs has also been reported, which can be enhanced through Förster resonance energy transfer (FRET) to photosensitising dyes near the QD surface.[7] Photo-induced electron transfer reactions of QDs and FRET interactions with dyes have generated considerable interest in their photocatalytic properties, and their potential for photodynamic therapy (PDT). [7-9] A further reason for studying ROS production is that prolonged illumination of QDs taken up in cells may induce phototoxic reactions.[10] Therefore quantum yields of ROS generation should be quantified.

The mechanisms by which ROS may be generated by QDs can be summarised by analogy with the well-known Type 1 and 2 classification used for organic photosensitisers. [11;12] In Type 1 photo-oxidative or photo-reductive pathways, photo-induced electron transfer reactions take place which

generate intermediates including ROS such as superoxide radical anions. In Type 2 reactions, energy transfer takes place directly with molecular oxygen generating highly reactive singlet oxygen ($^1O_2$). [13;14] Photo-induced formation of ROS by QDs in solutions and phototoxic effects on cells have been reported using a range of techniques, although contrasting results have been observed. Ipe and co-workers carried out studies on free radical generation in aqueous solutions by photoirradiation of CdS, CdSe and CdSe/ZnS QDs.[15] Using electron paramagnetic resonance (EPR) spectroscopy and a fluorometric assay, they showed that photoactivated CdS and CdSe QDs stabilized with mercaptoacetic acid ligands generated hydroxyl and superoxide radicals, whereas under the same conditions ROS generation using CdSe/ZnS QDs was not detectable. Green et al. reported the nicking of double-stranded DNA in the presence of CdSe/ZnS QDs after illumination and provided evidence for the involvement of free radicals using EPR spectroscopy, however no definitive assignment of the observed EPR signals was carried out. [16] Subsequently, Clarke et al. reported that UV irradiation of QD-dopamine conjugates (QDs: CdSe/ZnS) resulted in DNA damage of cells due to electron transfer from QDs to dopamine followed by oxidation of dopamine.[17] Similarly, Liang et al. demonstrated calf thymus DNA (ctDNA) damage with water soluble CdSe QDs and Rajendran et al. showed that protein-conjugated CdS QDs under UV illumination generated significant amounts of hydrogen peroxide. [18;19] Anas et al. reported the photosensitised damage of plasmid DNA using CdSe/ZnS.[20] Using CdTe QDs with various capping layers, Cho et al. showed that MCF-7 cells (breast adenocarcinoma) exhibited a decrease in cellular metabolic activity which was attributed to the presence of Cd^{2+} ions and ROS. [21] Nadeau and colleagues have recently reported phototoxic effects on cells treated with mercaptopropionic acid-capped CdSe/ZnS QDs and InP QDs. [22;23] In a systematic study using a range of techniques including EPR, they found evidence for superoxide ($O_2^{-\cdot}$) generation but not singlet oxygen ($^1O_2$). Using CdSe/ZnS QDs conjugated with dopamine, enhanced ROS generation was observed.

Encapsulation by polyethylene glycol (PEG) chains is widely used to confer water solubility and biocompatibility to QDs. We have recently demonstrated that PEGylated CdSe/ZnS QDs can interact via FRET with a phthalocyanine photosensitiser (PS) to generate singlet oxygen via the Type 2 mechanism using direct detection of singlet oxygen. [24] The present study also uses PEGylated CdSe/ZnS QDs. Although various types of QDs have been used to examine their potential for ROS formation, no study has performed quantitative studies of ROS quantum yields (QYs). A further limitation of many studies performed to date is that UV excitation is used instead of non-photolytic visible excitation. Photolytic generation of hydroxyl radicals from hydrogen peroxide could lead to misinterpretation of the origin of hydroxyl radical formation. Moreover for *in vivo* studies using QDs, UV is not employed owing to strong absorption by tissue chromophores and therefore limited light penetration. In this study we have investigated ROS formation in air-equilibrated aqueous solution using visible light excitation (460-510 nm) which avoids the potential photolytic processes that can be induced using UV irradiation.

2. Results

2.1 ROS production using EPR spin trapping

EPR spectroscopy in combination with spin trapping allows qualitative determination of ROS formation using a diamagnetic spin trap that forms a stable paramagnetic adduct with short-lived radicals. Here, the generation of oxygen free radicals with water soluble CdSe/ZnS QDs in aqueous buffered solutions under illumination was measured using 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) as spin trap. QD aqueous solutions (with (Figure 1A) and without (Figure 1B) NADH: nicotinamide adenine dinucleotide) in the presence of DEPMPO and under *in situ* illumination using a light-emitting diode (LED) light source (455 nm) during EPR measurements resulted in the generation of the DEPMPO-superoxide radical adducts with typical

hyperfine coupling constants characteristic for the superoxide anion ($O_2^{-\cdot}$) radical adduct (^{1}H = 1.11 mT, ^{14}N = 1.33 mT, ^{31}P = 5.00 mT. [25]

The addition of the electron donor NADH to the QD solution significantly increased (approximately 4 times) the intensity of the DEPMPO-superoxide adduct (Figure 1A and B). Both EPR spectra indicated traces of another radical species which originated from hydroxylamine contaminants in the spin trap DEPMPO and which are visible under oxidative conditions, as shown elsewhere. [26] The key advantage of *in situ* illumination is that nascent species are readily identified in contrast to external illumination followed by mounting of the sample in the spectrometer cavity. The use of DEPMPO also enables unambiguous identification of the superoxide species in contrast to the 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) spin trapping agent where the OH-adduct is rapidly formed from degradation of the superoxide adduct.[27]

Although hydroxyl radicals can be formed from the hydrogen peroxide dismutation product of superoxide, NADH is known to rapidly scavenge hydroxyl radicals, and no hydroxyl radical adduct was detected under these conditions. [28] The addition of superoxide dismutase (50 U/mL) removed the radical signals in the EPR spectrum by catalyzing dismutation of $O_2^{-\cdot}$ to water and oxygen (Figure 1C and D) and confirmed the generation of only superoxide anion radicals. Furthermore, the addition of NaN_3 (5 mM) as an $^{1}O_2$ quencher did not change the formation of the DEPMPO-superoxide adduct signal (data not shown), indicating that $^{1}O_2$ was not a precursor to $O_2^{-\cdot}$.

