Electronic Supplementary Information for

Quantum Dots Decorated with Pathogen Associated Molecular Patterns as Fluorescent Synthetic Pathogen Models

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Materials and Methods

Materials. All chemicals were obtained from commercial sources and used as received. *E. coli* LPS Kdo_2-lipid A was obtained from Avanti Polar Lipids (catalogue number: 699500). This material is a mixture of 6 LPSs from *E. coli* strain WBB06. Over 91% is Kdo_2-lipid A. Polymyxin B was purchased from Sigma. 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethylene glycol)-2000) (mPEG2000 PE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC) were purchased from Avanti Polar Lipids. (cat. no. 881601P and. 850457P respectively). Hoechst 33342 and DiOC18 were purchased from Biotium.

QD Synthesis

Hydrophobic core-shell CdSe-ZnS QDs were synthesized according to a published procedure.[1] QDs were characterised by HRTEM, EDS, UV-vis and fluorescence spectroscopy, NMR, FT-IR and ICP-OES.

The CdSe-ZnS QDs used in this study had an average diameter of 5.8 nm (based on HRTEM and position of the first excitonic peak (628 nm)[2]). The number of Cd, Se, Zn and S atoms per QD was calculated using the bulk densities and formula weights of CdSe and ZnS, and the volumes of 4.4 nm (CdSe core diameter) and 5.8 nm (total diameter) spherical nanoparticles, and was confirmed experimentally by ICP-OES: ~ 770 Cd and Se atoms, and ~ 1400 Zn and S atoms.

Synthesis of QD-LPS micelles

Hydrophobic QDs (2.5 mg) were combined with pure Kdo_2-lipid A (250 μL, 430 μM) and sonicated for 2 minutes. LPS-functionalized nanoparticles form a suspension after sonication. The suspension was kept for 4 h at room temperature, and contained both empty micelles and those containing QDs. The empty micelles were removed with centrifugation cycles (5 × 60 min) at 11,000 g. The QDs containing micelles formed a pellet while the empty micelles stayed suspended. The successive supernatants were discarded and the final QD-micelles were resuspended in 1 mL of water. The QD-LPS used in the *in vivo* studies were prepared as above but using 4.5 mg of QD and 250 μL of 43 μM LPS. The QD concentration was calculated by ICP-OES.

Synthesis of QD-PC/PEG-PE micelles

QD-PC/PEG-PE micelles were prepared according to a recently published procedure.[3] In a typical synthesis, in a 5 mL round-bottomed flask, 100 μL of hydrophobic QD (5 μM in chloroform) and 50 μL of chloroform solutions containing mPEG(2000) (2 mg, 0.72 μmol) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC) (1 mg, 1.3 μmol) were mixed together. The flask was left open at room temperature for 3 h in a fume hood to slowly evaporate the chloroform solvent. Any remaining chloroform was removed under vacuum using a rotary evaporator to form a thin film. The flask containing the QDs was then heated in a water bath set to 80 °C for 30 s, after which 250 μL of water was quickly added. This affords QD-PC/PEG-PE micelles and empty micelles. Large aggregates were removed by two centrifugation cycles at 11,000 g and empty micelles were removed by centrifugation cycles (2 × 45 min) at 300,000 g. The micelles containing QDs formed a pellet while the empty micelles stayed suspended. The successive supernatants were discarded and the final QD-PC/PEG-PE micelles were resuspended in 1 mL of water.

