

Supporting Information for:

“Targeting Triple-Negative Breast Cancer Cells Using Dengue Virus-mimicking pH-Responsive Framboidal Triblock Copolymer Vesicles”

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Experimental Details

Materials

All reagents were used as received unless otherwise stated. Tetrahydrofuran (THF), pyridine, benzoic anhydride, 2-(diisopropylamino)ethyl methacrylate (DPA, or D), deuterium chloride (DCI), trimethylamine, 2,6-di-tert-butyl-4-methylphenol (BHT), dextrose, tryptone, yeast extract, MTT-ESTA, isopropanol, hydrochloric acid, glycidyl methacrylate (GlyMA, or E) and 4, 4'-azobis-4-cyanopentanoic acid (ACVA) were purchased from Sigma-Aldrich (UK). DPA inhibitor was removed by passing this monomer through an inhibitor removal column prior to use. Ethanol, PBS buffer, dichloromethane, dimethyl sulfoxide (DMSO) and dimethyl formamide (DMF) were purchased from Fisher Scientific (UK). 2-Cyano-2-propyl dithiobenzoate (CPDB) was purchased from Strem Chemicals (Cambridge, UK). Glycerol monomethacrylate (GMA, or G) was kindly donated by GEO Specialty Chemicals (Hythe, UK) and used without further purification. 2-(Methacryloyloxy)ethyl phosphorylcholine (MPC, or M) was kindly donated by Biocompatibles Ltd. 2-Hydroxypropyl methacrylate (HPMA, or H) was purchased from Alfa Aesar (UK) and contained 0.07 % crosslinker impurity, as judged by high performance liquid chromatography (HPLC). Rhodamine B piperazine was prepared in-house by Dr. Jeppe Madsen. Deuterated methanol (CD₃OD) was purchased from Goss Scientific (UK). Deionized water was obtained using an Elga Elgastat Option 3A water purifier; its pH was approximately 6.2 and its surface tension was around 72.0 mN m⁻¹ at 20 °C. Primary human dermal fibroblasts (HDFs) and human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from LGC standards (Teddington, UK). DMEM was purchased from Biosera (UK). Foetal calf serum, L-glutamine, penicillin, streptomycin, amphotericin B, plate count agar and trypsin-EDTA were all purchased from Sigma-Aldrich (UK). The antibodies rat-anti-human scavenger receptor CD-36 IgG, rabbit-anti-human scavenger receptor B1 IgG, goat-anti-rat Cy3 IgG and goat-anti-rabbit Cy3 IgG were obtained from Abcam (UK).

RAFT synthesis of PGMA₅₈ macro-CTA in ethanol. A round-bottomed flask was charged with GMA (30.0 g; 187 mmol), CPDB (0.823 g; 2.97 mmol), ACVA (167 mg, 0.156 mmol) and ethanol (39.2 g). This sealed reaction vessel was purged with N₂ for 30 min and placed into a pre-heated oil bath at 70 °C for 135 min. The resulting PGMA macro-CTA (80 % GMA conversion; $M_n = 15,400$ g mol⁻¹, $M_w/M_n = 1.13$) was purified by precipitation into excess dichloromethane. A mean DP of 58 was calculated for this macro-CTA using ¹H NMR spectroscopy.

RAFT synthesis of PMPC₆₀ macro-CTA in ethanol. A round-bottomed flask was charged with MPC (15.0 g; 50.8 mmol), CPDB (0.165 g; 0.747 mmol), ACVA (52.3 mg, 0.187 mmol) and ethanol (12.8 g). This sealed reaction vessel was purged with N₂ for 30 min and placed into a pre-heated oil bath at 70 °C for 210 min. The resulting PMPC macro-CTA (98.6 % MPC conversion; $M_n = 30,200$ g mol⁻¹,

$M_w/M_n = 1.09$) was purified by precipitation into excess THF followed by dialysis against ethanol for 24 h. A mean DP of 60 was calculated for this macro-CTA using ^1H NMR spectroscopy.

Preparation of sterile PGMA₅₈-P(HPMA₃₀₀-*stat*-GlyMARh₁)-PDPA₄₈ triblock copolymer vesicles.