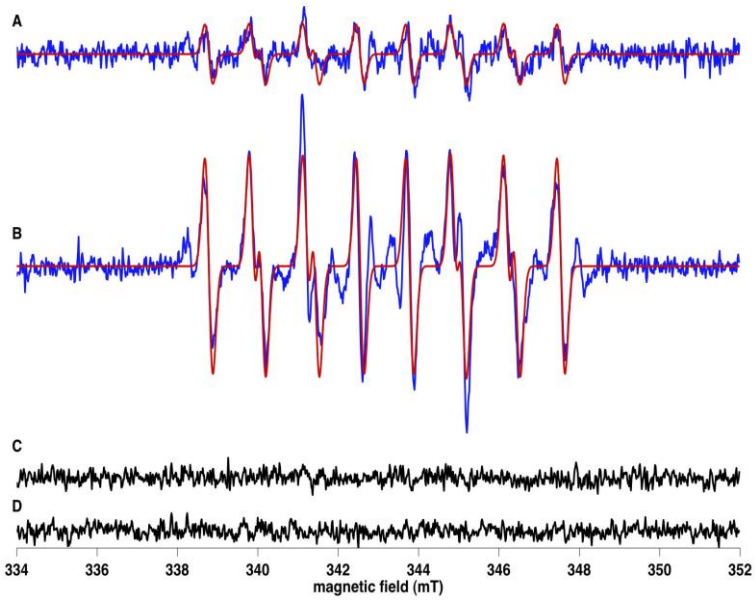

Figure 1. EPR spectra obtained upon *in-situ* visible light illumination of air-saturated buffered H_2O (pH 7.4, 0.01 M PBS) containing CdSe/ZnS QDs: 5 μM, and DEPMPO: 50 mM, (A) in the absence of NADH: experimental (cyan) and simulation of the DEPMPO-superoxide adduct (red), (B) with 1 mM NADH: experimental (blue) and simulation of the DEPMPO-superoxide adduct (red), (C) with 50 U/mL SOD (in the absence of NADH), (D) with SOD and NADH.

2.2 Oxygen consumption measurements

The use of oxygen consumption techniques to investigate the production of ROS in oxygenated solutions of photosensitisers is a well established technique.[29] Since ROS production can involve formation of a number of species, including hydroxyl radicals, singlet oxygen or superoxide anion radicals, the formation of these ROS and their subsequent reaction with substrate molecules results in depletion of the dissolved molecular oxygen. The oxygen consumption technique has previously been used to determine quantum yields of ROS production for organic photosensitisers. [29;30]

In this study, measurements of oxygen consumption were performed using a fiber-optic dissolved oxygen probe with exceptionally stable calibration properties compared to conventional Clark electrodes.[31] Quantum yields for singlet oxygen production via the Type 2 mechanism can now

routinely be determined using direct detection of singlet oxygen phosphorescence, but an advantage of the oxygen consumption technique is that it is sensitive to both Type 1 and 2 mechanisms, which is relevant to the photocatalytic properties of QDs. In order to identify the mechanism of oxygen consumption a number of different combinations were employed: (a) Addition of L-Tryptophan which is readily oxidized by ROS including singlet oxygen which will result in oxygen depletion; (b) Addition of a well-known electron donor, NADH. [32-34] A similar approach has been used to study superoxide and singlet oxygen formation from water soluble fullerenes. [35] A relatively high starting concentration of substrate for these experiments was used so that limited depletion of substrate concentrations occurs during illumination. NADH is also known to react rapidly with singlet oxygen. [36] Formation of superoxide ($O_2^{-\cdot}$) can lead to molecular oxygen consumption via dismutation to hydrogen peroxide (H_2O_2). Alternatively, if singlet oxygen or other ROS are produced, such as hydroxyl radicals through the Fenton mechanism, then they will also react with the substrates thus resulting in oxygen depletion. Replacement of H_2O by D_2O in order to prolong the singlet oxygen lifetime and thus the extent of reaction with the substrate, and addition of the singlet oxygen physical quencher, azide, were employed to confirm the involvement of singlet oxygen in oxygen depletion. At an azide concentration of 5 mM, over 95% quenching of $^1O_2$ should occur in deuterated solution. [37]

Air-saturated aqueous solutions of CdSe/ZnS QDs supplemented with NADH were prepared and oxygen consumption was compared to that without NADH. Illumination of the QD solution in the absence of NADH resulted in a small decrease in oxygen levels, whereas NADH alone resulted in no detectable decrease. However, when QDs and NADH were both present a significant increase in oxygen consumption was observed, as shown in Figure 2.