Quantification of LPS loading on QD

The number of LPS molecules per QD micelle was quantified using the zinc dipicolylamine (Zn-DPA) affinity ligand (**1**, Fig S4) originally developed by Hamachi et al.[4] for fluorescence detection of phosphorylated peptides and proteins. This anthracene-derived bis(Zn^{2+}–DPA) complex, and related Zn-DPA complexes have

been found to selectively target LPS-modified surfaces[5] and the surface of bacterial cells via recognition of the anionic phosphate esters within the lipid A component of the bacterial lipopolysaccharides.[6]

QD-LPS micelles were treated with increasing concentrations of **1** in HEPES (20 mM, pH 7.5). As expected, binding to the phosphate groups of Kdo_2-lipid A caused an increased in the fluorescence emission of **1** (Fig S4). The QD-LPS-**1** complexes were separated from the unbound molecules of **1** by passing these solutions through a Nanosep 100k Omega membrane filter (low protein-binding, modified polyethersulfone on polyethylene substrate). These solutions were then treated with EDTA (15 mM) to release the anthracene-$(DPA)_2$ ligand (Filtrate 1 in Fig. S5). The retenates containing the QD-LPS-bound molecules of **1** were also treated with EDTA to release the anthracene-$(DPA)_2$ ligand from the QD-LPS micelles, and then passed through a Nanosep 100k Omega membrane filter (Filtrate 2 in Fig. S5). Both filtrate fractions were analysed by UV-vis and the concentration of anthracene-$(DPA)_2$ was calculated from the extinction coefficient of **1** in the presence of EDTA (15 mM) at 260 nm (ε = 32,500 L • mol^{-1} • cm^{-1}) in HEPES (20 mM, pH 7.5). Each QD-LPS captured ca. 130 molecules of **1**. Because each LPS molecule carries two di-anionic phosphate ester groups, the number of LPS molecules per QD should be half (65 molecule of LPS per QD).

Cell culture

The J774A.1 macrophage/monocyte cell line was obtained from the European Collection of Cell Cultures (ECACC). Cell cultures were grown at 37 °C in a humidified atmosphere of 5% CO_2 in 30 mL of complete medium (Isocove's Modified Dulbecco's Modified Medium (IMDM) , containing phenol red, L-Glutamine (2 mM), Penicllin/Streptomycin (50 U/mL) (Pen/strep) and 5 % Foetal Calf Serum (FCS)). Cells were grown to confluence after which they were removed from the culture flask via gentle scraping and then resuspended in complete medium.

Cell viability was determined using Trypan blue (0.1% solution) exclusion. Cells were adjusted to a suspension of 2.5×10^5 cells mL^{-1} in medium and used for the subsequent experiments.

Preparation of Bone marrow-derived Dendritic cells (BMDCs)

C57BL/6 mice at 6 weeks of age were bred at The University of Edinburgh animal facility. All experiments were conducted according to the Guidelines for Animal Experimentation of Edinburgh University. All animals were maintained under specific pathogen-free conditions and routinely checked by the Edinburgh University Animal Resource Centre staff. Bone marrow cells were cultured in RPMI 1640 medium, containing phenol red, L-Glutamine (2 mM), Pen/Strep (50 U/mL) , 10 % FCS and 20 ng /mL of Granulocyte-monocytes colony-stimulating factor (GM-CSF). The DC culture medium was replaced every 2 days to replenish GM-CSF and remove non-adherent cells. Cells were harvested by gentle aspiration on day 7 and then were used as immature DC for *in vitro* assays.

IN-VITRO STUDIES

Cytokine Assay

In a 96 well-plate, 100 µl of BMDCs or J744 Macrophages in IMDM medium (2.5×10^5 cells mL^{-1}, 25,000 cells per well), 50 µL of IMDM medium and 50 µL of the QD solutions (15 nM), LPS (250 nM) or PBS alone were incubated for 16 h (macrophages) or 24 h (BMDCs) at 37 °C in a humidified atmosphere of 5% CO_2. Cultures were performed in triplicate in a final volume of 200 µL (100 µL cells, 50 µL compound and 50 µL PBS). An additional group of cultures were set up with the LPS inhibitor, polymyxin B (final concentration 250 µM) instead of PBS. The same studies were simultaneously carried out with ten-fold serially diluted samples (1/100, 1/1000, 1/10000) After 24 h, supernatants were harvested by centrifuging plates at 400g for 3 minutes and gentle removing medium from cells. Supernatants were then frozen at -20°C until used. Cytokine (IL-6) was quantified by capture ELISA. Plates were coated overnight with 50 µL (10 µg/mL) IL-6 capture antibody (clone MP5-20FS, BD Pharmingen, UK). The following morning plates were washed with PBS/Tween 20 (0.05%) (Sigma Aldrich) and non-specific binding blocked with 2% bovine serum albumin (BSA). Samples (and recombinant cytokine for standardisation at 20 ng/mL to 9.8 pg/mL) were then applied to the plate. Following the 1 h incubation at RT, plates were washed three times with PBS/Tween and detection antibody (biotin labelled MP5-32C11, BD Pharmingen) added. Following further 1 h