All equipment was rigorously cleaned with disinfectant and a 70 % aqueous ethanol solution. All glassware and ancillary apparatus were autoclaved for 20 min at 121 °C. PGMA₅₈ macro-CTA (0.501 g, 0.0527 mmol), HPMA monomer (2.28 g, 15.8 mmol), GlyMA monomer (7.49 mg, 0.0527 mmol), ACVA initiator (2.96 mg, 0.0105 mmol, CTA/ACVA molar ratio = 5.0) and deionized water (25.1 g, 10 % w/w) were weighed into a 50 mL round-bottomed flask and stirred until the ACVA and macro-CTAs had fully dissolved. This solution was then sterilized via ultrafiltration while transferring into a sterile 50 mL round-bottomed flask equipped with a sterile stirrer bar and rubber septum. This aqueous reaction solution was purged with N₂ for 20 min prior to immersion of the round-bottomed flask in an oil bath set at 70 °C for 2 h. The HPMA polymerization was quenched by cooling to room temperature with subsequent exposure to air. The amine-functionalized fluorescent label, rhodamine piperazine (1.00 mL, 28.8 mg/mL, 0.0527 mmol), was sterilized by filtration and added to the PGMA₅₈-P(HPMA₃₀₀-*stat*-GlyMA₁) diblock copolymer (25.1 mL of 10% w/w dispersion, 0.0527 mmol GlyMA) in a 50 mL round-bottomed flask. The epoxy-amine reaction between the fluorescent label and the pendent epoxy groups on the GlyMA repeat units was allowed to proceed overnight before the resulting solution was transferred into a gamma-irradiated 30 mL slide-a-lyzer and placed in a 2 L beaker filled with autoclaved deionized water (~2 L) and equipped with an autoclaved stirrer bar. The dialysis fluid was changed regularly (twelve times over a six-day period) until the dialysate was no longer pink/purple. Visible absorption spectroscopy analysis indicated no characteristic absorption band at 564 nm in this dialysate, indicating that all excess unreacted rhodamine piperazine had been removed via dialysis. The contents of this 30 mL slide-a-lyzer were emptied into a sterile 50 mL beaker. Precursor PGMA₅₈-P(HPMA₃₀₀-*stat*-GlyMARh₁) diblock copolymer vesicles (12.0 mL of a 10 % w/w dispersion, 1.20 g copolymer, 0.0225 mmol), ACVA (1.26 mg, 0.00449 mmol, CTA/ACVA molar ratio = 5.0) and DPA monomer (0.479 g, 2.25 mmol, target DP = 100) were weighed into a 25 mL round-bottomed flask and purged with N₂ for 20 min prior to immersion of this flask into an oil bath set at 70 °C for 24 h. The DPA polymerization was quenched by cooling to room temperature followed by subsequent exposure to air. The reaction solution was transferred into a gamma-irradiated 15 mL slide-a-lyzer and placed in a 2 L beaker filled with autoclaved deionized water, equipped with an autoclaved stirrer bar. The dialysis fluid was changed periodically (ten times over a five-day period) to remove unreacted DPA monomer.

Preparation of sterile (97 PGMA₅₈ + 3 PMPC₆₈)-P(HPMA₃₀₀-*stat*-GlyMARh₁)-PDPA₅₂ triblock copolymer vesicles.

All equipment was rigorously cleaned with disinfectant and a 70 % aqueous ethanol solution. All apparatus was autoclaved for 20 min at 121 °C. PGMA₅₈ macro-CTA (0.486 g, 0.0511 mmol), PMPC₆₀ macro-CTA (0.0237 g, 0.00158 mmol), HPMA monomer (2.28 g, 15.8 mmol), GlyMA monomer (7.49 mg, 0.0527 mmol), ACVA initiator (2.96 mg, 0.0105 mmol, CTA/ACVA molar ratio = 5.0) and deionized water (25.1 g, 10 % w/w) were weighed into a 50 mL round-bottomed flask and stirred until the initiator and macro-CTAs were fully dissolved. The solution was sterilized by ultrafiltration during its transfer into a sterile 50 mL round-bottomed flask equipped with a sterile stirrer bar and a rubber septum. The solution was then purged with N₂ for 20 min prior to immersion of the flask in an oil bath set at 70 °C. After 2 h, the HPMA polymerization was quenched by cooling to room temperature with subsequent exposure to air. Rhodamine piperazine (1.00 mL, 28.8 g dm⁻³, 0.0527 mmol) was sterilized by filtration and added to the (97 PGMA₅₈ + 3 PMPC₆₀)-P(HPMA₃₀₀-*stat*-GlyMA₁) diblock copolymer (25.2 mL of a 10% w/w copolymer dispersion containing 0.0527 mmol GlyMA) in a 50 mL round-bottomed flask. The epoxy-amine reaction was allowed to proceed overnight

before transferring the reaction solution into a gamma-irradiated 30 mL slide-a-lyzer. This was then placed in a 2 L beaker containing autoclaved deionized water (2 L), equipped with an autoclaved stirrer bar. The dialysis fluid was changed regularly (twelve times over a six-day period) until the dialysate was no longer pink/purple. Visible absorption spectroscopy analysis indicated no characteristic absorption band at 564 nm in this dialysate, indicating that all excess unreacted rhodamine piperazine had been removed via dialysis. The contents of the slide-a-lyzer were emptied into a sterile 50 mL beaker. Precursor (97 PGMA₅₈ + 3 PMPC₆₀)-(H₃₀₀-*stat*-ERh₁) diblock copolymer vesicles (12.0 mL of a 10 % w/w copolymer dispersion, 1.20 g copolymer, 0.0225 mmol), ACVA (1.26 mg, 0.00448 mmol, CTA/ACVA molar ratio = 5.0) and DPA monomer (0.477 g, 2.24 mmol, target DP = 100) were weighed into a 25 mL round-bottomed flask and purged with N₂ for 20 min prior to immersion of this flask into an oil bath set at 70 °C for 24 h. The DPA polymerization was quenched by cooling to room temperature followed by exposure of the flask's contents to air. The reaction solution was transferred into a gamma-irradiated 15 mL slide-a-lyzer and then placed in a 2 L beaker filled with 2 L autoclaved deionized water and equipped with an autoclaved stirrer bar. The dialysis fluid was changed regularly (ten times over a five-day period) to remove unreacted DPA monomer.

Benzoate-protection of hydroxyl groups for GPC analysis. Triblock copolymers were dissolved in pyridine prior to addition of benzoic anhydride (four equivalents based on GMA and HPMa residues). Esterification of the hydroxyl groups was allowed to proceed for 24 h at room temperature. The benzoate-protected copolymers obtained by this method were always fully soluble in THF (unlike their precursors).