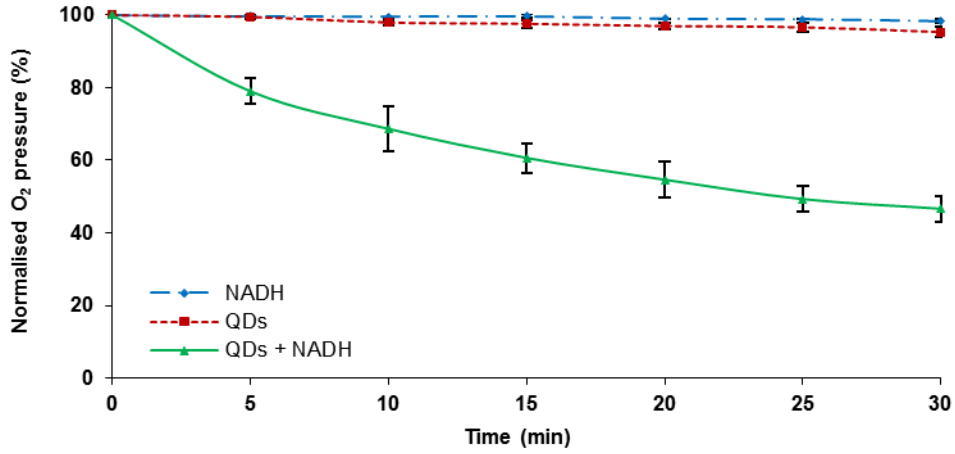

Figure 2. Oxygen partial pressure measurements in air-saturated solution as a function of illumination time for QDs normalised to % values QD alone (50 nM), NADH (1 mM) alone and QD + NADH (QD: 50 nM, NADH: 1 mM) solutions in buffered H_2O (pH 7.4, 0.01 M PBS). All samples were illuminated with a halogen lamp equipped with a bandpass filter (460-510 nm).The data points are the mean +/- SD for three readings.

Oxygen consumption increased with increasing concentrations of QDs (25-100 nM) and NADH from 0.5 - 2 mM (supporting information, Figure S1). In order to determine whether singlet oxygen was generated, the effects of an $^1O_2$ scavenger (azide) and $^1O_2$ stabilizer (D_2O) were tested. Figure 3A shows the % oxygen consumption measured at 15 minutes illumination of QD-NADH solutions. The lack of involvement of $^1O_2$ was supported by the finding that azide did not inhibit the oxygen consumption and the decrease in oxygen levels at 38% was very similar within experimental error to that without azide. Likewise, the use of deuterated solutions to prolong the lifetime of $^1O_2$ did not enhance the oxygen consumption rates, arguing against the involvement of $^1O_2$. [38;39] Since NADH can rapidly scavenge singlet oxygen, these results indicate that other ROS are involved and not singlet oxygen. We postulate that oxygen depletion is due to superoxide formation via an electron-transfer mechanism as discussed later.

To further investigate the photo-induced generation of ROS by QDs including singlet oxygen, aqueous deuterated solutions of CdSe/ZnS QDs were prepared with L-Tryptophan and illuminated for various times. A slower rate of oxygen depletion was observed compared to NADH with a linear dependence versus illumination time. Figure 3B shows a histogram of the % oxygen consumption observed upon illumination of QD-tryptophan solutions. The oxygen depletion was found to be significantly smaller (17%) using tryptophan (5 mM) compared to NADH at 1 mM. No change in oxygen depletion was observed when singlet oxygen quenching azide was added to the solution.

For comparison we measured the oxygen consumption using a system we have investigated previously where we showed that singlet oxygen can be efficiently generated from PEGylated CdSe/ZnS-QDs complexed with a disulfonated aluminium phthalocyanine ($AlPcS_{2a}$) via the FRET mechanism. Direct detection of singlet oxygen near-IR phosphorescence at 1270 nm was employed. [24] In this previous study, we were not able to detect any direct generation of singlet oxygen from CdSe/ZnS-QDs alone. In the present study, we were not able to detect singlet oxygen either but were able to determine an upper limit to the quantum yield of singlet oxygen production as < 0.003 (supporting information, Figure S2) using the same near-IR detection technique, with and without addition of azide. To demonstrate the effect of singlet oxygen production on oxygen consumption, we therefore illuminated deuterated solutions of QD-phthalocyanine (PS) complexes, where the phthalocyanine concentration was sufficient to quench the QD emission by 90% giving a singlet oxygen yield of 0.15 (supporting information, Figure S2), which is a factor of fifty higher than the upper limit for QDs alone.

