Supporting Information

A Facile Dye-Initiated Polymerisation of Lactide-Glycolide Generates Highly Fluorescent PLGA for Enhanced Characterisation of Cellular Delivery

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EXPERIMENTAL

MATERIALS

Lactide (LA), glycolide (GA) 99%, 5-aminofluorescein (AF), rhodamine 6G (R6G), 2-aminoanthracene (AA), tin(II) 2-ethylhexanoate ($Sn(Oct)_2$) 92.5-100.0%, deuterated chloroform ($CDCl_3$), deuterated dimethyl sulfoxide (DMSO-d6), Roswell Park Memorial Institute (RPMI) 1640 medium, heat-inactivated fetal bovine serum (FBS), penicillin, streptomycin, L-glutamine, phorbol 12-myristate 13-acetate, paraformaldehyde and Resomer® RG 502, poly(D,L-lactide-co-glycolide) lactide:glycolide 50:50, ester terminated (7,000-17,000 MW) were purchased from Sigma-Aldrich (Gillingham, UK). Ammonium carbonate was purchased from Fluka (Bucharest, Romania). Alexa Fluor™ 488 Phalloidin (AF488) and Alexa Fluor™ 647 Phalloidin (AF647) were purchased from Thermo Fisher Scientific (Loughborough, UK). Dichloromethane (DCM), methanol, diethyl ether and acetone were HPLC grade provided by Fisher Scientific (Loughborough, UK). All the materials and solvents were used as received unless stated otherwise.

METHODS

Polymer synthesis - PLGA-2-aminoanthracene (PLGA-2AA, blue), PLGA-5-aminofluorescein (PLGA-5AF, green) and PLGA-rhodamine-6G (PLGA-R6G, red) were synthesised by ROP of LA and GA as shown in Figure 1A, where 2-aminoanthracene, 5-aminofluorescein and rhodamine-6G and were used as initiators.

2-Aminoanthracence labelled PLGA (PLGA-AA) - The synthesis was carried out by melting LA (30 mmol, 4.323 g, 30 eq), GA (20 mmol, 2.32 g, 20 eq) and 2-aminoanthracence (1 mmol, 0.193 g, 1eq) at 130°C under a nitrogen atmosphere. Sn $(Oct)_2$ (0.5% wt/wt) was added and the reaction continued for 3 hours. Hereafter, the reaction mixture was cooled to room temperature. The synthesised co-polymer was dissolved in acetone and precipitated in methanol four times to remove remaining unreacted monomers, free dye and short polymer chains. The filtered precipitate was dried under reduced pressure to produce a grey solid (4 g, 60% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.4-8.5 (ArC-*H*, 9H), 5.25 (m, 1H, OCO-*CH*(CH_3)-COO), 4.91 (m, 2H OCO-*CH_2*-COO), 1.61 (q, 3H, OCO-CH(C*H_3*)-COO).

5-Aminofluorescein labelled PLGA (PLGA-5AF) - The synthesis was carried out by melting LA (30 mmol, 4.323 g, 30 eq), GA (20 mmol, 2.32 g, 20 eq) and 5-aminofluorescein (1 mmol, 0.347 g, 1eq) at 130°C under a nitrogen atmosphere. $Sn(Oct)_2$ (0.5% wt/wt) was added and the reaction was proceeded for 3 hours after which, the reaction mixture was cooled to room temperature. The synthesised co-polymer was dissolved in acetone and precipitated in cold methanol four times to remove remaining unreacted monomers, free dye and short polymer chains. The filtered precipitate was dried under reduced pressure to generate of a deep orange product (4.3 g, 64% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.5-7.25 (ArC-*H*, 9H), 5.25 (m, 1H, OCO-*CH*(CH_3)-COO), 4.91 (m, 2H OCO-*CH_2*-COO), 1.61 (q, 3H, OCO-CH(C*H_3*)-COO).

Rhodamine 6 G labelled PLGA (PLGA-R6G) - The synthesis was carried out by melting LA (30 mmol, 4.323 g, 30 eq), GA (20 mmol, 2.32 g, 20 eq) and rhodamine-6G (1 mmol, 0.479 g, 1eq) at 130°C under a nitrogen atmosphere. $Sn(Oct)_2$ (0.5% wt/wt) was added and the reaction continued for 3 hours after which, the reaction mixture was cooled to room temperature. The synthesised co-polymer was dissolved in acetone and precipitated in methanol four times to remove the unreacted monomers, free dye and short polymer chains. The filtered precipitate was dried under reduced pressure to generate of a light red product (1.7 g, 26% yield).

^{1}H NMR (400 MHz, DMSO-d6) δ (m, 1H, OCO-*CH*(CH_3)-COO), 4.91 (m, 2H OCO-*CH_2*-COO), 1.61 (q, 3H, OCO-CH(C*H_3*)-COO), 1.3 (q, 3H, CH-C(C*H_3*)-C).

NPs formation - Green and blue fluorescent PLGA NPs were prepared using PLGA-AF and PLGA-AA respectively by nanoprecipitation [24]. Briefly, a solution of each labelled PLGA (25 mg) in 10 ml of acetone was added to 10 ml of the anti-solvent (Milli-Q deionised water, 18.2 mΩ,) using a syringe pump (0.7 mL/min). The mixture was stirred at room temperature overnight, to ensure the complete evaporation of acetone. Whereas, PLGA-R6G NPs were prepared using a solution of PLGA-R6G (25 mg) in 10 ml of DMSO, which was added to 10 ml of the anti-solvent (deionised water) using a syringe pump (0.7 mL/min). DMSO was removed with dialysis against 1 L of deionised water for 24 hrs. The NPs suspensions were filtered using 0.45 µm syringe filters to remove any aggregates and maintain system sterility.

