SUPPLEMENTARY INFORMATION

Novel hydrophilic copolymer-based nanoparticle enhances the therapeutic efficiency of Doxorubicin in cultured MCF-7 cells

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CONTENTS

1. MATERIALS

All materials used in the study were obtained from Sigma-Aldrich unless specified otherwise.

2. METHODS

2.1 Characterization by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) and Gel Permeation Chromatography (GPC)

^{1}H NMR (^{1}H Nuclear Magnetic Resonance) spectra were measured using Bruker 500 MHz spectrometer, using CD_3OD as the solvent for the copolymer and ME-Br initiator used in this study. All chemical shifts were referenced to the solvent peak for CD_3OD (δ3.31 ppm). Gel Permeation Chromatography (GPC) was used to determine the weighted average molecular weight ($\overline{M_w}$) and polydispersity index (PDI) of the polymer (Waters Styragel HR 3 7.8 x 300 mm column, Waters Styragel HR 4 7.8 x 300 mm column, 5 µm). Agilent Technologies 1100 Series GPC and Agilent GPC software were used for measurements and data analysis respectively. Measurements were taken using dimethylformamide (DMF) as the eluent at the flow rate of 0.7 mL/min at 40 °C, and calibrated against poly (methyl methacrylate) (PMMA) standard.

2.2 Synthesis and Characterization of 2-(4-morpholino)-ethyl-2-bromoisobutyrate Initiator (ME-Br) for Atom Transfer Radical Polymerisation (ATRP)

4-(2-hydroxyethyl) morpholine (9.1 mL; 75.5 mmol) and triethylamine (15.8 mL; 113.3 mmol) were dissolved in 250 mL of dry methylene chloride in an inert environment. After cooling the mixture for 20 min

on ice, 2-bromoisobutyryl bromide (10.3 mL; 83.1 mmol) was added drop-wise under nitrogen and the reaction was stirred at room temperature over 48 h. 0.1 M sodium carbonate solution was added to the reacted mixture to remove triethylammonium bromide salt by phase separation. The organic layer was retrieved and washed 5 × with 200 mL portions of deionised water. After drying over anhydrous magnesium sulphate, solvent was removed from the final product under reduced pressure at room temperature and stored in the absence of light in a desiccator.

2.3 Synthesis and Characterization of *p*(HEMA-*ran*-GMA) copolymer *via* ATRP

The monomers, 2-hydroxyethyl methacrylate (HEMA; 3.4 mL; 28 mmol) and glycidyl methacrylate (GMA; 1.6 mL; 12 mmol), were dissolved in methanol (MeOH) at a volumetric ratio of 1:3 (monomer : MeOH) in a Schlenk flask. 2-(4-morpholino)-ethyl-2-bromoisobutyrate (ME-Br; 210 µL; 1 mmol) was added and the mixture was degassed 3 × using the standard "freeze-pump-thaw" method and backfilled with argon gas. Copper (I) bromide (CuBr; 143 mg; 1 mmol) and 2,2-bypyridine (bpy; 392 mg; 2 mmol) was added and the reaction was carried out at 80 °C under standard Schlenk conditions for 1 h. Upon completion, the reaction was opened to air and additional MeOH (15 mL) was added. The *p*(HEMA-*ran*-GMA) copolymer product was collected by precipitation in excess diethyl ether and dried overnight under vacuum. Co-polymer composition was determined by ^{1}H NMR (500 MHz, CD_3OD) where peaks at δH 2.551 (1H, br), δH 2.688 (1H, br) and δH 2.868 (1H, br) corresponded to the epoxide moiety, confirming the presence of GMA in the polymer chain. Weighted average molecular weight ($\overline{M_w}$) and polymer polydispersity (PDI) of *p*(HEMA-

ran-GMA) were measured by Gel Permeation Chromatography (GPC). *p*(HEMA-*ran*-GMA) characterization results can be found in Figure S2.