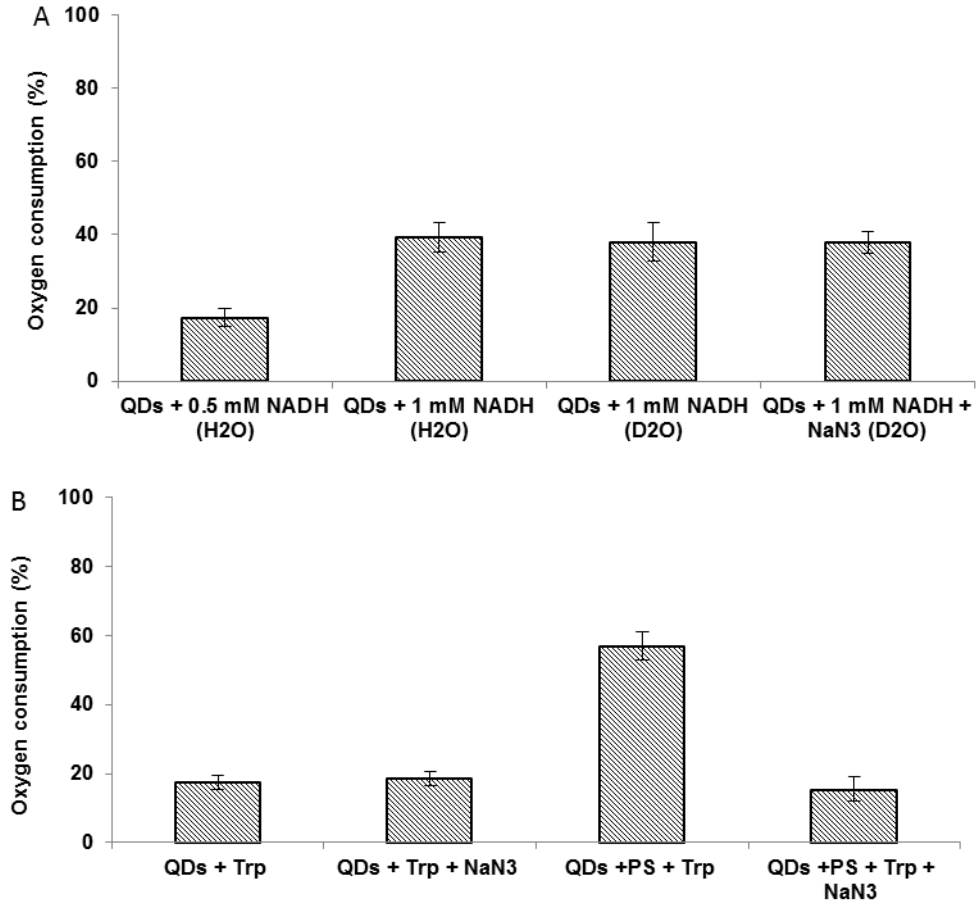

Figure 3. Histogram plot of oxygen consumption in air-saturated solutions (A): after 15 minutes light exposure of QD + NADH buffered H_2O solutions (QDs: 50 nM, NADH: 0.5 mM and 1 mM) versus deuterated solutions (D_2O) in the presence and absence of 5 mM NaN_3. (B): after 15 minutes light exposure of deuterated solutions containing QDs (50 nM) + L-Tryptophan (5 mM) or QDs + $AlPcS_{2a}$ (PS) + L-Tryptophan (QDs: 50 nM, $AlPcS_{2a}$: 500 nM, + L-Tryptophan (Trp): 5 mM) solutions in the presence and absence of 5 mM NaN_3. All samples were illuminated with a halogen lamp equipped with a bandpass filter giving output over 460-510 nm. Data show mean +/- SD of three readings.

As shown in Figure 3B, a substantial and larger decrease in oxygen levels was observed at 57% in the presence of tryptophan using the QD complexes compared to absence of the phthalocyanine.

Control experiments without QDs showed no oxygen consumption as would be expected since the phthalocyanine exhibits negligible absorption at the illumination wavelengths. [24] Addition of 5 mM azide reduced the oxygen consumption to the same levels observed without addition of the phthalocyanine, within experimental error. This result is consistent with the generation of singlet oxygen by the QD-phthalocyanine complexes, where the singlet oxygen generated via a Type 2 mechanism is then scavenged by tryptophan resulting in depletion of molecular oxygen. The oxygen consumption in the presence of the phthalocyanine and azide was very close to that observed for the QD and tryptophan alone. The rate constant for singlet oxygen quenching by tryptophan in aqueous solution is 3.2 $x10^7$ M^{-1} s^{-1}, and in D_2O the lifetime of singlet oxygen is 68 microseconds therefore at the tryptophan concentration used (5 mM) over 90% of the singlet oxygen would be removed through chemical and physical quenching. [34]

2.3 Superoxide generation by QDs quantified using cytochrome c reduction

Optical absorption measurements of ferricytochrome c reduction have been widely used for quantitative analysis of superoxide radical anions. [40;41] Superoxide reduces ferricytochrome c ($Cyt.Fe^{3+}$) to ferrocytochrome c ($Cyt.Fe^{2+}$) which exhibits stronger optical absorption at 550 nm. Inhibition of this reaction can be confirmed using superoxide dismutase (SOD). This method has been extensively used to study formation of $O_2^{-\cdot}$ from conventional organic photosensitisers and determine superoxide quantum yields. [42-46] EPR has also been used for superoxide quantum yield measurements and gives comparable results to the cytochrome c technique, but is more difficult to perform due to degradation of the adduct signal than the optical method. [47] We have therefore used ferricytochrome c reduction to quantitate superoxide formation and determine the quantum yield, as carried out previously for riboflavin. [46]

In the case of PEGylated QDs, a potential advantage of using this macromolecular substrate is that the steric hindrance resulting from the presence of the PEG chains covering the QD surface may inhibit interaction of the ferricytochrome with the QD surface. Although we have no direct support for this argument, PEGylation of nanoparticles is widely used to inhibit binding of serum proteins through steric hindrance. The reaction of superoxide anion with cytochrome c involves the transfer of a single electron to produce ferrocytochrome c from ferricytochrome c (Equation 1), which is identified on the basis of the increase in absorbance at 550 nm and SOD inhibition.

$$Cyt.Fe^{3+} + O_2^{-} \rightarrow Cyt.Fe^{2+} + O_2 \qquad (1)$$

In our study, air-saturated buffered solutions of QDs with cytochrome c were prepared and illuminated with a filtered halogen lamp over the 460-510 nm range as used for the oxygen consumption experiments. Upon illumination typical absorption spectra of ferricytochrome c and ferrocytochrome c were evident and the changes in absorption at 550 nm were calculated with reference to the isobestic point (where no change in absorption takes place) at 556 nm. As shown in Figure 4A, Cyt.Fe^{3+} was reduced as a function of exposure time with concurrent formation of ferrocytochrome c (Cyt.Fe^{2+}) shown by the increasing absorbance at 550 nm, which is consistent with the generation of $O_2^{-\cdot}$. The addition of SOD prior to illumination inhibited the formation of ferrocytochrome c by 90%, providing additional support for the production of the $O_2^{-\cdot}$ (Figure 4B). Illumination of ferricytochrome c alone solutions did not induce any changes. In order to provide further evidence for the involvement of $O_2^{-\cdot}$ formation, NADH was added and as expected the addition of the electron donor greatly increased the reduced ferrocytochrome c signal which was suppressed following the addition of SOD (data not shown). However a slow dark reaction in the presence of NADH was also noted as previously reported therefore no quantitative analysis was made. [46]