Nanoparticles loaded with 5-aminofluorescein - A solution of PLGA (25 mg) and AF (2mg) in 10 ml of acetone was added to 10 ml of the anti-solvent (Milli-Q water) using a syringe connected to a pump (rate of addition 0.7 mL/min). The mixture was stirred at room temperature, overnight, to ensure the complete evaporation of acetone.

Nanoparticles loaded with doxorubicin – Nanoparticles were synthesised using a double emulsion–solvent evaporation method. Solutions of PLGA-AF (1 mL, 10 mg/ml, DCM) and doxorubicin (100 µl, 10mg/ml, DI water) were prepared. The aqueous doxorubicin (0.1 mL) phase was added to the organic PLGA-AF phase (1 mL) and sonicated for (1 min, 3W, Sonicator 3000, Misonix, USA) by a microtip probe to produce an emulsion. This emulsion was added to a PVA solution (0.5% w/v, deionised water) and sonicated (5 min, 3 W). The organic solvent was eliminated *via* mechanical stirring (4 hrs) and purified using PD10 columns. The production process yield was evaluated by weighing the solid residue after freeze-drying known volumes of nanoparticle suspension.

Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy - ^{1}*H*-NMR spectroscopy was performed at 400 MHz using a Brüker DPX400 Ultrashield spectrometer and deuterated chloroform ($CDCl_3$) as a solvent. All the chemical shifts were recorded in ppm with reference to $CDCl_3$. The spectra were analysed using MestRENova (6.0.2, Mestrelab Research S.L., 2009).

Particle size and zeta potential measurement - Mean NP hydrodynamic diameters were obtained by measuring the light scattering at 173° angle to incident radiation at 25 °C after diluting the samples with DI water using Malvern (TM) Zetasizer equipped with 10 mW He-Ne laser operating at a wavelength of 633 nm. The zeta potential for the NPs was measured in water using the same Malvern (TM) Zetasizer.

Transmission electron microscopy (TEM) - NPs morphology was evaluated by using a Tecnai G2 (FEI, Oregon, USA) Transmission Electron Microscopy (TEM). 17µl of 2m/ml NPs suspended in water were pipetted onto a copper grid and wicked off after 10 minutes, subsequently the grid was treated with 3% w/v of uranyl acetate.

Differential scanning calorimetry (DSC) - Polymer thermal properties were investigated by differential scanning calorimetry (DSC) (Q2000, TA Instruments) at a heating rate of 10 C/min. Thermal Analysis Software (Version 4.5.05A) was used to analyse the resulting data. Pans with pin holed lids (TA Instruments, Brussels, Belgium) were utilized for the analysis of the samples, using empty pan as the reference. The DSC cell was purged with nitrogen gas at a flow rate of 50 mL/min. Glass transitions were analysed performing two heating/cooling cycles from 0 up to 150 ˚C.

Impact of NPs on cells viability - The AlamarBlue® metabolic activity assay was used to assess the effects of the green, red and blue PLGA NPs on the cells. Activated cells were incubated with media containing 50, 100, 250 and 500 µg/ml of NPs for 24 hours. Hereafter, the media was removed and the cells were washed with phosphate buffered saline (PBS) and incubated in fresh media containing 10% AlamarBlue® for 3 hours. Supernatants (100µL) were transferred to 96 opaque black well plates and the fluorescence was recorded (Optima FLUOstar®) at excitation/emission of 540/580 nm. The results were plotted as mean % viability vs control ± standard error of the mean (SEM).

Cellular uptake of NPs by cells - Cells were seeded on 8 well chambered (37 °C, 5% CO_2, 24 hrs). The media was then changed with fresh media or media containing 100 µg/mL of green, red and blue NPs (37 °C, 5% CO_2, 3 hrs). Following treatment, cells were washed twice with PBS and then exposed to 5 µM DRAQ5™ (blue NPs) or 1µM Hoechst® (green NPs & red NPs) for 15 min in the dark at 37 °C to stain the nucleus. After nucleic acid staining, cells were washed twice with PBS and then exposed to AF488 (blue NPs) or AF647 (green & red NPs) for 30 min in the dark at 37 °C to stain the cytoskeleton. The cells were washed twice with PBS and fixed in paraformaldehyde (4% v/v, PBS, 300 µL) for 15 min in the dark at room temperature. The plates were finally washed twice with PBS and stored with mounting media (Fluoromount™ Aqueous, SigmaAldrich, 4 °C) ready for imaging.

Fluorescence imaging - Cells were imaged using an inverted Nikon Eclipse T1 and QIMAGING optiMOS camera equipped with CoolLED pE-4000 fluorescence illumination and pE-100 brightfield illumination and Nikon Plan Fluor 10 x (0.30 NA) objective and 40 x (0.75 NA). Fluorescence was captured through excitation at 405, 490, 550, 635 nm collecting at emission at 410-500, 500-550, 550-650, and 650-750, using an exposure time of 100 ms. Bright field was captured at an exposure time of 10 ms. Blank sample of deionised water was imaged for control. All fluorescence and bright field images were corrected to a 12-bit image (0-4095).

In vitro release –Freeze-dried doxorubicin loaded NPs were resuspended in PBS (5mg, 0.5 mL) and placed in a dialysis tube (Slide-A-Lyzer™ MINI Dialysis Devices, 3.5K membrane MWCO, Thermo Scientific) and dialysed against 14 mL of PBS at 37 °C for 24 hours. Samples from the release media were collected after 3 and 24 hours. The amount of doxorubicin release was calculated using fluorimeter (λex 480 nm, λem 590 nm) using a calibration curve of doxorubicin in PBS.