2.4 Nanoparticle Synthesis

2.4.1 Synthesis of poly (glycidyl methacrylate) (PGMA) nanoparticles

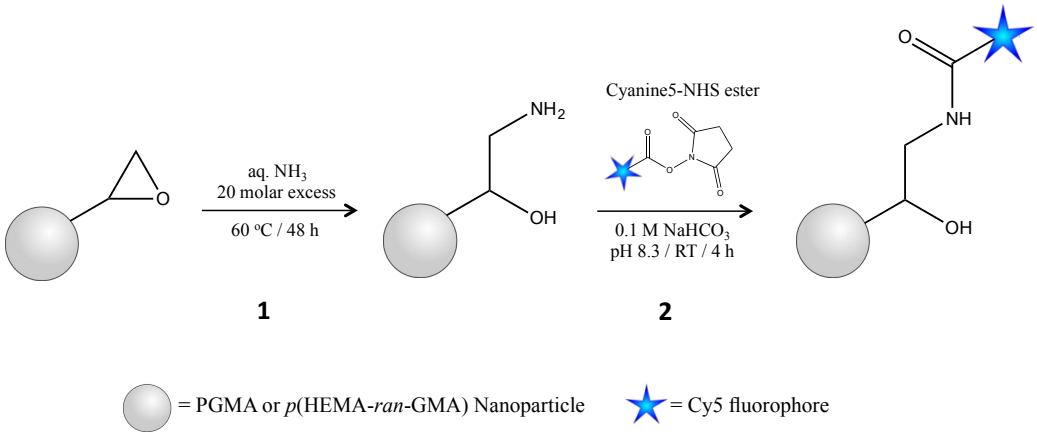
The synthesis of poly glycidyl methacrylate (PGMA) and the solvent evaporation oil-in-water (O/W) emulsion method to synthesise PGMA nanoparticles, along with the justifications for their use for therapeutic purposes have been previously described extensively.[1-3] In brief, 100 mg of PGMA was dissolved in 1 : 3 mixture of chloroform and methyl ethyl ketone respectively to form 8 mL of organic phase. This was added drop-wise into vigorously stirring aqueous phase made up of 30 mL 1.25 w/v% Pluronic F-108 in MilliQ water and sonicated extensively. An aqueous suspension of PGMA nanoparticles was retrieved by removing all solvents under reduced pressure at 40 °C. Known volumes of the nanoparticle suspension were lyophilised for yield assessment, while the remaining suspension was stored at room temperature prior to analyses and further functionalization.

2.4.2 Synthesis of p(HEMA-ran-GMA) nanoparticles

p(HEMA-*ran*-GMA) nanoparticles were synthesized *via* spontaneous "water-in-oil" (W/O) inverse nano-emulsion. *p*(HEMA-*ran*-GMA) (131.7 g/mol; 100 mg; 758 µmol) was completely dissolved in 4 mL of MilliQ water. The surfactant, sodium dioctyl sulfosuccinate (AOT; 17 g) was completely dissolved in 250 mL of dry hexane. The aqueous copolymer solution was then added to the hexane-AOT mixture with moderate stirring which resulted in an optically clear and homogenous emulsion. 1:100 dilution of ethylene diamine in MilliQ water (42 µL;

6.17 µmol) and a few drops of triethylamine (TEA) was added to the stable emulsion to enable *p*(HEMA-*ran*-GMA) nanoparticle formation within the aqueous micelles in the W/O emulsion *via* cross-linking of epoxide groups present on the copolymer backbone, overnight at room temperature. To retrieve the cross-linked *p*(HEMA-*ran*-GMA) nanoparticles as an aqueous suspension, the W/O micro-emulsion was disrupted by the addition of MilliQ water and centrifuged (10000 g; 30 min). The aqueous phase consisting of *p*(HEMA-*ran*-GMA) nanoparticles was carefully removed and dialysed using regenerated cellulose membrane (Fisherbrand™ MWCO 12-14 kDa) against MilliQ water over 48 h to remove unreacted reagents and/or remnant AOT.