incubation at RT and washing, streptavidin-alkaline phosphatase was added to the plates. Plates were again incubated and washed and then 50 μL (1.35×10^{-4} M) PNPP substrate added to the plate. Optical density at 405 nm was measured and cytokine concentration determined by extrapolation from the recombinant cytokine standard curve.

Live cell imaging. J774 Macrophages were seeded overnight (37 °C, 5% CO_2) onto the surface of Ibidi microslides (Ibidi GmbH, Munich) at a concentration of $1x10^6$ cells per ml in phenol red-free medium (RPMI 1640 with L-G, P/S, 5% FCS and without phenol red). The following morning, cells were stained with the nuclear dye, Hoechst 33342 (Biotium), at a concentration of 5 μg/mL in phenol red-free RPMI-1640 for 30 min at 37 ^{0}C in a final volume of 150 μL. Cells were then washed 3 times with phenol red-free medium and the cell membrane stained with DiOC18 (Biotium). DiOC18 staining was carried out for 45 min at 37 ^{0}C, at a concentration of 20 μg/mL. Cells were again washed 3 times with phenol red-free medium and QD micelles in medium were added immediately prior to confocal analysis. All cell staining steps were carried out in the dark, in a humidified atmosphere, at 37 ^{0}C, 5% CO_2.
Macrophage/QD interactions were imaged in the xyz dimensions using a Leica (Leica Microsystems UK Ltd, Milton Keynes MK5 8LB) TCS SP5 laser scanning confocal microscope. From these images a 3D reconstruction was produced using Image Pro 3D Constructor version 7.0 (Media Cybernetics UK, Bucks, SL7 1HA) and the degree of QD internalisation was assessed visually.

Binding studies (FACS)

Solutions of QD-LPS were mixed with IMDM medium to give QD concentrations of 60 nM (for QD-LPS) or 90 nM (for control QD micelles) (1/10 dilution). These solutions (50 μL) were mixed with IMDM (450 μL) medium to give 1/100 solutions.
In a 24 well-plate, 450 μL of J744 macrophages in IMDM medium (4×10^5 cells mL^{-1}, 180,000 cells per well), and 50 μL of the above QD solutions were incubated for 3 h at 37 °C in a humidified atmosphere of 5% CO_2. Cultures were performed in triplicate in a final volume of 500 μL (450 μL of cells, 50 μL of compound).

After 3 h, the cells were harvested by gentle scraping and 500 µL of the cell suspension was transferred into tubes. The cells were washed 3 times by centrifugation (400 g for 3 min) and decantation in 200 µL FACS buffer (PBS +2% FCS, 0.1 % sodium azide).

Cell pellets were resuspended in 100 µL of FACS buffer and 100 µL of paraformaldehyde (2%), and incubated for 20 minutes on ice.