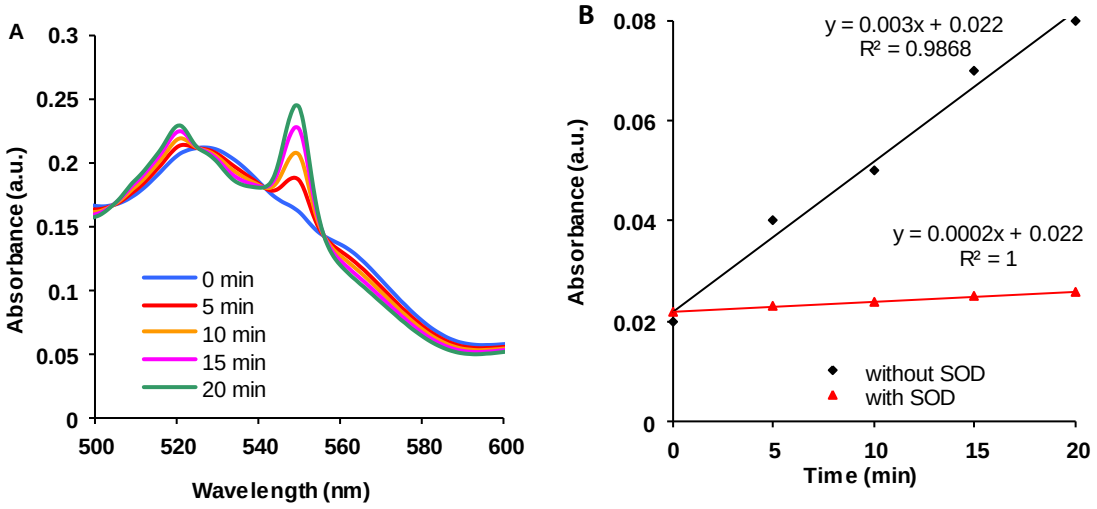

Figure 4. Reduction of ferricytochrome c upon illumination of air-saturated aqueous solutions containing CdSe/ZnS QDs. (A) Absorbance spectra of solutions of 80 nM QDs and 15 μM ferricytochrome c after different illumination times. (B) Plot of the cytochrome c absorption changes (15 μM) mediated by QDs (80 nM) in the presence and absence of SOD (50 U/mL), as a function of illumination time. The absorbance is displayed as the value recorded at 550 nm minus the value at the isobestic point (556 nm). All samples were prepared in buffered H_2O (pH 7.4, 0.01 M PBS) and illuminated with a filtered halogen lamp (460-510 nm).

The quantum yield or efficiency for superoxide generation was determined according to the standard photometric method used previously for riboflavin where 1:1 stoichiometric conversion to the reduced cytochrome c form by superoxide is assumed. [41;46] The number of reduced molecules of cytochrome c per unit volume was calculated from the absorbance data and then divided by the number of photons absorbed by the QDs per unit volume, as described in the supporting information. The value for the QD superoxide quantum yield was found to be 0.0035. It is assumed that this value is wavelength invariant but since we used a polychromatic light source (460-510 nm range) in principle the use of the more general term 'quantum efficiency' can be applied, i.e. 0.35%.

3. Discussion

Photocatalytic generation of reactive oxygen species by nanoparticles including metal oxides, quantum dots and fullerenes is the subject of considerable interest due to potential medical applications such as antibacterial photodynamic therapy. [48;49] Phototoxicity resulting from ROS generation may also place constraints on other biomedical and diagnostic applications. QDs in particular are known to generate ROS as summarized in the introduction although there are some discrepancies between studies of CdSe/ZnS QDs, which are frequently used in biomedical studies owing to their high photoluminescence quantum yields. Another potential problem, particularly in cellular studies, is that UV radiation is often used to excite the QDs where they most strongly absorb. However interaction of UV light with endogenous chromophores can itself result in ROS generation and hydroxyl radicals can be generated by photolysis of hydrogen peroxide.[40]

In this work, the potential of photoexcited QDs to produce ROS in aqueous solution was studied using visible light and a range of complementary techniques. Previous solution phase studies on ROS generation by QDs have employed either EPR or a combination of EPR and chemical trapping agents where their fluorescence and/or absorption properties are modified through oxidation. Since some studies have shown that several ROS species such as superoxide and hydroxyl radicals may be produced by QD photoexcitation, we employed EPR using DEPMPO as the spin-trapping agent which provides specific identification of the ROS species. There have been several studies employing colorimetric or fluorogenic agents such as dichlorodihydrofluorescein derivatives but there is concern that many such agents are not sufficiently specific to the ROS species and that the same optical signal can result from oxidation by different ROS. [22;50] The ferricytochrome c assay that we employed however is specific for superoxide detection and owing to its high sensitivity we were able to use this technique for quantitative studies. The use of the oxygen consumption assay is complementary as well since this technique is able to discriminate between Type 1 processes (eg

free radical ROS) and the Type 2 mechanism (singlet oxygen) as previously demonstrated for fullerenes. [35]

Spin trapping in combination with electron paramagnetic resonance (EPR) spectroscopy demonstrated the generation of superoxide anions from photoexcited PEGylated CdSe/ZnS QDs (Figure 1). Addition of superoxide dismutase (SOD) removed the superoxide signal. No spin trap-adduct signal attributable to hydroxyl radicals was observed. These results can be explained through the reaction of photoexcited QDs with molecular oxygen which results in the generation of ROS other than $^1O_2$. In the presence of the electron donor, NADH, a significant enhancement of superoxide yield was observed. In a previous EPR study using DMPO (5, 5-dimethyl-pyrroline N-oxide) as a spin-trapping agent by Ipe et al. no ROS formation could be detected in aqueous solution including hydroxyl radical generation using a chemical trapping assay. [15] This difference from our data may simply be due to the experimental setup: the observed superoxide signal in our study was only just detectable despite using a different spin trap (DEPMPO) in combination with *in situ* illumination, which enabled prompt spectral acquisition without any delay while transferring the sample into the microwave resonator.