2.4.3 *Cyanine5 (Cy5) functionalisation of nanoparticles*



Schematic S1: Schematic representation of Cyanine5 fluorophore functionalisation of PGMA and *p*(HEMA-*ran*-GMA) nanoparticles *via* two stages: **(1)** Epoxide ring opening reaction to functionalise nanoparticles with amine groups **(2)** Cy5 functionalisation *via* the amine groups on nanoparticle surface by substitution with NHS-ester.

Aqueous ammonia (aq. 25 % NH_3) was added in 20-fold excess with respect to the epoxide functional groups present in the nanoparticle suspension and reacted over 48 h at 60 °C to enable amine-functionalization of the nanoparticles *via* epoxide ring opening. The amine-functionalized nanoparticles were purified by dialysis overnight (Fisherbrand™ Regenerated Cellulose MWCO 12-14 kDa) against

MilliQ water and lyophilized for yield assessment. Cyanine5-N-hydroxysuccinimide (Cy5-NHS) ester (Lumiprobe) (0.27 mg; 433.6 µmol) was dissolved in 1 : 10 reaction volume of dimethyl sulfoxide (DMSO) and added to a 9 : 10 reaction volume of amine-functionalized nanoparticles in amine-free buffer (50 mM HEPES; pH 8.3) making up a total volume of 10 mL. The reaction mixture was briefly sonicated and stirred over 4 h at room temperature. The chemical reaction scheme for the functionalizations mentioned above can be found in Schematic S1. Unbound fluorophore was removed to purify the Cy5 functionalised nanoparticles by dialysis (MWCO 12-14 kDa) against MilliQ water overnight.

2.5 Nanoparticle Characterisation

Mean hydrodynamic size of the nanoparticle variants were characterized using dynamic light scattering (Zetasizer Nano ZS) using a 4 mW He-Ne laser operating at 633 nm with a scattering angle of 173°. Surface charges of the nanoparticle variants (zeta potential) were also determined. Measurements were taken in triplicate after an initial equilibrium of 1 min. The samples were calibrated against measurements for recorded for PGMA (refractive index 1.515; viscosity 0.05) in water at 25 °C as dispersant (refractive index 1.33; viscosity 0.887). The intensity-weighted hydrodynamic sizes and zeta potentials of the nanoparticle variants were presented as mean ± standard deviation. All zeta potential measurements were taken at physiological pH (7.4). 10 µL nanoparticle suspensions were deposited on carbon-coated copper grids and dried overnight in preparation for Transmission Electron Microscopy (TEM) imaging. All TEM images were obtained at 120 kV using JEOL JEM-2100. Thermal analysis to assess the cross-linking efficiency of lyophilized *p*(HEMA-*ran*-GMA)

nanoparticles was conducted by thermogravimetric analysis (TGA) (PerkinElmer Thermal Analyzer TGA 7) and differential scanning calorimetry (DSC) (PerkinElmer DSC 7) at a uniform heating-rate of 5 °C/min over a range of 22 °C to 350 °C.

2.6 Doxorubicin (DOX) Loading into Cy5-Functionalised Nanoparticles

Both nanoparticle variants were loaded with DOX by a "back-filling" method.

For Cy5-p(HEMA-ran-GMA) nanoparticles: A known mass of lyophilised Cy5-*p*(HEMA-*ran*-GMA) nanoparticles was mixed with an equal amount of DOX in 5 mL MilliQ water. The suspension consisting of nanoparticles and DOX was sonicated and allowed to stir at room temperature for 2 h. DOX-loaded *p*(HEMA-*ran*-GMA) nanoparticles were then retrieved as a pellet by centrifugation (20000 g; 30 min; 3×).