After the incubation the cells were washed and resuspended in 200 µl of FACS buffer. The binding abilities of QDs by macrophages were analyzed by flow cytometry using a BD FACScalibur flow cytometer (BD Biosciences). Data was analysed using FlowJo software (TreeStar Inc., Portland USA)

IN VIVO STUDIES

Animal vaccination

C57BL/6 mice at 6-8 weeks of age were bred at The University of Edinburgh animal facility. All experiments were conducted by trained personnel under a Project Licence granted by the UK Home Office under the Animal (Scientific Procedures) Act 1986. This licence was approved locally by the University of Edinburgh's Ethical Review Committee.. All animals were maintained under specific pathogen-free conditions and routinely checked by the Edinburgh University Animal Resource Centre staff.

For intraperitoneal (i.p) vaccination with the DNP-OVA antigen, four groups of animals (n = 5 per group) were injected into the peritoneal cavity with:

- a 200 µL solution containing a single dose of 100 µg of DNP-OVA (20 µL, 5 mg/mL) co-administrated with QD-LPS nanoparticles (40 µL, 235 nM, 95 pmol) in PBS (140 µL);
- a 200 µL solution containing a single dose of 100 µg of DNP-OVA (20 µL, 5 mg/mL) co-administrated with LPS (40 µL, 43 µM, 4 µg) in PBS (140 µL);
- a 200 µL solution containing a single dose of 100 µg of DNP-OVA (20 µL, 5 mg/mL) co-administrated with 180 µL PBS.

For subcutaneous (s.c.) vaccinations with the DNP-OVA antigen, animals (n = 5) received a single dose of 100 µg of DNP-OVA antigen (20 µL, 5 mg/mL), co-administrated with *E. coli* LPS (10 µL, 43 µM, 10 µg) and 70µl PBS, emulsified with 100 µL of incomplete Freund's Adjuvant (IFA), with 100 µL injected in each flank. Statistical analysis was performed by using GraphPad, Prism, version 4.0b.

Analysis of serum DNP-OVA antigen-specific antibodies

Blood was collected via the dorsal tail vein at 7 day intervals. Samples were allowed to coagulate by overnight incubation at 4 ^{o}C . Serum was separated from coagulated blood by centrifugation at 13,000 g for 3 min and stored at −20 ^{o}C for later analysis. Antigen-specific IgG titers were analyzed by ELISA.

Plates were coated with 50 μL DNP-BSA antigen (10 μg/mL) per well at 4 ^{o}C overnight. The following morning the plates were washed with PBS/Tween and blocked using PBS with 2% BSA (100 μL). Serum was diluted (1:10) in the blocking buffer and added to the plates. After 2 h incubation at room temperature, plates were again washed and incubated with 50 μL of detection antibodies (Polyclonal Goat-mouse IgG streptavidin-alkaline phosphatase conjugate, 1:2500 dilution, anti-mouse IgG1 alkaline phosphatase conjugate, 1:2000 dilution and IgG2c alkaline phosphatase conjugate, 1:1000 dilution) for 2 h. Following the incubation 50 μL (1.35 x 10^{-4} M) p-nitrophenyl phosphate (PNPP) substrate was added and the plates were read at 405 nm. End-point antibody titer was determined by extrapolating the dilution which gave an OD of 0.2 for the titration curve.

Fluorescence studies. Measurements were made with an Edinburgh instrument FS900 fluorimeter. Excitation was at 395 nm with bandwidths of 2 nm for excitation (unpolarised) and emission (unpolarised). Temperature was maintained at 25°C.

Transmission electron microscopy. High-resolution transmission electron microscopy (HRTEM) and energy-dispersive X-ray spectroscopy (EDS) studies were conducted on a JEOL JEM-2011 electron microscope operating at 200 kV. The samples were prepared by depositing a drop of a solution of nanocrystals onto a copper specimen grid coated with a holey carbon film and allowing it to dry.