Measurements of oxygen consumption were also carried out in aqueous solutions with and without the addition of biomolecular substrates, NADH or tryptophan. With photo-excitation of QDs *alone*, only a small decrease in oxygen levels was observed, as shown in Figure 2. We attribute this oxygen consumption to $O_2^{-\cdot}$ formation via *electron transfer* between photoexcited QDs and ground state molecular oxygen. However the rate of oxygen consumption could be significantly enhanced in the presence of the electron donor NADH. These results are consistent with the EPR data, shown in Figure 1, where a much larger superoxide signal was detected in the presence of NADH. No significant change in oxygen consumption was observed when carried out in deuterated solution (Figure 3). Likewise the addition of azide did not change the rate of oxygen consumption (Figure

3A). The extent of dynamic or 'collisional' quenching of the QDs was minimal in the presence of NADH, and the mean QD photoluminescence lifetime was shortened from 22 to 20.8 ns at 1 mM NADH (supporting information, Table S1). In the presence of 5 mM tryptophan, which may also act as an electron donor, an increase in oxygen consumption was likewise observed although to a smaller extent than for a much lower concentration of 1 mM NADH (Figure 3B). Addition of azide did not affect oxygen consumption in deuterated solutions containing QD and tryptophan. The lack of any change in oxygen consumption in the presence of azide shows that generation of singlet oxygen is negligible.

We have previously observed that PEG encapsulated CdSe/ZnS QDs complexed with a sulfonated phthalocyanine photosensitiser can generate singlet oxygen efficiently using the near-IR phosphorescence detection technique, in agreement with other studies with different QDs and photosensitisers. [24] Using the same near-IR detection technique PEG encapsulated CdSe/ZnS QDs *alone* did not generate any detectable $^1O_2$ in aerated aqueous solution (supporting information, Figure S2) with an upper limit to the quantum yield estimated as < 0.003. This observation is also consistent with the oxygen consumption experiments (Figure. 3A, B) as discussed above. As discussed in the introduction, there have been reports of direct singlet oxygen production by QDs but our data shows that the yields must very small indeed for CdSe/ZnS QDs. In fact the sensitivity of the near-IR technique is limited by the background noise from the relatively strong QD photoluminescence tail even out to 1270 nm compared to the weak singlet oxygen phosphorescence which has a very small quantum yield. Thus this upper limit is higher than it might be without the interference from the QD emission, so we cannot completely rule out any residual singlet oxygen generation. We note that no singlet oxygen from photoexcitation of mercaptopropionic acid-capped CdSe/ZnS QDs was detectable using indirect detection based on a singlet oxygen fluorescence probe, Sensor Green.[22]

It has been proposed that dismutation of superoxide to hydrogen peroxide can also generate singlet oxygen instead of ground state oxygen alone. Our results on oxygen consumption rule out any significant contribution from this mechanism. Moreover Foote and colleagues showed many years ago in a study, which is often overlooked, that this was a very minor process with an upper limit of 0.2%; a conclusion which is supported by a more recent study. [51;52]

For quantitative measurements of the superoxide quantum yield, measurements of ferricytochrome c reduction were carried out, as shown in Figure 4. In air-saturated aqueous solutions, the production of superoxide with a quantum yield of 0.0035 was measured PEG encapsulated CdSe/ZnS QDs. In a previous study by Lu et al. cytochrome c reduction by photoexcited QDs was reported but not quantified in terms of a quantum yield. [53] The superoxide yield reported here is lower than observed for Riboflavin (0.009) but higher than for hypericin at pH 7 (4.7×10^{-4}) measured using the same technique, and also higher compared to a series of porphyrin photosensitisers where yields typically lower than 10^{-4} were measured using EPR. [45;46;54] A palladium bacteriopheophorbide photosensitiser has also been shown to generate ROS (superoxide and hydroxyl but not singlet oxygen) with an estimated yield of 0.002. [27] In a further study by the same laboratory on a palladium bacteriochlorophyll photosensitiser (WST11), which is effective for in vivo photodynamic therapy, a significant increase in oxygen consumption was observed in the presence of human serum albumin which was ascribed to a Type 1 mechanism through binding of the photosensitiser to the protein.[55]

Our results are in agreement with the conclusions of Cooper and colleagues who studied CdSe/ZnS QDs capped with mercaptopropionic acid synthesized in their laboratory, whereas we have used commercially synthesized PEG encapsulated QDs dissolved in aqueous solution. [22] In their study conjugation of the QDs with dopamine, an electron donor, increased superoxide production measured using a chemical colorimetric trapping assay. In our study the electron donor (NADH)

was admixed with the QDs. Our data using DEPMPO as spin trap with EPR spectroscopy and cytochrome c reduction which are both specific to superoxide detection therefore provide further confirmation that despite variation in structural properties between commercial and laboratory samples, superoxide formation is possible for CdSe/ZnS QDs. In addition, we have determined the quantum yield for superoxide formation which has not been attempted previously. The redox properties of CdSe and CdSe/ZnS QDs have been examined by Amelia et al. [56] Their results indicate that redox reactions are likely to be dominated by surface traps for electrons and holes.