Caenorhabditis elegans growth –C. elegans nematodes (Bristol N2) were maintained on nematode growth medium (NGM) agar and *Escherichia coli* (OP50) at 20 °C[26]. Synchronized growth cycles of *C. elegans* were prepared by harvesting eggs from gravid females, which were collected by rinsing an NGM growth plate with sterile deionized water (3.5 mL). Sodium hydroxide (5 M, 0.5 mL) and sodium hypochlorite (5%, 1 mL) were added to the worm suspension and vortexed (10 min) to separate nematodes from the eggs. The eggs were initially collected using centrifugation (1500 rpm, 1 min), and subsequently washed once with 5 mL of sterile deionized water and collected with centrifugation (1500 rpm, 1 min). The centrifuged egg suspension was aspirated to 0.1 mL and plated onto a fresh plate of NGM agar, seeded overnight with an *E. coli* lawn. The generation time of *C. elegans* using these conditions was 4 to 5 days.

C. elegans NPs uptake - NGM agar plates containing synchronized cycles of *C. elegans* (L_4-young adult stages) were harvested using sterile ultra-pure deionised water (18.2MΩ). The nematodes were washed with sterile deionised water (1 mL, 3 times) and collected using centrifugation (2500 rpm, 1 min). *C. elegans* (∼300 nematodes) were dosed with PLGA nanoparticles at 100 µg/mL (n = 3) for 12 hours (PLGA NPs containing encapsulated rhodamine B) or 12 hours (PLGA-2AA, PLGA-5AF and PLGA-R6G) and washed prior to imaging (3 times, 5 mL of sterile deionized water, centrifugation 1500 rpm, 1 min). Regions of interest containing *C. elegans* that had ingested fluorescent nanoparticles were imaged using a Nikon Eclipse TS100, Nikon 4× 0.10 NA (air) objective.

Supplementary Figures S1-S7

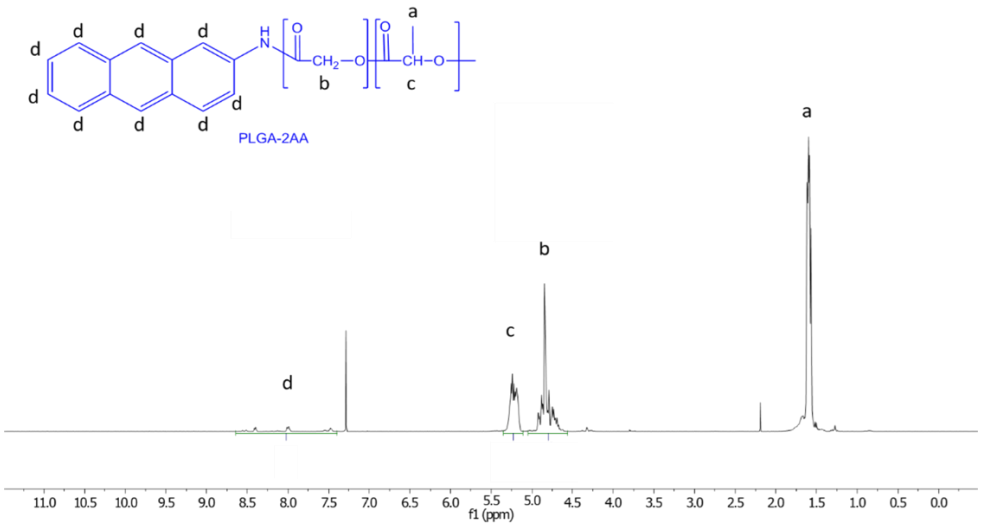

Figure S1. ^{1}H NMR spectrum of PLGA-2AA in $CDCl_3$, prepared by ROP with $Sn(Oct)_2$ as catalyst and 2-AA as initiator.

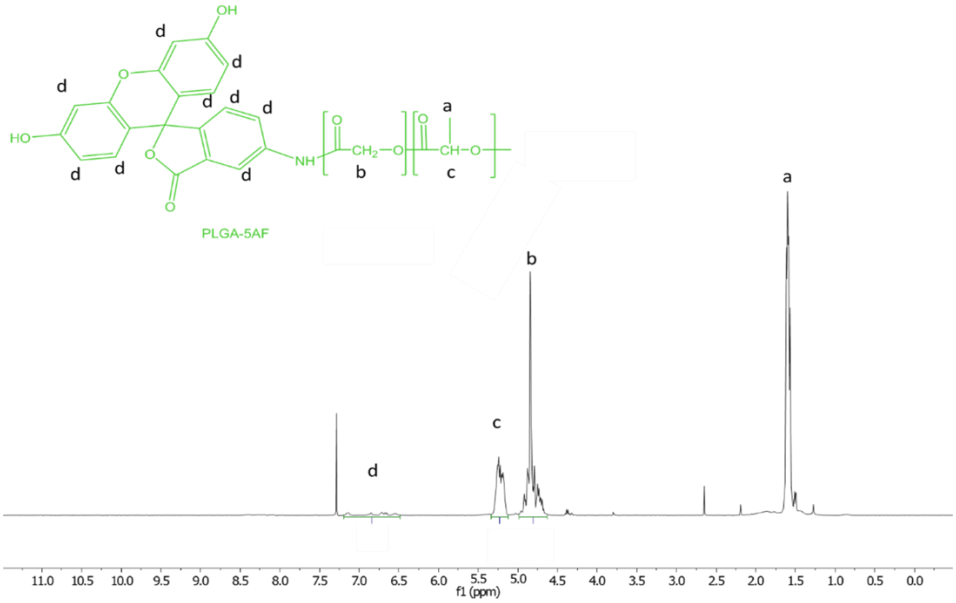

Figure S2. ^{1}H NMR spectrum of PLGA-5AF in $CDCl_3$, prepared by ROP with $Sn(Oct)_2$ as catalyst and 5-AF as initiator.