For Cy5-PGMA nanoparticles: A known volume of the Cy5-PGMA nanoparticle suspension was centrifuged to pellet the required mass of nanoparticles. An equal mass of DOX in 5 mL MilliQ water was added to the pellet and sonicated. The suspension was allowed to stir at room temperature for 2 h. The volume of the suspension of Cy5-PGMA nanoparticles and DOX was reduced to ~1 mL under reduced pressure at room temperature. DOX-loaded Cy5-PGMA nanoparticles were retrieved by centrifugation of the concentrated suspension of nanoparticles and DOX (20000 g; 30 min; 3×).

The DOX-loaded nanoparticle pellets for both variants were reconstituted in 1× PBS (pH 7.4) to 5 mg/mL nanoparticle concentration and stored at 4 °C until required.

2.7 DOX Loading and Release Profile Assessment by High Performance Liquid Chromatography (HPLC)

DOX loading and release experiments were performed in pre-warmed 1× PBS (37 °C; pH 7.4). Quantification of the drug was determined by HPLC analysis. Standard concentrations of DOX in 1× PBS (10 µL injections in triplicate) were run on a Waters 2695 separations module coupled with Waters 2489 UV/Vis detector with a 50 : 50 isocratic solvent system consisting of 0.02 M phosphate buffer (pH 5.4) and acetonitrile (ACN) at flow-rate of 10 mL/min. Monitoring the eluent at 233 nm produced a peak at the retention time of 2 min. Each standard sample of known concentration was run for 10 min and the integrated area of the detected peak corresponded to the assessed DOX concentration to produce a standard curve. The limit of detection for DOX using this method was 0.1 µg/mL. For DOX loading assessments of each nanoparticle variant, a known mass of drug-loaded nanoparticles were dispersed in 1 mL methanol, sonicated in an ultrasonic bath for 15 min and left to stand at room temperature for 1 h. The samples were then centrifuged (20000 g, 30 min) to collect a known volume of the supernatant without disrupting the nanoparticle pellet and the solvent was extracted completely under reduced pressure at room temperature. The dried DOX from each sample was reconstituted in 1 mL of 1× PBS (pH 7.4) and assessed for concentration by the above-mentioned HPLC method to determine the DOX loading efficiency per mg of nanoparticles. DOX release experiments were carried out in pre-warmed 1× PBS (37 °C; pH 7.4) by dispersing DOX-loaded nanoparticles to make up a final nanoparticle concentration of 1 mg/mL in a sink volume of 10 mL. 200 µL of the sinks were sampled in triplicate over a course of 3 days at specific time points. No fresh 1× PBS was added. Each sample was centrifuged (20000 g, 30 min) and the supernatant was assessed for DOX concentration using the HPLC method as-above. The results of the DOX loading and release experiments were reported as means ± SEM.

2.8 *In-Vitro* Assessment of Nanoparticle Variants

2.8.1 Culture of MCF-7 cells

MCF-7 cells (human breast adenocarcinoma cell line, ATCC) were cultured in Minimum Essential Media α (MEM α, Gibco) supplemented with 0.15 % sodium bicarbonate, 10 % fetal bovine serum (FBS) and 1× GlutaMAX, and were grown in a humidified incubator at 37 °C with 5 % CO_2. *In-vitro* assessments of nanoparticle cytotoxicity (Section 2.8.2) and IC_{50} assessment of DOX-loaded nanoparticles (Section 2.8.4) were conducted using this biologically relevant cancer model with the above-mentioned cell culture media.