Bioburden test. A typical bioburden test was performed to ensure the sterility of the copolymer dispersions. Samples were tested both in liquid media and also using agar count plates. For the former assay, sterile copolymer nanoparticles (10 % w/w dispersions) were inoculated (1 mL in 2000 mL) into Lurias-Bertani (LB) bacteria growth medium (dextrose, 1.0 g dm⁻³, tryptone, 5.0 g dm⁻³, yeast extract, 2.5 g dm⁻³ to a final pH of 7.0 ± 0.2 and grown overnight at 37 °C with continuous shaking. An increase in turbidity over time is taken to be an indication of bacterial growth. A negative control (bacterial growth medium in PBS buffer) and positive controls (copolymers prepared under conventional non-sterile conditions) were also prepared for comparison. In the case of the agar plate experiments, 10 % w/w copolymer dispersions were diluted by 1:2000 using sterile PBS, inoculated and then spread onto agar petri dishes (100 µL per 35 mm petri dish) for plate counting. This is a non-selective protocol for the detection and plate count of microorganisms (both aerobic and anaerobic) in aqueous solution. A negative control plate (comprising bacteria growth medium plus PBS buffer only) and positive controls (copolymer nanoparticles prepared under conventional non-sterile conditions) were also inoculated. One plate was prepared per batch and sample vial, and each sample vial was tested in triplicate. The plates were incubated in a 37 °C oven overnight. When sterile conditions are compromised, colonies grew on the plates after inoculation for 16-24 h. If plates tested negative for bioburden after 24 h, they were incubated for a further 24 h to ensure their sterility (in case the titre reading was low).

MTT-ESTA. This assay uses 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to assess cellular metabolic potential as an eluted stain bioassay (ESTA) of treated cells after vesicle treatment. In brief, 3–4 × 10⁴ HDF or HeLa cells were cultured per well in 24-well plates until 70% confluence (typically 48 h). Cells were incubated for 24 h with varying concentrations of the aqueous vesicle dispersions, as prepared via PISA according to the above synthetic protocols. After such exposure, cell cultures were thoroughly washed using PBS and then incubated with MTT solution (0.50 g dm⁻³ MTT in PBS, 1.0 mL per well for a 24-well plate) for 45 min at 37 °C and in a 95% air/5% CO₂ environment. Intracellular dehydrogenase activity reduces MTT to give a purple-colored formazan salt. After 45 min, the solution was aspirated and the insoluble intracellular formazan product was

solubilized and released from cells by adding acidified isopropanol (0.30 mL per well for a 24-well plate or 1.0 mL per cm² of cultured tissue) and was incubated for 10 min. The optical density (absorbance) at 570 nm was then measured with the aid of a 630 nm reference filter using a plate-reading spectrophotometer. For statistical analysis (paired student's t-test), experiments were performed in triplicate wells for N = 3 independent experiments.

Cell culture. Primary human dermal fibroblasts (HDFs) were maintained in DMEM supplemented with 10 % v/v foetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 g dm⁻³ streptomycin and 0.625 mg dm⁻³ amphotericin B. Cells were sub-cultured routinely using 0.02 % w/v trypsin-EDTA and used for experimentation between passages 4 and 8. Human breast cancer cell lines MCF-7 and MDA-MB-231 were maintained in DMEM supplemented with 10 % v/v foetal calf serum and 2 mM L-glutamine and sub-cultured using 0.02 % w/v trypsin-EDTA for just ten consecutive passages.

Detection of scavenger receptor expression by flow cytometry. HDFs, MCF-7 and MDA-MB-231 cells were seeded at a density of 1×10^5 cells per well in 1 ml of cell medium and cultured in 6-well plates for two days until 70% confluence. Cells were then detached with trypsin-EDTA and washed using PBS. Aliquots of 2.5×10^5 cells were blocked for 10 min at 4°C in 5% BSA-PBS and then incubated for 1 h at 4°C with either rat-anti-human CD-36 IgG or rabbit-anti-human scavenger receptor B1 IgG primary antibodies (both 1:100 in 1% BSA-PBS). Cells were then washed three times using PBS and incubated for 30 min at 4°C with the secondary antibody (either goat-anti-rat Cy3 IgG or goat-anti-rabbit Cy3 IgG; both 1:1000 in 1% BSA-PBS). Afterwards, cells were washed three times with PBS, pelleted, resuspended in 200 µL PBS at 4°C and plated in 96-well plates in preparation for automated sampler flow analysis using a BD *FACSArray*TM bioanalyzer. A total number of 10,000 events (cells) per sample were assayed for Cy3 signal detection. Cy3 fluorescent IgGs were subsequently energized with a green (532 nm) laser and their signals, along with particle-scattered light, were detected and then processed using BD *FACSArray*TM electronics. Relevant negative control cell aliquots (no antibody-staining) were used to establish background noise. Flow data were collected from N = 3 independent experiments.