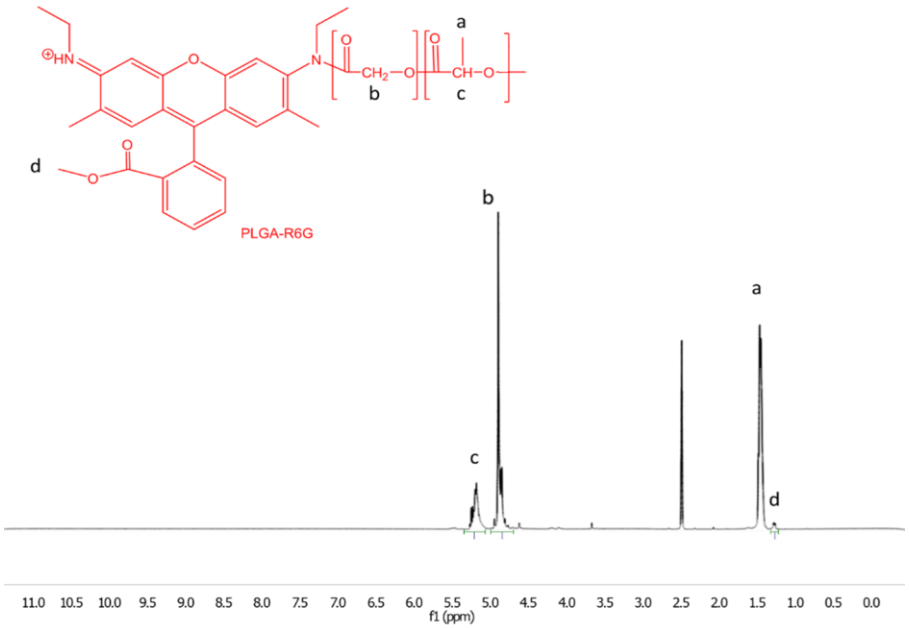

Figure S3. ^{1}H NMR spectrum of PLGA-R6G in DMSO-d_6, prepared by ROP with $Sn(Oct)_2$ as catalyst and R6G as initiator.

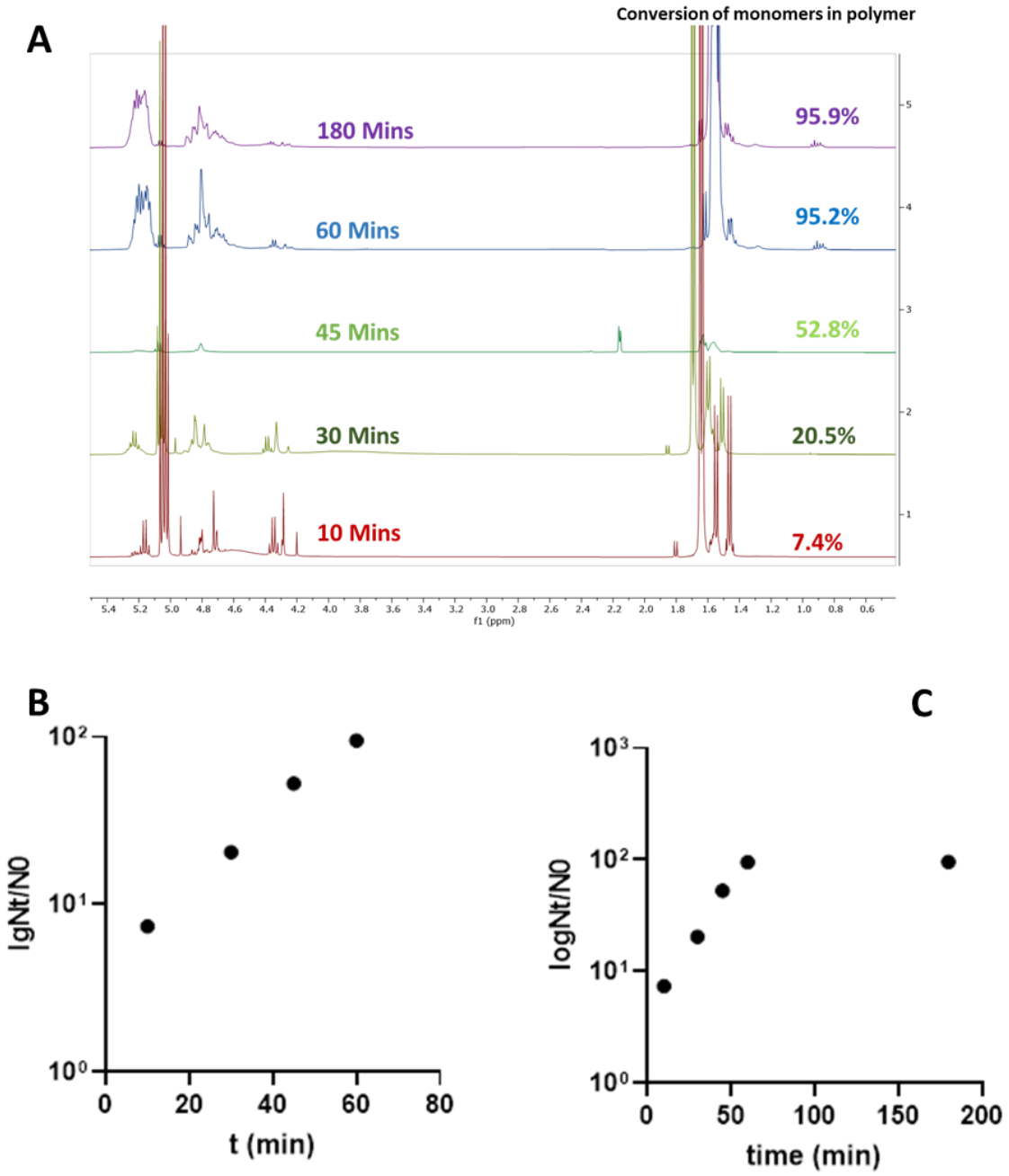

Figure S4. (A) Stacked ^{1}HNMR spectra recorded at 10-30-45-60 and 180 mins for the 5AF-initiated ROP of PLGA. (B) conversion vs time in the linear region (up to 60 mins of reaction). (C) lost in linearity in the conversion of monomers into polymer after 60 mins.