2.8.2 Cytotoxicity assessment by MTS Assay

Nanoparticle cytotoxicity was quantified by cell metabolism, often referred to as viability, assessed by MTS assay (Promega CellTiter 96® AQueous One Solution Cell Proliferation Assay). In brief, MCF-7 cells were seeded in triplicate (10^4 cells/well) in 96-well plates (Corning® Clear Polystyrene Microplate: clear flat bottom) in 50 µL culture media per well and were allowed to settle overnight. 50 µL of respective Cy5-conjugated nanoparticle suspensions at varying final concentrations (0 – 1000 µg/mL nanoparticles in culture media) were then added and allowed to incubate with the cells for 24, 48 or 72 h in 96-well plates. Control wells (with no nanoparticle treatment) were topped up with 50 µL culture media such that the final volume of all test wells was 100 µL. The MTS assay was conducted according to the manufacturer's protocol (20 µL MTS reagent per test well) at the specified time points and the absorbance at 490 nm resulting from the coloured formazan product generated by viable cells due to the reduction of the MTS

tetrazolium compound was assessed on a plate reader (PerkinElmer EnSpire™ Multimode Plate Reader). Measured values were normalised to blank wells with culture media only.

2.8.3 *Confocal imaging of Cy5-functionalised nanoparticles in MCF-7 cells*

Cells were seeded (5 x 10^4 cells/well) on poly-L-lysine treated cover slips (10 mm) in 24-well plates ((Corning® Clear Polystyrene Microplate: clear flat bottom) with 500 µL culture media per well and allowed to settle overnight. Cy5-conjugated nanoparticle variants (20 µg/mL in 100 µL culture media) were added in duplicate to the respective wells and incubated for 24 h. To fix the cells in each well, media was removed, cell layers were washed with 1× PBS twice and then 4 w/v% paraformaldehyde in 1× PBS was added for 15 min. After removing the fixative, the cover slips in each well were washed twice with 1× PBS before permeablizing the cells in each well with 0.5 v/v% Triton® X-100 in 1× PBS for 15 min at room temperature. The permeabilization solution was removed and each well was washed twice with 1× PBS before the addition of 2 v/v% donkey serum in 0.5 % Triton® X-100 solution (2 % DKS) to block the cells for 1 h at 4 °C. Antibodies recognising Mouse βIII-tubulin (1 : 500) in 2 % DKS was added to each well and incubated overnight at 4 °C. After removing the primary antibodies, Hoechst stain (1:2000) and AF555 goat anti-mouse secondary antibody (1 : 1000) in 2 % DKS was added to each well and incubated in the dark at room temperature for 20 min. Prepared samples on cover slips were washed twice with 1× PBS and mounted onto glass slides for confocal imaging (Nikon Confocal Microscope C2) using spectral properties of DAPI (cell nucleus), AF555 (microtubules in cytoskeleton) and Cy5 (nanoparticles).

2.8.4 Assessment of MCF-7 cell growth inhibition by DOX-loaded Cy5-functionalised nanoparticle variants

MCF-7 growth inhibition was assessed in the presence of free DOX and DOX-loaded nanoparticles in varying concentrations using the MTS assay as described previously in Section 2.8.2. Cells (10^4 cells/well in 50 µL culture media) were allowed to incubate overnight in 96-well plates in triplicate before various concentrations of DOX and UV-sterilised DOX-loaded nanoparticle variants were added and the cells incubated for a further 24 h before the MTS assay. The respective inhibitors (free DOX and DOX-loaded nanoparticle variants) were added in the required drug concentrations in volumes of 50 µL, making the total volume of each test well 100 µL. DOX availability from each nanoparticle variant was calculated from the drug loading assessment and the required concentration of DOX-loaded nanoparticle variants to deliver specific DOX concentrations was delivered to the test wells. Blank nanoparticle variants were added to the analysis matrix to serve as controls, alongside test wells containing untreated MCF-7 cells.

2.9 Statistical Analysis

All statistical analyses were conducted using GraphPad Prism 6.0. All presented data is illustrated as the mean, with error bars that represent standard error of the mean (SEM). Significant differences are denoted by $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). All data are the means of technical triplicates and post-hoc analyses are specified where required.