Nanoparticle uptake experiments. HDFs, MCF-7 and MDA-MB-231 cells were seeded at a density of 5×10^4 cells per well in 1 ml of cell medium and cultured in 24-well plates for two days until 80% confluence (typically within 24h). The cells were then treated with the aqueous dispersion of rhodamine labelled copolymer vesicles (0.5×10^{-4} g L⁻¹) in the cell growth medium nanoparticles and incubated for 12h. Cell uptake of nanoparticles was detected using an EVOS[®] epifluorescence imaging system. Briefly, the nanoparticle medium with the framboidal vesicles was removed and cells were washed with PBS prior to inspection under the epifluorescence EVOS[®] microscope using a RFP LED cube (Ex: 531/40 Em: 593/40). Afterwards, the cells were detached using trypsin-EDTA and washed using PBS. Cells were pelleted and resuspended in 100 ml flow buffer (PBS with 5% v/v of FBS) and uptake of the fluorescent particles was confirmed using a CyAn Flow Cytometer using the PE/PI/Texas Red signal detection.

DNA plasmid transfection with framboidal vesicles

Bacterial strains, plasmids, and culture conditions. The pEGFP plasmid used for the EGFP expression and transfection efficiency experiments was obtained from Clontech Laboratories (Palo Alto, CA). *Escherichia coli* strain JM109 (Promega, Southampton, UK) was used for propagation and the selection of transformed bacteria was maintained by growing cultures at 37°C in Luria-Bertani (LB) medium in the presence of ampicillin (100 g dm⁻³). Plasmid DNA (pDNA) was isolated from *E. coli* JM109 using Qiagen affinity chromatography columns (Clontech Laboratories). Plasmids were

resuspended in sterile deionized water. The pDNA concentration was calculated using a *NanoDrop*TM UV spectrophotometer 2000 (Thermo-Scientific).

Loading of plasmid DNA within framboidal vesicles by electroporation. Briefly, ~10 μ l of a 0.50 g L⁻¹ aqueous solution of plasmid DNA (5.0 μ g) was added via micropipet to 1.0 ml of a 0.5×10^{-3} g L⁻¹ aqueous dispersion of copolymer vesicles in PBS. This aqueous mixture of plasmid DNA and vesicles was then added to sterile electroporation cuvettes (VWR, UK; 4 mm gap between electrodes) prior to electroporation under the above conditions. Plasmid loading was performed using a BTX Genetronics electroporator (San Diego, CA, USA) with the following settings: two 500 V pulses per sample, a pulse duration of 10 ms with each pulse separated by an interval of 100 ms, polarity (unipolar) and mode (lv). Electroporated samples were recovered under sterile conditions and stored at 4°C prior to transfection experiments. To confirm loading, gel shift assays were performed using 0.8% TBE agarose gels run at 100 v for 30 mins. Loaded samples were compared with free plasmid DNA and unloaded vesicles for gel mobility shifts.

MD-MB-231 cell transfection with pEGFP-loaded framboidal vesicles. MD-MB-231 cells were plated onto 24-well plates at a density of 3×10^4 cells per well per ml in cell medium. When they reached 60% confluence (typically within 24 h), they were treated with the pEGFP-loaded framboidal vesicles. Accordingly, the cell medium was replenished and the vesicles loaded with the electroporated plasmid DNA were diluted ten-fold by volume to afford plasmid DNA (500 ng) plus aqueous dispersion of copolymer vesicles (0.5×10^{-4} g L⁻¹) in the cell growth medium. Transfection experiments were carried out using Lipofectamine 2000TM as a positive control according to the manufacturer's protocol. A negative control (non-transfected cells alone) was also included. An additional negative control was performed whereby empty framboidal vesicles were mixed with the free EGFP plasmid but not electroporated (i.e. not loaded) prior to cell treatment. Cells were incubated overnight (typically 16 h) with the plasmid DNA-loaded framboidal vesicles (or as described in the controls). Expression of EGFP was determined using an EVOS[®] epifluorescence imaging system. Briefly, the transfecting medium with the framboidal vesicles was removed and cells were washed with PBS prior to inspection under the epifluorescence EVOS[®] microscope using a GFP cube (ex 470/22 Em 51).

[N.B. Fibroblasts undergo endocytosis at a relatively moderate rate of 1% of its plasma membrane per minute. In receptor-mediated endocytosis, about 25% of the fibroblast cell membrane undergo invagination (which is the crucial first step for endocytosis) at any given time point. Hence substantial intracellular uptake should be achieved within 16 h. Formation of the endosome takes about 1 min and within 5–15 min the endocytosed cargoes are transported to late endosomes, which is close to the cell nucleus. Thus it typically takes about 16 min for the framboidal vesicles to reach the nucleus. This time frame has also been observed for other tumor cells (see B. Tycko et al., *J. Cell Biology*, **1983**, 97, 1762-1776). In our experiments, the vesicles were always present in large excess during cell uptake experiments. Cells remained in contact with the vesicles for the entire duration of all experiments. We deliberately chose a 16 h time frame that was below the 'doubling time' (approximately 18-20 h for cancer cells and 24 h for HDF cells), which is standard practice for such uptake experiments].

Characterization

¹H NMR Spectroscopy. All ¹H NMR spectra were recorded in either CD₃OD or CD₃OD/DCI using a 400 MHz Bruker Avance-400 spectrometer (64 scans averaged per spectrum).