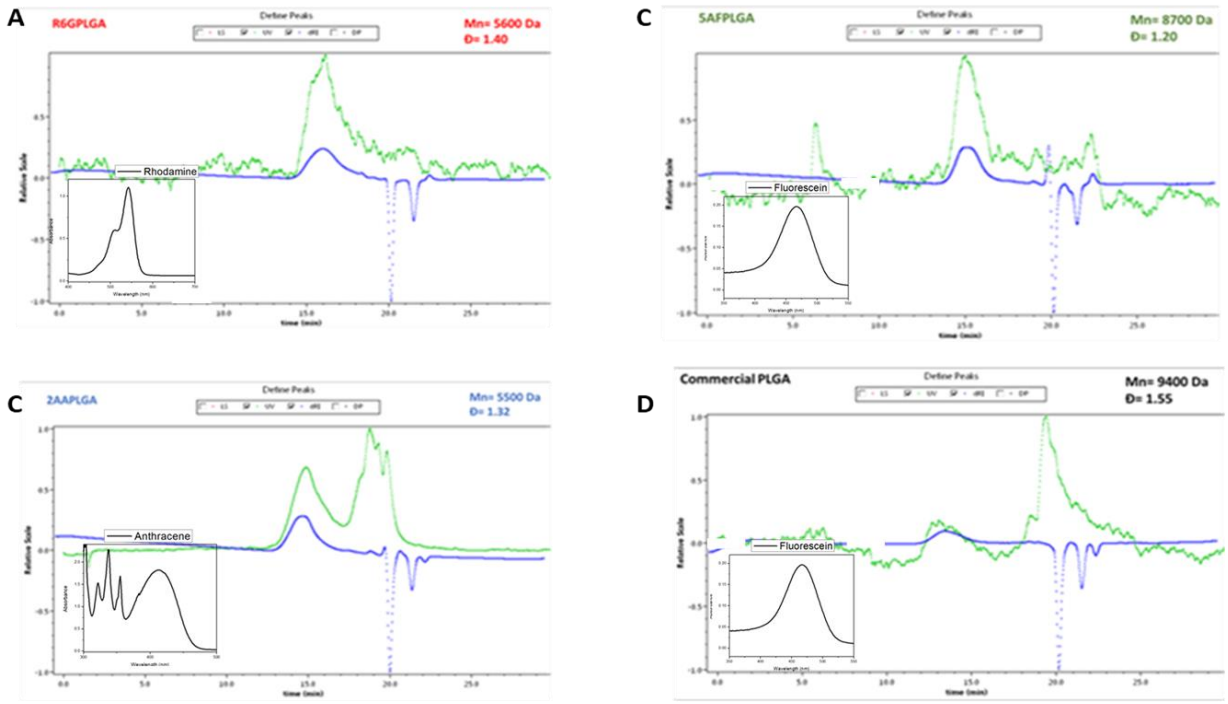

Figure S5. GPC RI (blue) and UV-vis (green) traces of (A) PLGA-R6G, (B) PLGA-AF (C) PLGA-AA and (D) commercial coloured PLGA in THF (PMMA calibrants) (Insets) UV-vis scans of dyes.