Based on our results a possible mechanism for the interaction of photoexcited QDs with oxygen and the electron donor, NADH, (or NADPH) is now considered by analogy with known mechanisms for conventional organic photosensitisers through the following reactions (Equations 2-5) [57-59] :

$$QD^{*} + NADH \longrightarrow QD^{e-} + NAD^{\bullet} + H^{+} \quad (2)$$

$$QD^{e-} + O_2 \longrightarrow QD + O_2^{-\bullet} \quad (3)$$

$$2O_2^{-\bullet} + 2H^{+} \longrightarrow H_2O_2 + O_2 \quad (4)$$

$$NAD^{\bullet} + O_2 \longrightarrow NAD^{+} + O_2^{-\bullet} \quad (5)$$

Photoexcitation of the QD produces a long-lived metastable excited ‘dark’ state denoted by QD^{*} which is then reduced by electron transfer from NADH (Equation 2). The anionic QDs bearing the donated electron (denoted as $QD^{\bullet -}$) may subsequently be reoxidised by reaction with molecular

oxygen, forming the superoxide anion (Equation 3). The latter undergoes *dismutation*, with two superoxide anions combining to form hydrogen peroxide (H_2O_2) and molecular oxygen (Equation 4) which results in net depletion of dissolved molecular oxygen. The $NAD^{\cdot}$ radicals formed through Equation 2 are strongly reducing species and can reduce molecular oxygen, yielding NAD^+ and more superoxide anion (Equation 5). However there will be mechanistic differences between the PEG encapsulated QDs and organic dyes. For example, in the case of Rose Bengal triplet-triplet state annihilation and charge recombination processes can occur, and $NAD^{\cdot}$ radicals can reduce the dye to form the semireduced dye intermediate which can generate more superoxide.

The catalytic chain of reactions (Equations 2-5) amplifies the rate of oxygen consumption and explains why much higher oxygen consumption and EPR signals are observed in the presence of NADH. This series of reactions is analogous to that proposed for Rose Bengal and metalloporphyrin photosensitisers where the photoexcited triplet state can accept an electron from NADH to generate the semireduced Rose Bengal radical anion which can then interact with oxygen to form superoxide anions and subsequently hydrogen peroxide. [57-59] These reactions all involve electron transfer (Type 1) rather than energy transfer (Type 2), emphasizing the importance of electron donating molecules in ROS radical formation. [58]

The much higher rate of oxygen consumption observed using NADH at 1 mM (Figure 3A) compared to tryptophan at 5 mM (Figure 3B) is consistent with the strong electron-donating properties of NADH. Lambert et al. observed that the radical ion yield from photo-reduction of Rose Bengal was twenty times lower in the presence of tryptophan compared to NADH. [57]

A critical assumption is that NADH can interact with the QD by penetrating the PEGylated layer encapsulating the QD since electron transfer can only take place over very short distances. Since the NADH concentration is high (1 mM) compared with the 50 nM QD concentrations employed, it is

reasonable to assume that NADH can be present within the PEGylated layer at sufficient concentrations to participate in electron-transfer interactions with the QD surface.

With the addition of a phthalocyanine photosensitiser to form a complex with the QDs, the rate of oxygen consumption is further increased through generation of singlet oxygen via FRET. [24] The oxygen consumption was inhibited upon the addition of azide (Figure 3B). The oxygen consumption is attributed to the chemical reaction of singlet oxygen with tryptophan. Since the quantum yield of singlet oxygen generation at 0.15 is much higher (supporting info, Figure S2) than for superoxide the rate of oxygen consumption is correspondingly higher.

The mechanistic studies of QD photosensitized ROS properties documented here can be compared with studies of the photochemical properties of fullerene nanoparticles, where a Type 1 mechanism was observed in the presence of electron donors, including NADH. [35] The nature of electronic states involved are different since QDs are inorganic nanoparticles whereas fullerenes are organic, but close parallels may be drawn in the mechanisms involved for producing ROS in solution by analogy with several organic photosensitisers including Rose Bengal. However unlike QDs, fullerenes are capable of generating significant amounts of singlet oxygen directly via the Type 2 mechanism.

4. Conclusions

In this work we identify the nature of the ROS initially produced by non-photolytic visible light excitation of PEG-encapsulated CdSe/ZnS QDs in aerated aqueous solution as the superoxide radical anion. PEGylated CdSe/ZnS QDs are widely used in biomedical applications for in vitro labeling and in vivo since PEGylation confers water solubility and minimizes uptake by organs of the reticuloendothelial system. The potential of CdSe/ZnS QDs to participate in Type 1

photochemical processes to generate superoxide was shown by oxygen consumption, EPR spectroscopy in combination with spin trapping and cytochrome c reduction. All three methods confirmed the formation of superoxide anion radicals in the presence and absence of NADH and excluded the formation of $^1O_2$ as a precursor for $O_2^{-\cdot}$. Although various types of QDs have been used to examine their potential for ROS formation, no previous study has carried out quantitative studies of superoxide quantum yields, but herein we have determined the yield using the cytochrome c reduction assay. Direct formation of singlet oxygen from photoexcited QDs was not observed. Significantly enhanced levels of superoxide formation were observed in the presence of the electron donor, NADH. Since the techniques we used are all designed for solution phase studies we can only infer the relevance to in vivo ROS generation. However the concentration of NADH employed in these experiments, c. 1 mM, is comparable to cellular levels of NAD(P)H therefore it is possible that Type 1 photochemistry involving PEG-encapsulated CdSe/ZnS QDs can be driven catalytically by intracellular NADH. The same process has been proposed to occur with organic dye photosensitisers thereby enhancing their phototoxicity in cells.[35;55;58] Hitherto it has been assumed that the quantum efficiency of ROS generation in cells will be too low but this mechanism involving NAD(P)H and other cellular reductants may lead to a reassessment of the role of QD cellular phototoxicity, especially when surface ligands such as cell-penetrating peptides are employed to enhance cellular uptake. PEGylation is believed to counteract cadmium toxicity although in the long-term with slow cadmium leaching this may not be the case. [60] Moreover from our results it appears that photoexcited QDs will still be able to generate reactive oxygen species despite the presence of PEG encapsulation.