Gel Permeation Chromatography (GPC). Molecular weights and dispersities were determined using three different GPC set-ups, depending on the copolymer type. All chromatograms were analyzed using Varian Cirrus GPC software (version 3.3) provided by the instrument manufacturer (Polymer Laboratories). The DMF GPC set-up operated at 60 °C and comprised two Polymer Laboratories PL gel 5 μm Mixed C columns connected in series to a Varian 390 LC multi-detector suite (only the refractive index detector was utilized) and a Varian 290 LC pump injection module. The GPC eluent was HPLC-grade DMF containing 10 mM LiBr at a flow rate of 1.0 mL min⁻¹. DMSO was used as a flow-rate marker. Calibration was conducted using a series of ten near-monodisperse poly(methyl methacrylate) standards ($M_n = 645 - 618,000 \text{ g mol}^{-1}$). The THF GPC set-up comprised two 5 μm (30 cm) Mixed C columns and a WellChrom K-2301 refractive index detector operating at $950 \pm 30 \text{ nm}$. The mobile phase contained 2.0 % v/v triethylamine and 0.05 w/v % butylhydroxytoluene (BHT) and the flow rate was 1.0 mL min⁻¹. A series of ten near-monodisperse poly(methyl methacrylate) standards (M_p values ranging from 645 to 2,186,000 g mol⁻¹) were used for calibration. The aqueous GPC set-up comprised an Agilent 1260 Infinity series degasser and pump, and two Agilent PL Aquagel-OH 30 8 μm columns in series. This column set-up was calibrated with twelve near-monodisperse poly(ethylene oxide) standards with M_p values ranging from 1,080 to 905,000 g mol⁻¹. Copolymer solutions were analyzed in an acidic aqueous buffer containing 0.5 M acetic acid (pH 2), 0.3 M NaH₂PO₄ and acidified with concentrated HCl at a flow rate of 1.0 mL min⁻¹.

Dynamic Light Scattering (DLS). Intensity-average hydrodynamic diameters were measured at 25°C using a Malvern Zetasizer NanoZS model ZEN 3600 instrument at a fixed scattering angle of 173°. Aqueous dispersions of 0.10 % w/w were analyzed using disposable cuvettes and all data were averaged over three consecutive runs. The Stokes-Einstein equation was used to calculate the mean hydrodynamic diameter.

Visible absorption spectroscopy. This technique was used to estimate the extent of reaction of the rhodamine B piperazine dye label with the epoxy groups. The (97 PGMA₅₈ + 3 PMPC₆₈)-P(HPMA_{300-stat}-GlyMARh₁) diblock copolymer precursor vesicles was molecularly dissolved in methanol after the epoxy-amine reaction (conducted using an epoxy/amine molar ratio of 1.0) and diluted two-fold to afford a copolymer concentration of 5.0% w/w. The visible absorption spectrum of this copolymer solution recorded using a Shimadzu UV1800 spectrophotometer gave an absorbance of 0.71. Using the molar extinction coefficient of 96,100 M⁻¹ cm reported for the rhodamine B dye label in the literature (see T. Nguyen and M. B. Francis, *Org. Lett.*, **2003**, 5, 3245-3248) indicated a mean degree of dye functionalization of approximately 80%.

Transmission Electron Microscopy (TEM). As-synthesized copolymer dispersions were diluted fifty-fold at 20 °C to generate 0.10 % w/w dispersions. Copper/palladium TEM grids (Agar Scientific, UK) were surface-coated in-house to yield a thin film of amorphous carbon. The grids were then plasma glow-discharged for 30 s to create a hydrophilic surface. Aqueous droplets (12 μL) of 0.1 % w/w copolymer dispersions were placed onto freshly glow-discharged grids using a micropipette for 20 s and then blotted with filter paper to remove excess solution. To stain the diblock copolymer vesicles, uranyl formate (9 μL of a 0.75 w/v % solution) was soaked on the vesicle-loaded grid for 20 s and then carefully blotted to remove excess stain. To stain the triblock copolymer vesicles, a 9 μL droplet of a 1.0 w/v % phosphotungstic acid solution was placed on each vesicle-loaded grid for 5 s using a micropipette and then carefully blotted to remove excess stain. Each grid was then carefully dried using a vacuum hose. Imaging was performed using a FEI Tecnai Spirit TEM instrument equipped with a Gatan 1kMS600CW CCD camera operating at 120 kV.

Small-Angle X-ray Scattering (SAXS). Synchrotron SAXS patterns were recorded at station I22 of the Diamond Light Source (Didcot, UK). A monochromatic X-ray radiation (wavelength $\lambda = 0.1001$ nm) and 2D SAXS detector (Pilatus 2M, Dectris Ltd., Baden-Daettwil, Switzerland) were used for these experiments. The SAXS camera length set-up covered the q range from 0.01

nm⁻¹ to 1.9 nm⁻¹, where $q = \frac{4\pi\sin\theta}{\lambda}$ is the modulus of the scattering vector and θ is one-half of the scattering angle. Glass capillaries with a diameter of 2.0 mm were used as sample holders. 2D X-ray scattering data were reduced to 1D scattering patterns using Dawn software available at station I22 and were further analyzed (including normalization, background subtraction and model fitting) using Irena SAS macros for Igor Pro.¹ SAXS experiments were conducted on 1.0 % w/w aqueous copolymer dispersions.