3. SUPPORTING FIGURES

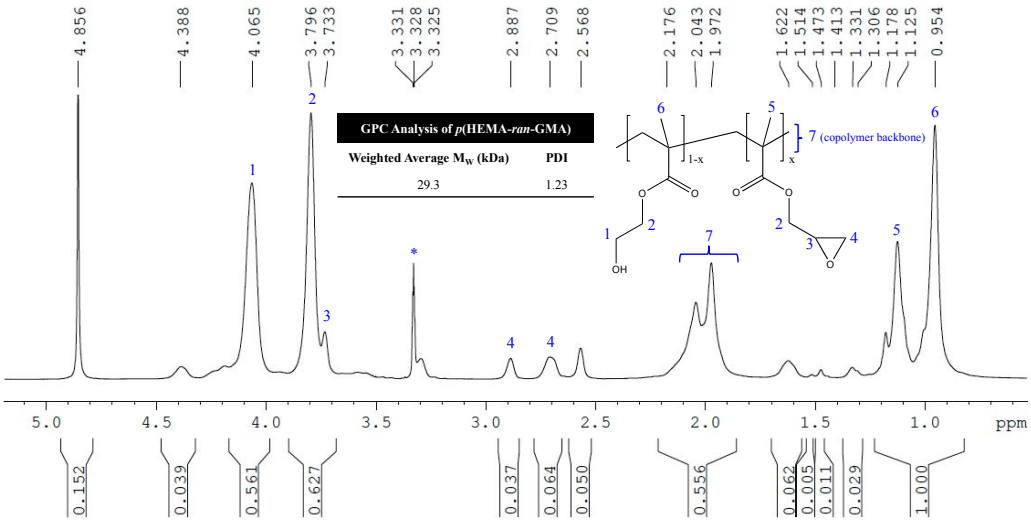


Figure S1. Copolymer Characterisation. ^{1}H Nuclear Magnetic Resonance (NMR) (500 MHz, CD_3OD, δ3.33) spectrum of *p*(HEMA-*ran*-GMA) copolymer synthesized by atom-transfer radical polymerization (ATRP) using 2-(4-morpholino)-ethyl-2-bromoisobutyrate (ME-Br) as initiator. The numbers in blue on the NMR spectrum correlate to the peaks responsible for the respective protons from the structure of the copolymer (inset schematic), in reference to the solvent peak marked by the blue asterisk on the spectrum. Gel Permeation Chromatography (GPC) analysis of *p*(HEMA-*ran*-GMA) provided the copolymer's weighted average molecular weight ($\overline{M_W}$) and polydispersity index (PDI) which are tabulated in the inset table. Confirmation of successful co-polymerization is derived from the presence of epoxide group at δ2.71 (1H, s), δ2.89 (1H, s) and δ3.73 (1H, s) and the GPC analysis of the product.