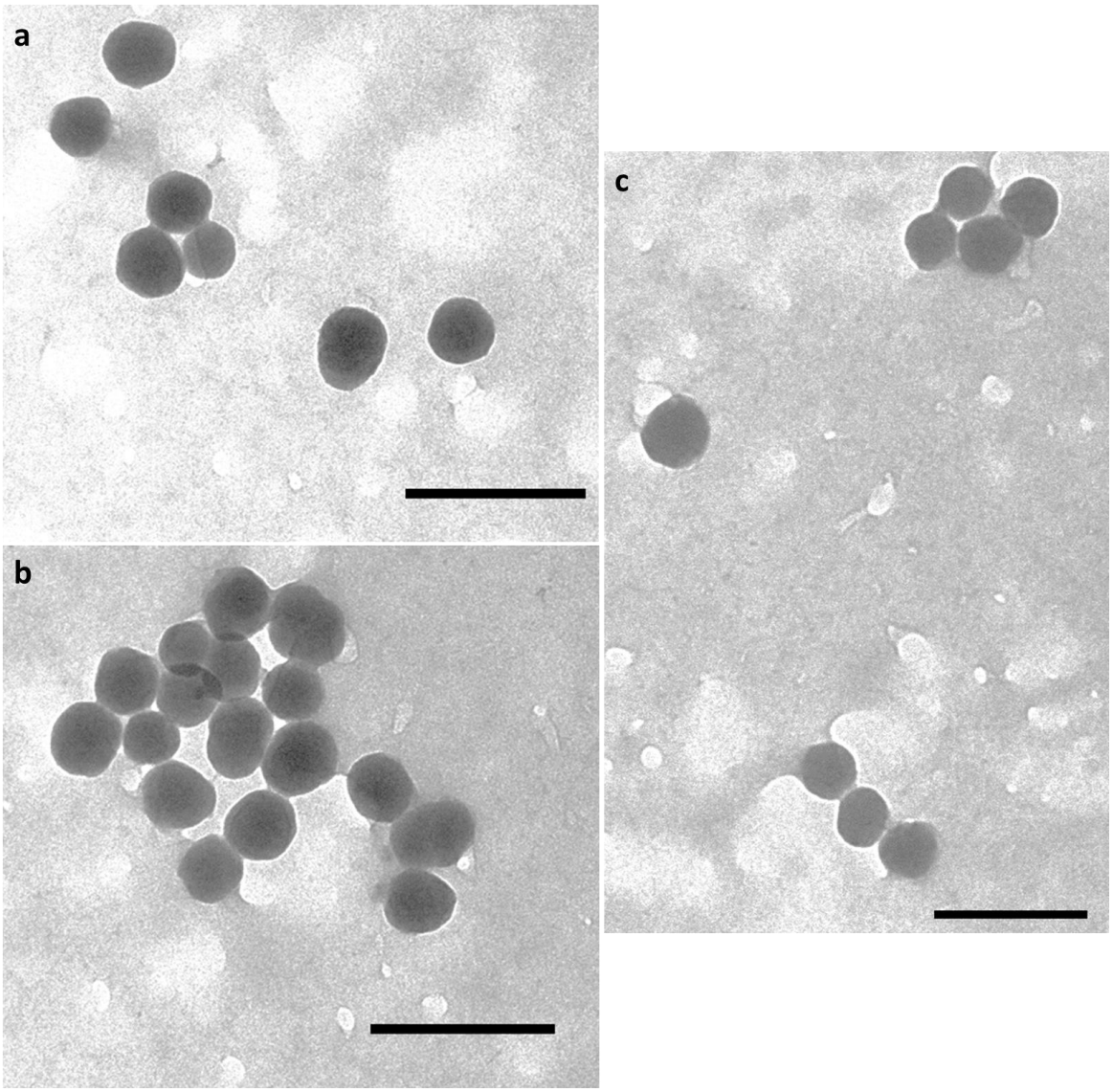

Figure S6. Transmission electron micrographs of a) PLGA-2AA NPs, b) PLGA-5AF and c) PLGA-R6G, where scale bar = 200 nm.

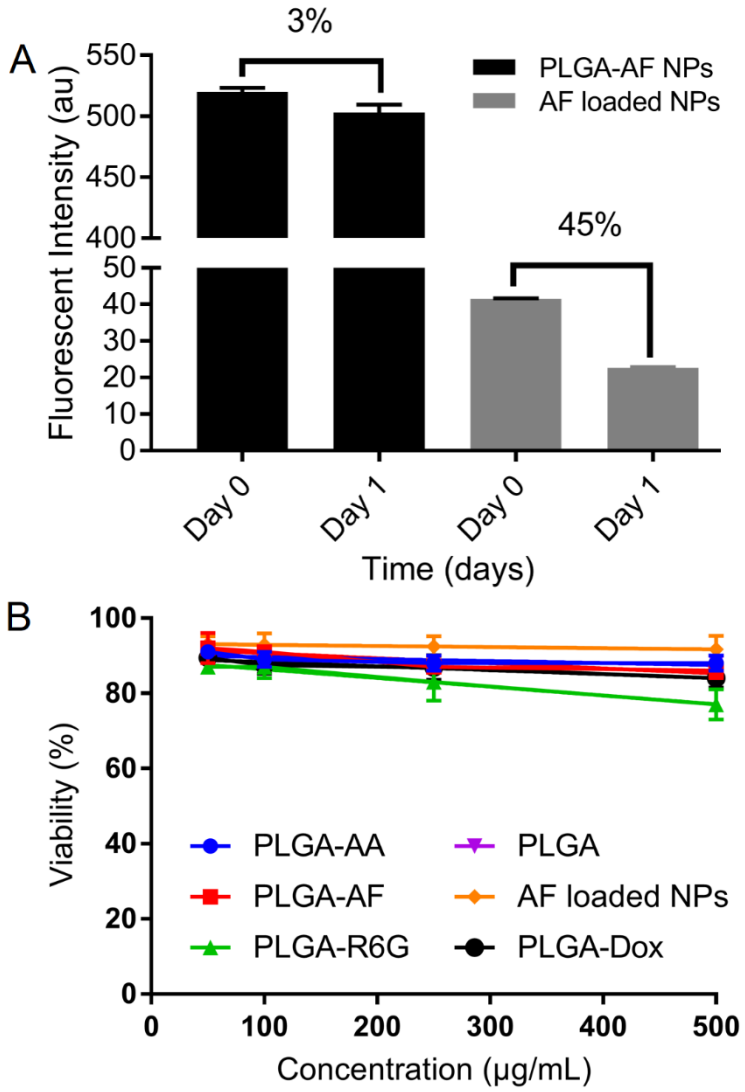

Figure S7. (A) Comparison of fluorescence intensity of PLGA-5AF vs PLGA loaded with 5-AF immediately after synthesis and purification and after 24 hrs of storage. Error bars represent standard deviation (n=3). (B) Viability of activated THP-1 macrophages following exposure to PLGA NPs (50-500 µg/mL), fabricated from newly synthesised fluorescent polymers and commercially available PLGA. Cells were exposed to NPs for 24 h, cell viability was assessed *via* AlamarBlue assay. Data are expressed as the average percentage of cell viability ± SEM (n = 3).