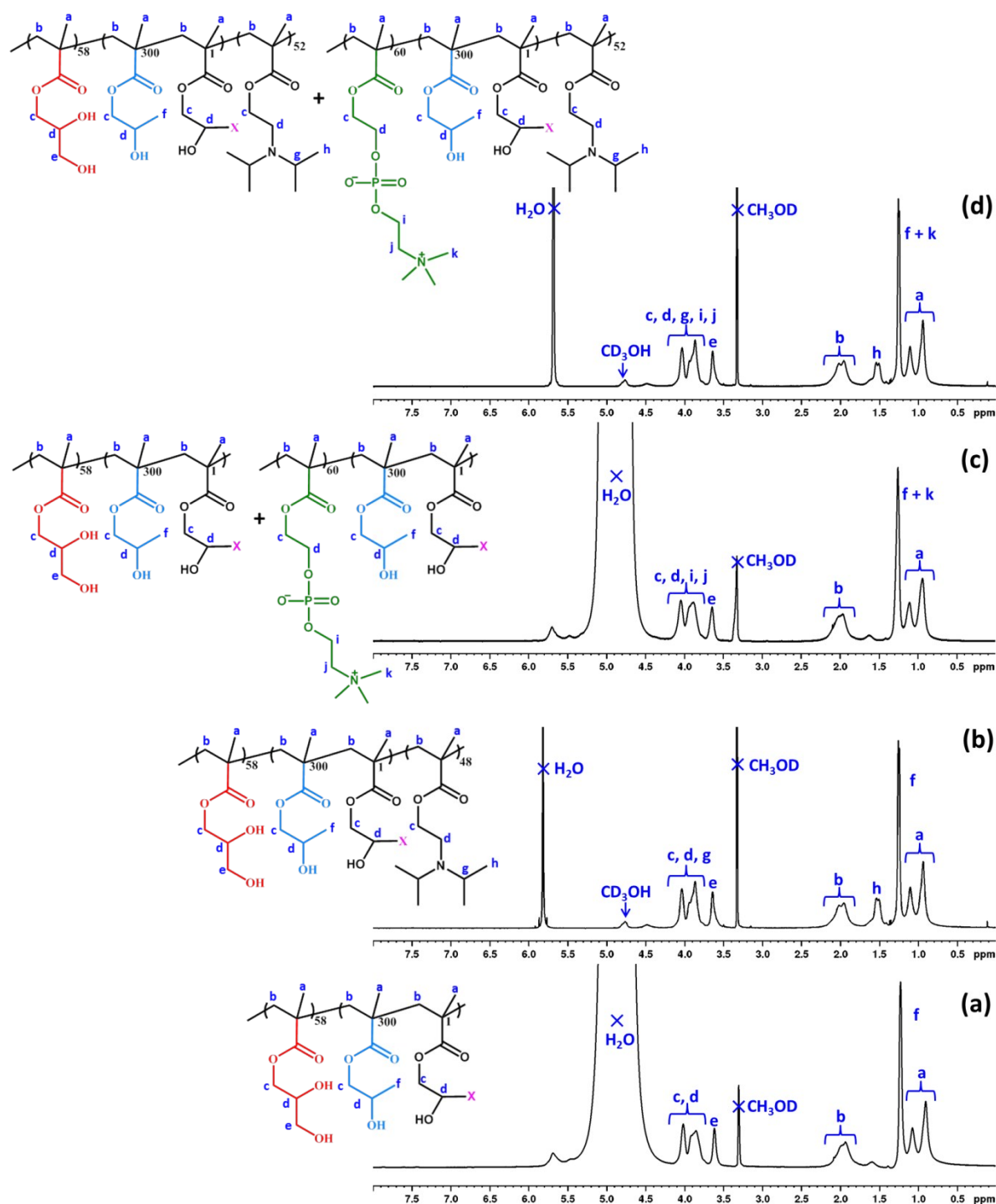


Figure S1. Assigned ^1H NMR spectra recorded in $\text{CD}_3\text{OD}/\text{DCl}$ for: (a) $\text{G}_{58}-(\text{H}_{300}\text{-stat-ERh}_1)$ diblock copolymer, (b) $\text{G}_{58}-(\text{H}_{300}\text{-stat-ERh}_1)\text{-D}_{48}$ triblock copolymer, (c) $(97 \text{ G}_{58} + 3 \text{ M}_{60})-(\text{H}_{300}\text{-stat-ERh}_1)$ diblock copolymer and (d) $(97 \text{ G}_{58} + 3 \text{ M}_{60})-(\text{H}_{300}\text{-stat-ERh}_1)\text{-D}_{52}$ triblock copolymer. In each case these copolymers were prepared under rigorously sterile conditions. [N.B. 'X' represents the conjugated rhodamine dye molecule in each of the six chemical structures shown above. The remaining unreacted epoxy groups are gradually converted into glycerol groups via slow hydrolysis during the long-term storage of such aqueous dispersions. As such, they become spectroscopically indistinguishable from the glycerol monomethacrylate residues on the stabilizer block].