5. Experimental Section

Materials: Amine-functionalized PEG encapsulated red-emitting CdSe/ZnS QDs (emission peak: 625 nm) were purchased from eBioscience Inc., US. The diameter of the QD core itself was < 10

nm but with the PEG coating the resulting hydrodynamic diameter was ~25 nm, according to the specifications provided by eBioscience. USA. L-Tryptophan, NADH (nicotinamide adenine dinucleotide), SOD (superoxide dismutase from bovine erythrocytes), Cytochrome c, PBS (0.01M Phosphate Buffered Saline), NaN_3 (sodium azide), D_2O (deuterated water) were obtained from Sigma Aldrich Ltd., UK. DEPMPO (5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide) was obtained from Calbiochem Inc., US. Aluminum disulfonated phthalocyanine ($AlPcS_{2a}$) was obtained from Frontier Scientific Inc.,US.

EPR Spectroscopy: Aqueous aerated solutions of CdSe/ZnS QDs (5 μM) were prepared in pH 7.4 0.01 M PBS with 50 mM DEPMPO (5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide) as spin trapping agent. In separate experiments, 1 mM NADH and 50 U/mL SOD were added. The solutions were transferred into small capillaries (micropipettes, Blaubrand ® intraMark, Brand GmbH + Co KG) and irradiated *in situ* in the EPR resonator using a light-emitting diode (LED) lamp with peak emission at 455 nm (M455L2, Thorlabs Inc) coupled with a liquid light guide to the sample in the cavity to provide direct *in situ* illumination. EPR measurements were carried out using a Bruker EMX plus spectrometer operating at X-band frequencies (9 GHz), equipped with a rectangular TE102 (Bruker ER 4104OR) cavity. All spectra were acquired using the following parameters: 20 mW microwave power, 0.15 mT modulation amplitude, and 100 kHz modulation frequency. The individual radical components of the spectra were identified by determination of the hyperfine coupling constants (hfc) arising from the interaction of the unpaired electron with the nuclei of ^{1}H, ^{14}N and ^{31}P from DEPMPO based on simulations using the Easyspin software. [61] No background signals were detected in the solutions of QD, DEPMPO and NADH in the dark. All solutions were air-saturated and experiments were carried out at 298 K as for all other experiments.

Oxygen Consumption Measurements: Air-saturated aqueous solutions containing CdSe/ZnS QDs (50 nM) were prepared in a quartz cuvette, stirred continuously and illuminated with a collimated

halogen lamp (Schott KL 1500 LCD) equipped with a bandpass filter with peak emission at 485 nm (480DF30, Omega Optical Inc.). NADH (0.5, 1 mM) or L-Tryptophan (5 mM) was added to the QD solutions to determine their effects on oxygen consumption. The lamp spectrum is shown in the supporting information, Figure S3. The top of the cuvette was sealed to prevent reoxygenation of the solutions. The changes in oxygen levels were recorded at various time intervals using a fibre-optic dissolved oxygen probe (Oxford Optronix, Oxford, UK), which was inserted into the centre of the cuvette through the cuvette seal. The sensing tip incorporates an oxygen-sensitive luminescent probe within an oxygen permeable matrix and exhibits a highly stable calibration unlike electrochemical probes for dissolved oxygen. [31] The probe recorded the oxygen level in the solutions as a function of the illumination time and data were recorded using an OxyLab pO2E^{TM} system (Oxford Optronix, Oxford, UK), coupled to an A/D converter (12 bit). The results were displayed on a PC computer using Labview software, and recorded as partial pressure values, *ie*, pO_2 in mmHg. NaN_3 (5 mM) for use as an $^1O_2$ scavenger was added to the above-described solutions and its effect on oxygen consumption rate was assessed. For the studies in the presence of a phthalocyanine photosensitiser (PS) aqueous deuterated solutions of samples containing QD-PS complexes (CdSe/ZnS QDs: 50 nM PS : 500 nM) were prepared and L-Tryptophan (L-Trp) with a final concentration of 5 mM was added to the solutions.[24]

Cytochrome c Reduction Measurements: The superoxide detection agent, cytochrome c (15 μM in PBS) was added to air-equilibrated aqueous solutions of CdSe/ZnS QDs (80 nM) in pH 7.4 0.01 M PBS. Samples were transferred into a 1 cm pathlength quartz cuvette for absorption spectrometry. Prior to illumination, the baseline absorbance of the cytochrome c at 550 nm was recorded, using a Perkin Elmer Lambda 25 UV/Vis spectrometer (Perkin-Elmer, UK). The solutions were illuminated with light for up to 20 minutes and the increase in absorbance at 550 nm of the irradiated solutions indicating the $O_2^{-\cdot}$ formation was monitored at 5 minutes intervals. The superoxide anion

scavenger, superoxide dismutase (SOD, 50 U/mL), was added to the solutions prior to illumination. The spectrum of each sample was monitored as a function of irradiation time. Control experiments were performed to check for any dark reactions. Samples were prepared and kept in the dark for over 30 minutes with a spectrum being recorded subsequently. An additional control experiment was carried out with only cytochrome c to exclude the autooxidation of cytochrome c upon light illumination. Exposure of the all samples to visible light irradiation was carried out, using a collimated halogen lamp (Schott, KL 1500 LCD), equipped with a bandpass filter with peak emission at 480 nm (480DF30, Omega Optical Inc.) as for the oxygen consumption measurements.

Acknowledgements

Elnaz Yaghini is supported by the Biotechnology and Biological Science Research Council. Katharina Pirker was supported by the FWF Austrian Science Fund (Erwin Schrödinger Fellowship J2959-N17 for K.F.P.).

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