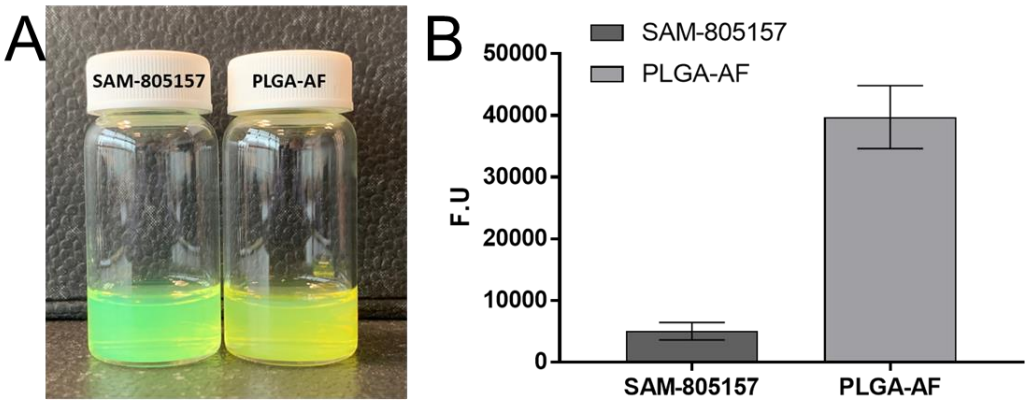

Figure S8. Comparison of Green Fluorescent PLGA NPs (805157) from Sigma-Aldrich Merck (SAM-805157) and PLGA-AF nanoparticles at the concentration applied for in vitro imaging. (A) Samples of nanoparticle suspensions (2.5 mg/ml). (B) Fluorescence intensity values of 100 µg/ml SAM-805157 and PLGA-AF nanoparticles following excitation at 470 nm and detection at 520 nm (integration time 40 µs). Data displayed as mean ± S.D from three independent values.

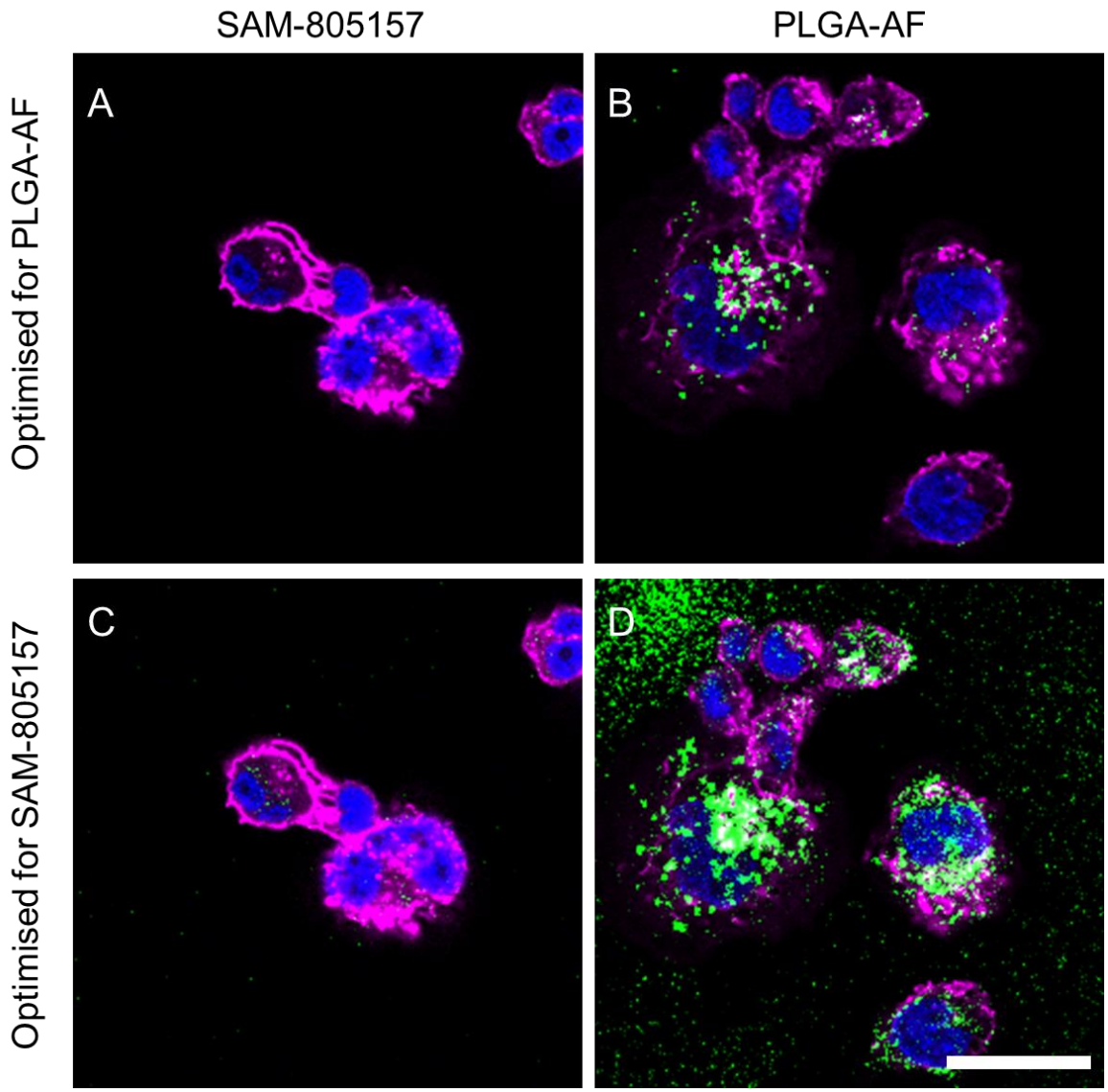

Figure S9: Comparative fluorescence confocal microscopy images for THP-1 cells treated with PLGA-AF and SAM-805157 100 nm particles. (A&B) Optimised images for PLGA-AF fluorescence intensity, where scale is Blue 202-28238 Green 2476-2728 and Magenta 256-24320. (C&D) Optimised images for SAM-805157, where scale is Blue 202-28238 Green 194-593 and Magenta 256-24320. Scale bar = 10 µM.