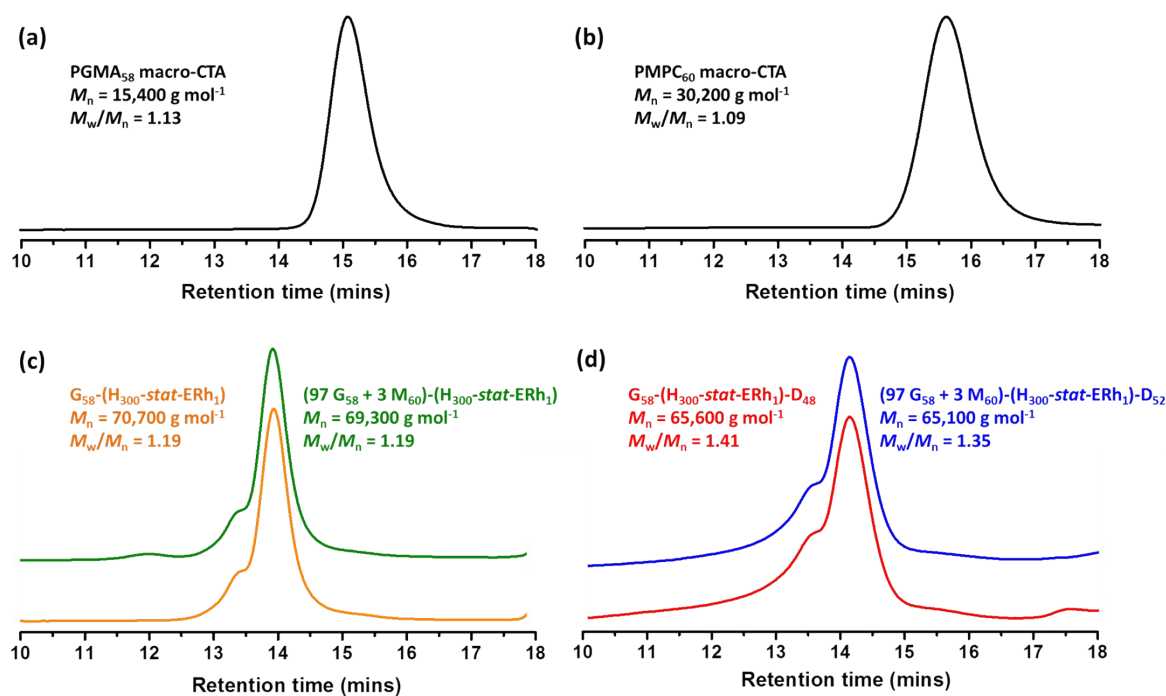


Figure S2. (a) DMF GPC curve (vs. PMMA standards) obtained for the PGMA₅₈ macro-CTA. (b) Aqueous GPC curve (vs. PEO standards) obtained for the PMPC₆₀ macro-CTA. (c) DMF GPC curves (vs. PMMA standards) obtained for the $G_{58}-(H_{300}\text{-stat-ERh}_1)$ diblock copolymer (orange) and the $(97 G_{58} + 3 M_{60})-(H_{300}\text{-stat-ERh}_1)$ diblock copolymer (green), which were both prepared under sterile conditions. (d) THF GPC curves (vs. PMMA standards) obtained for the benzoate-modified $G_{58}-(H_{300}\text{-stat-ERh}_1)\text{-D}_{48}$ triblock copolymer and the benzoate-modified $(97 G_{58} + 3 M_{60})-(H_{300}\text{-stat-ERh}_1)\text{-D}_{52}$ triblock copolymer, which were prepared under rigorously sterile conditions.