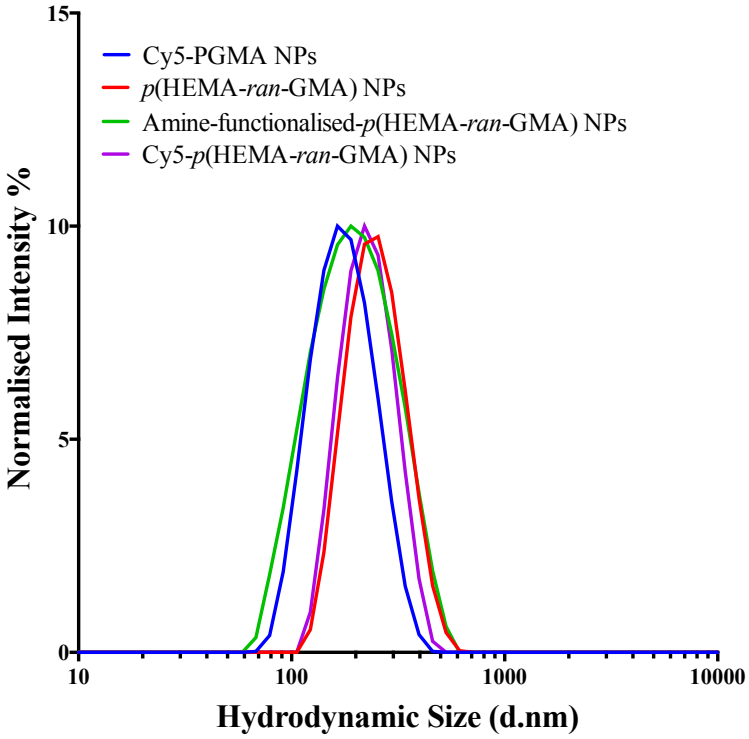


Figure S2. Hydrodynamic size distribution of nanoparticle (NP) variants assessed by Dynamic Light Scattering (DLS) represented as scattering intensity %.

Added information on nanoparticle surface charge (Refer to Table 1 in manuscript):

The unfunctionalized and cross-linked *p*(HEMA-*ran*-GMA) nanoparticles were highly negatively charged at -75 mV which made them very stable in aqueous solution, such that it was not possible to pellet them by centrifugation at 20000 g. Ring-opening reaction of the epoxide functional group on the nanoparticle surface to produce primary amine-terminated chains indicated an increase in the surface charge to -48 mV, which could be explained by the protonation of the amine functional group in aqueous medium. After nucleophilic substitution of the terminal amine groups by Cy5-N-hydroxysuccinimide (NHS) ester, the surface charge of Cy5-*p*(HEMA-*ran*-GMA) nanoparticles increased to -12.5 mV, at which point it became possible to retrieve the nanoparticles as a pellet by centrifugation at 20000 g.

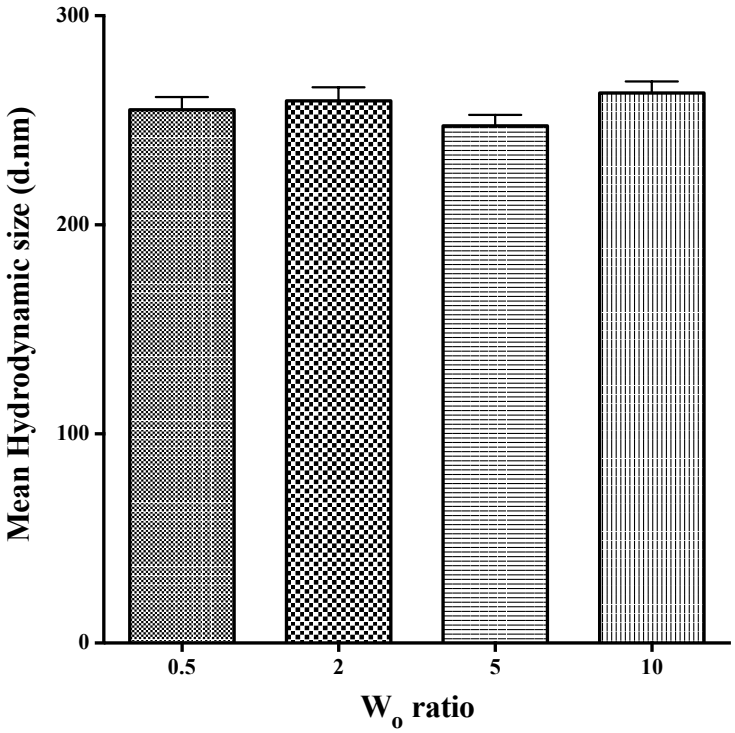


Figure S3. Mean hydrodynamic size of cross-linked *p*(HEMA-*ran*-GMA) nanoparticles with respect to changes in W_o ratio. Ordinary one-way ANOVA with post hoc analysis by Tukey's Multiple Comparisons Test. (n = 3)

No significant changes or trends observed in hydrodynamic size of *p*(HEMA-*ran*-GMA) nanoparticles was observed with changes in W_o ratio, while cross-linker was kept consistent at 50 mol% in the emulsion.