References:

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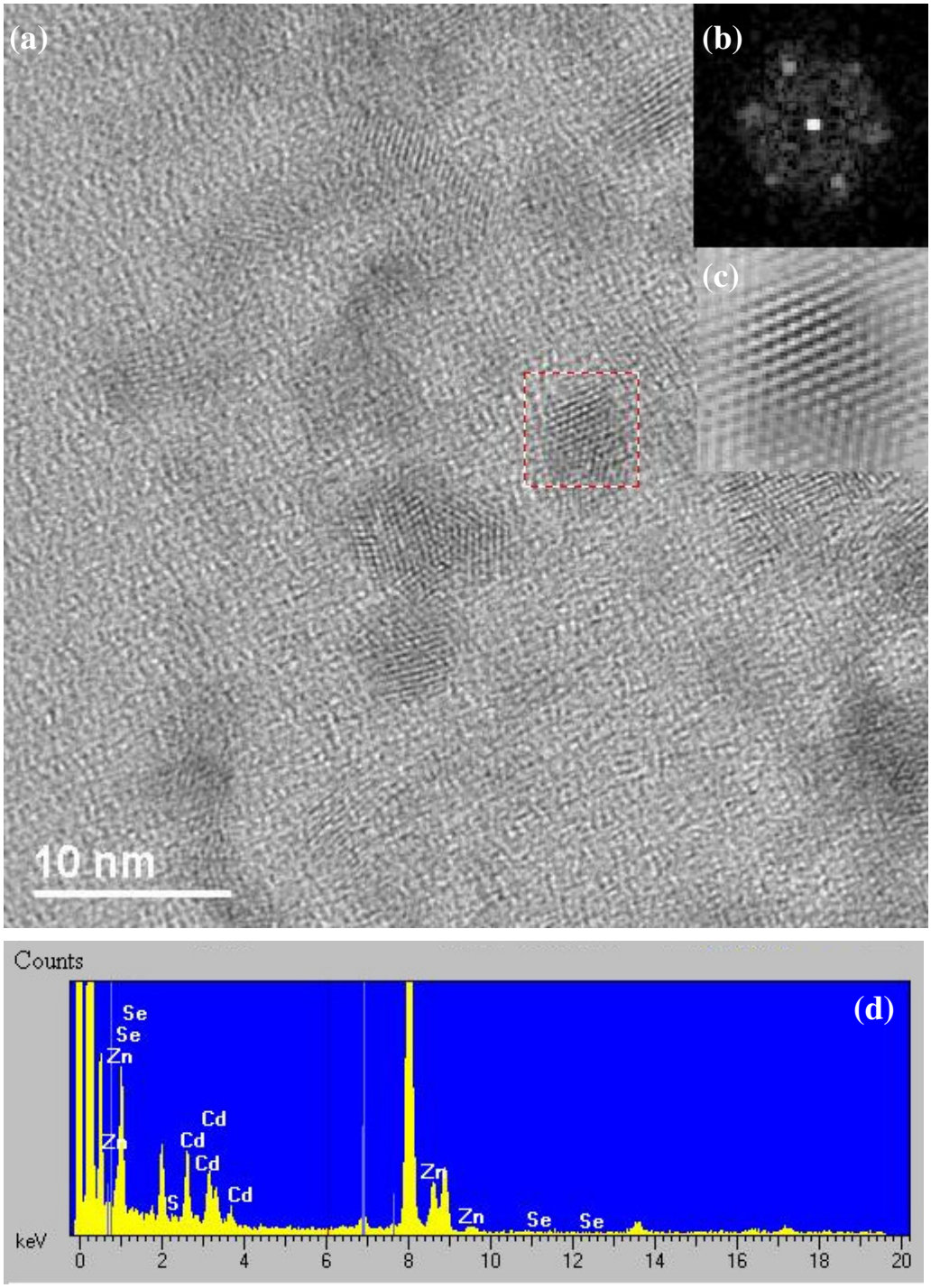

Fig. S1. High-resolution transmission electron microscopy (HRTEM) image of several CdSe-ZnS QDs (a), fast Fourier transform (FFT) of the QD inside the box (5.72 × 5.72 nm) (b) and corresponding inverse FFT (IFFT) image, (c) EDX spectra of these QDs (d).