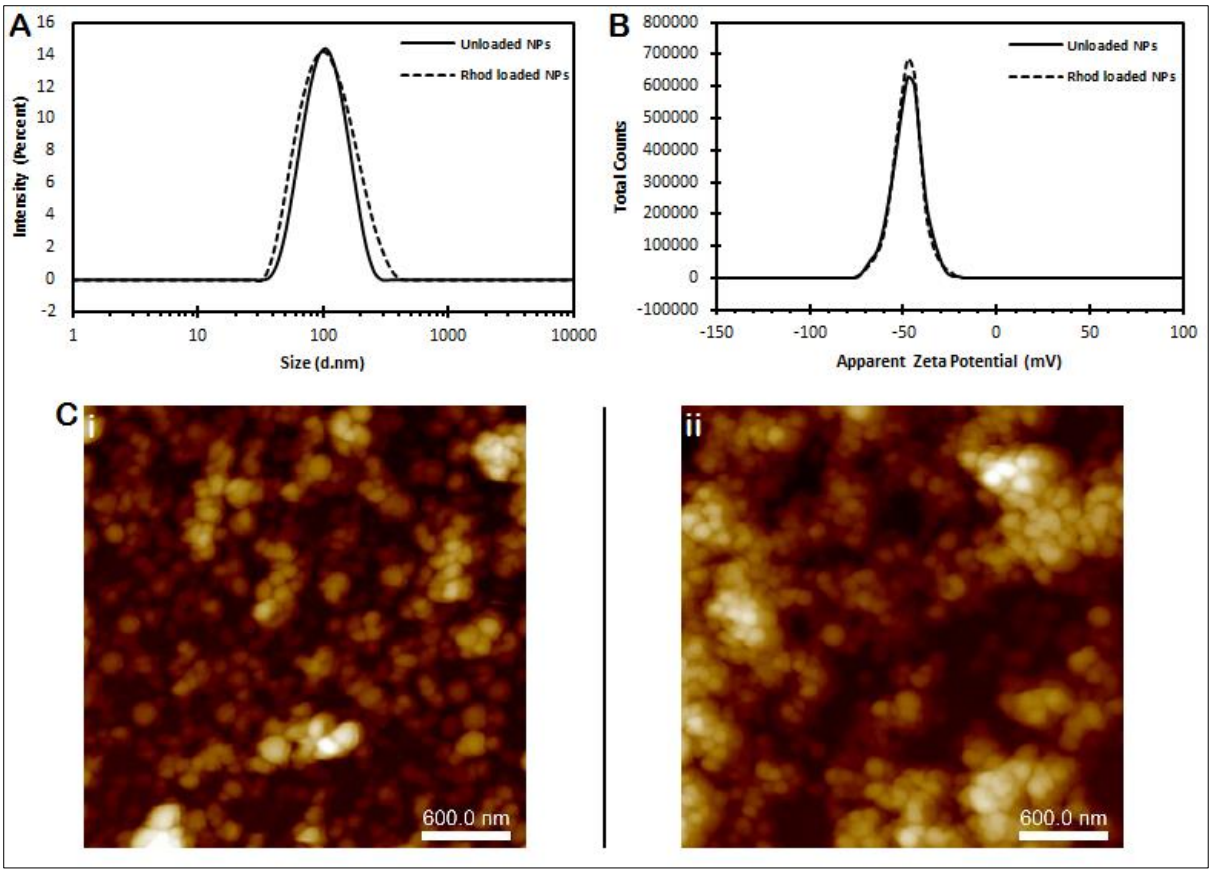

Figure S10 Comparison of size (A) and surface charge (B) between unloaded fluorescent PLGA NPs (solid lines) and Rhodamine-B loaded NPs (dashed lines). In (C) are AFM tapping mode images for (i) unloaded fluorescent PLGA NPs and (ii) Rhodamine-B loaded NPs.

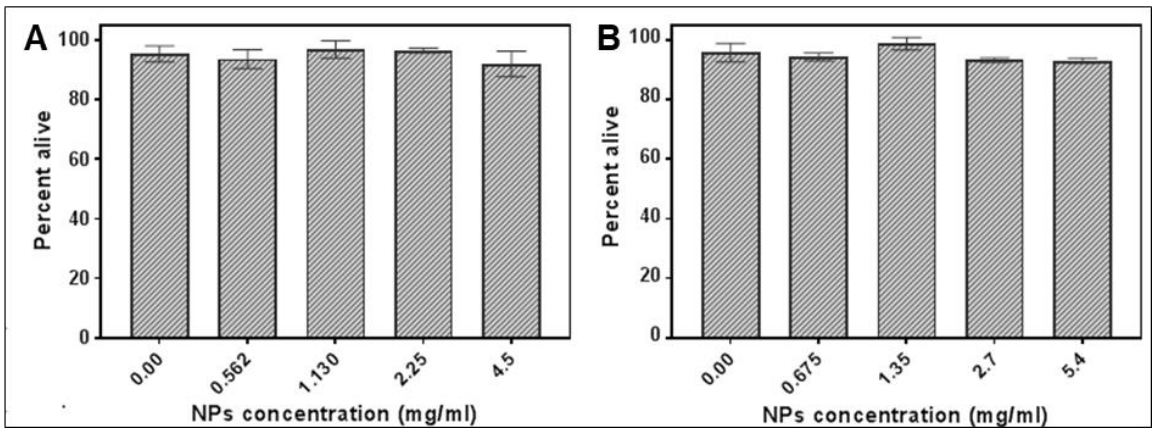

Figure S11. Viability of *C. elegans* after exposure to (A) unloaded fluorescent PLGA NPs and (B) Rhodamine-B base loaded NPs. Viability of the nematodes was determined by motility, using the ratio of motile worms to total population in the sample. Experiments were conducted at least three times. Error bars represent standard deviation.

Table S1. Size and zeta potential of dye-initiated PLGA NPs (n=3).

Polymer	Mean Diameter (nm) (±SD)	Zeta potential (mv) (±SD)
PLGA-2AA	101 ± 5	-43 ± 3
PLGA-5AF	103 ± 6	-40 ± 4
PLGA-R6G	98 ± 5	-45 ± 4
Unlabelled PLGA	120 ± 2	-47± 3