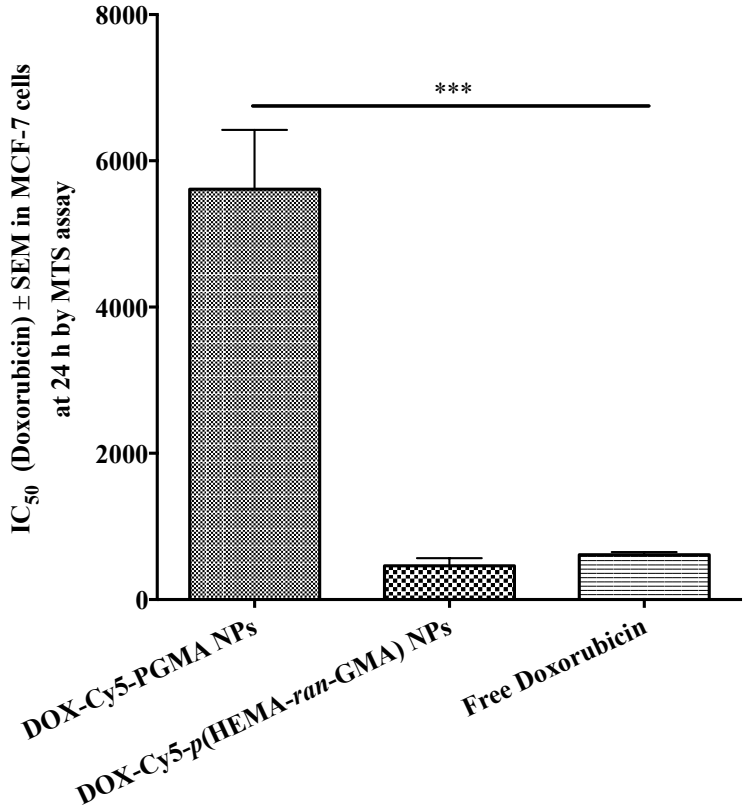


Figure S4. Extrapolated IC_{50} values ± standard error of measurement (SEM). Ordinary one-way ANOVA with post hoc analysis by Tukey's Multiple Comparisons Test. (n = 3, ***p < 0.0005)

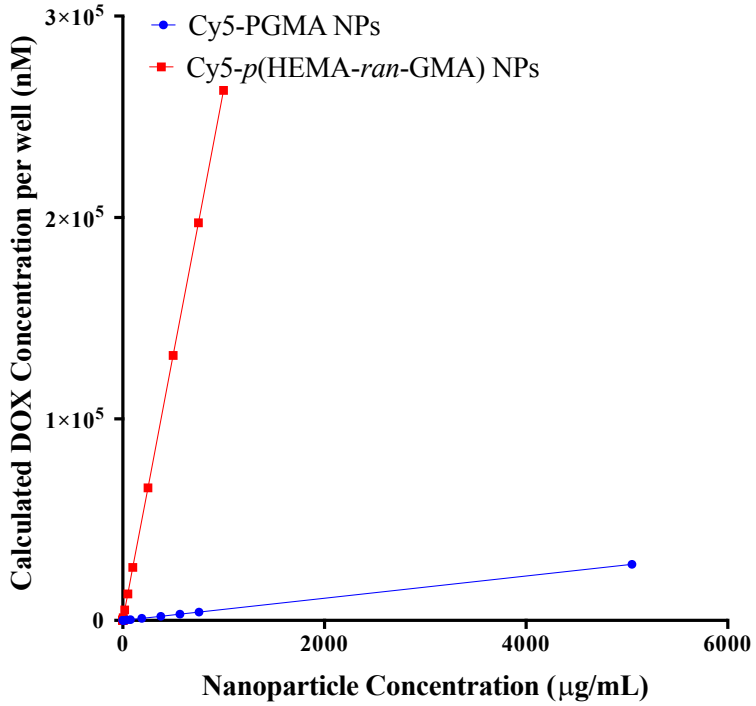


Figure S5. Relationship between nanoparticle concentration and respective DOX loading per test well

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