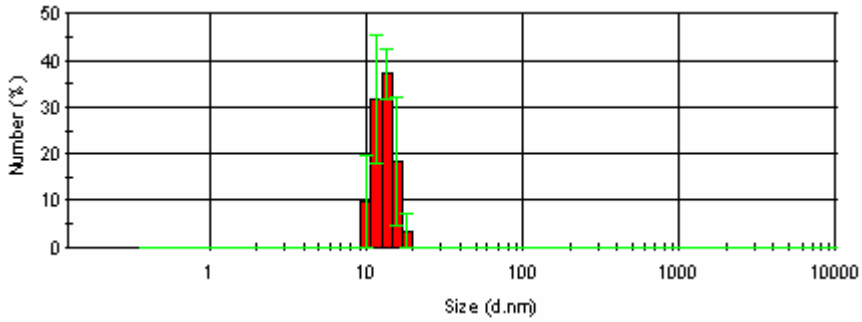

Fig. S2. Size distribution (dynamic light scattering) of QD-LPS (LPS = Kdo_2-lipid A)

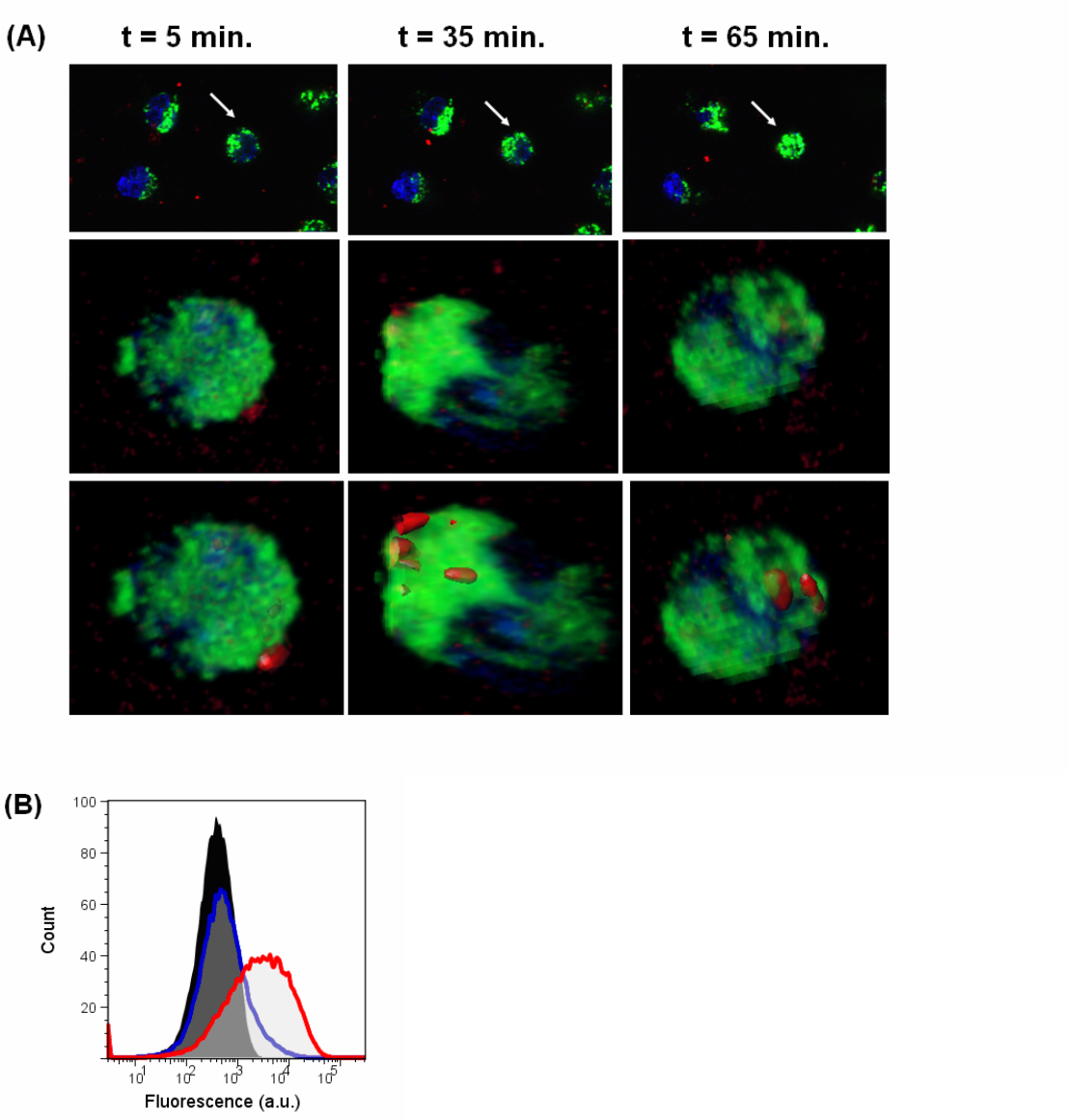

Fig S3. (A) Confocal images of J774 macrophages after staining with Hoechst 33342 (blue, nuclei) and DiOC18 (green, membrane) at 5 min, 35 min and 65 min after incubation with 20 nM QD micelles (red) containing a mixture of phosphatidylcholine (PC) and n-poly(ethyleneglycol) phosphatidylethanolamine (PEG-PE) and the 3D reconstituted images of the cell marked with an arrow. (B) Flow cytometry analysis of macrophages before (black) and after incubation with QD-PC/PEG-PE (0.9 nM, red line; 90 pM, blue line)

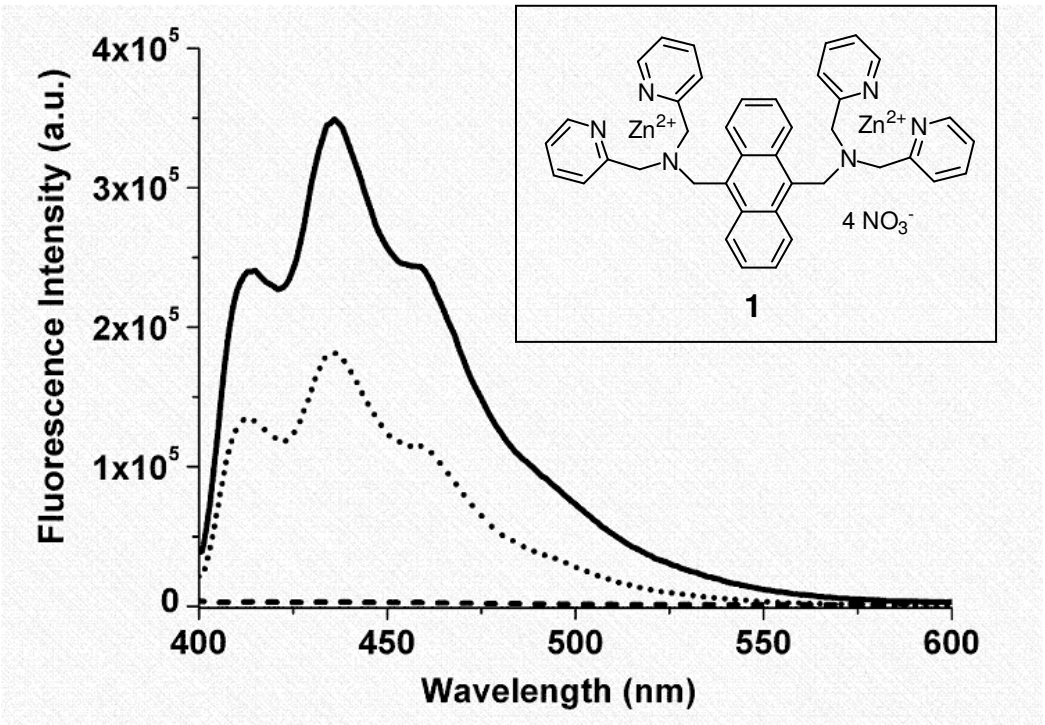

Fig. S4. Fluorescence spectra of QD-LPS (50 nM) alone (dashed line), **1** (6.5 μM) alone (dotted line) and **1** (6.5 μM) in the presence of QD-LPS (50 nM) in HEPES buffer (20 mM, pH 7.5). Excitation wavelength = 380 nm. The inset shows the structure of **1**.

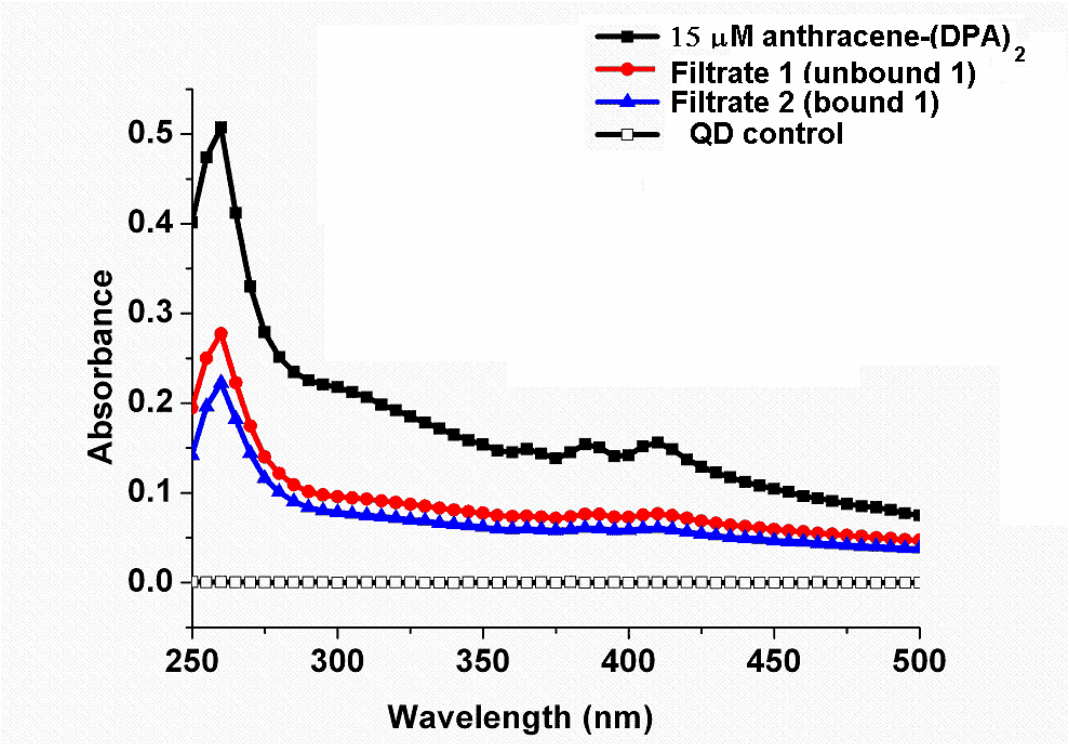

Fig S5. UV-vis spectra of **1** in HEPES (20 nM, pH 7.5) after addition of EDTA (15 mM) to release anthracene-$(DPA)_2$ from **1** (closed squares; 15 μM) and mixtures containing **1** (15 μM) + QD-LPS (50 nM). The molecules of **1** which did not bind to QD-LPS are in filtrate 1 (solid circles; 8.5 μM) and those which did bind to QD-LPS in filtrate 2 (solid triangles; 6.5 μM). The control QD micelles (coated with PEG-PE or PC/PEG-PE mixtures) did not bind **1** (open squares).