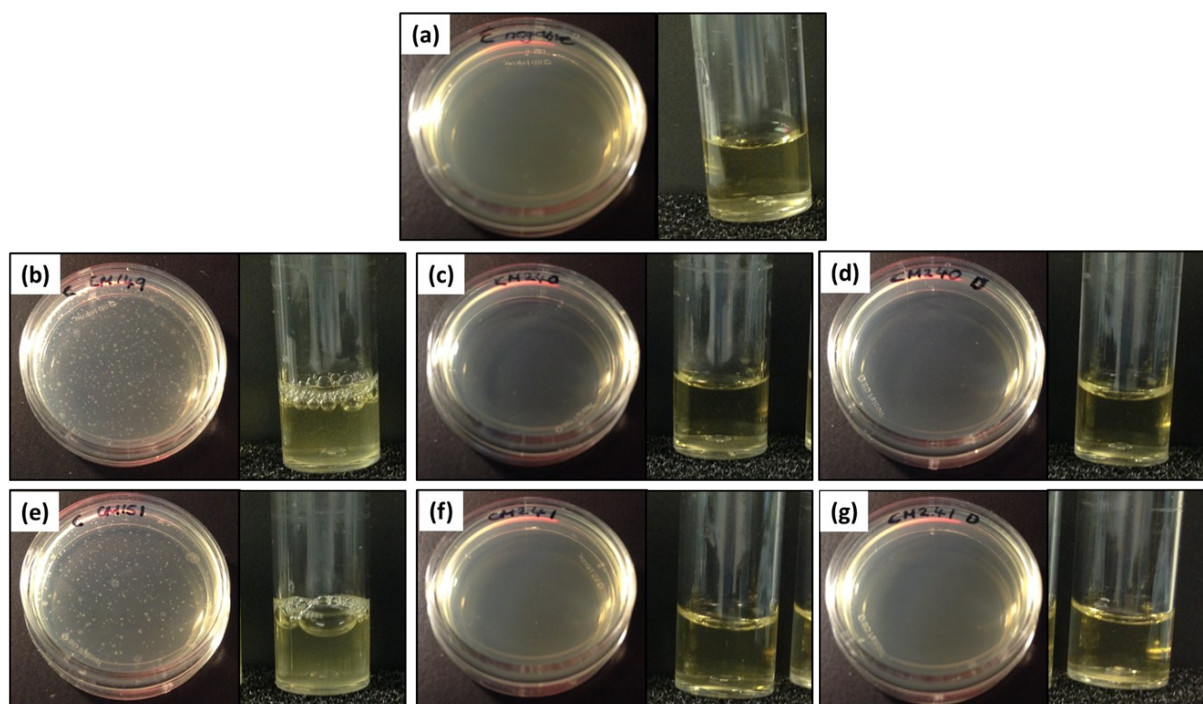


Figure S3. Bioburden tests undertaken using agar count plates (left) and liquid LB medium (right) for (a) a negative control sample containing no nanoparticles, (b) G_{58} - H_{300} diblock copolymer vesicles prepared under normal (non-sterile) conditions, (c) G_{58} -(H_{300} -*stat*-ERh₁) diblock copolymer vesicles prepared under sterile conditions, (d) G_{58} -(H_{300} -*stat*-ERh₁)-D₄₈ triblock copolymer vesicles prepared under sterile conditions, (e) G_{58} - H_{300} -D₈₆ triblock copolymer vesicles prepared under normal (non-sterile) conditions, (f) (97 G_{58} + 3 M_{60})-(H₃₀₀-*stat*-ERh₁) diblock copolymer vesicles prepared under sterile conditions and (g) (97 G_{58} + 3 M_{60})-(H₃₀₀-*stat*-ERh₁)-D₅₂ triblock copolymer vesicles prepared under sterile conditions. Note the slightly greater turbidity (and bubbles) in the liquid LB medium and the discernible colony growth on the agar plates for copolymers (b) and (e) prepared under non-sterile conditions.

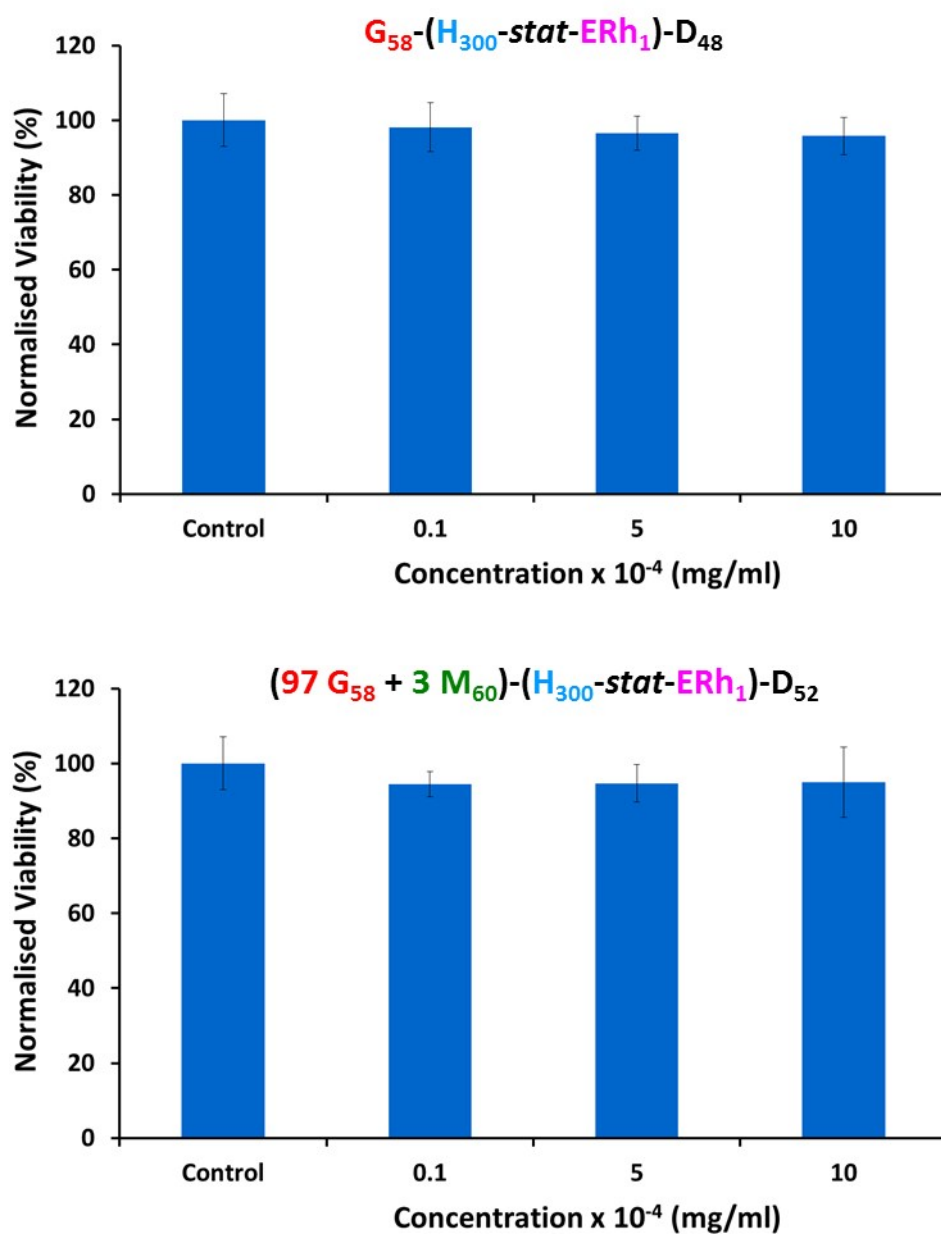


Figure S4. MTT tests for $G_{58}-(H_{300}\text{-stat-ERh}_1)\text{-D}_{48}$ triblock copolymer vesicles (left) and $(97 G_{58} + 3 M_{60})-(H_{300}\text{-stat-ERh}_1)\text{-D}_{52}$ triblock copolymer vesicles (right), which were both prepared under sterile conditions. N.B. N = 3 experiments performed in triplicate wells.

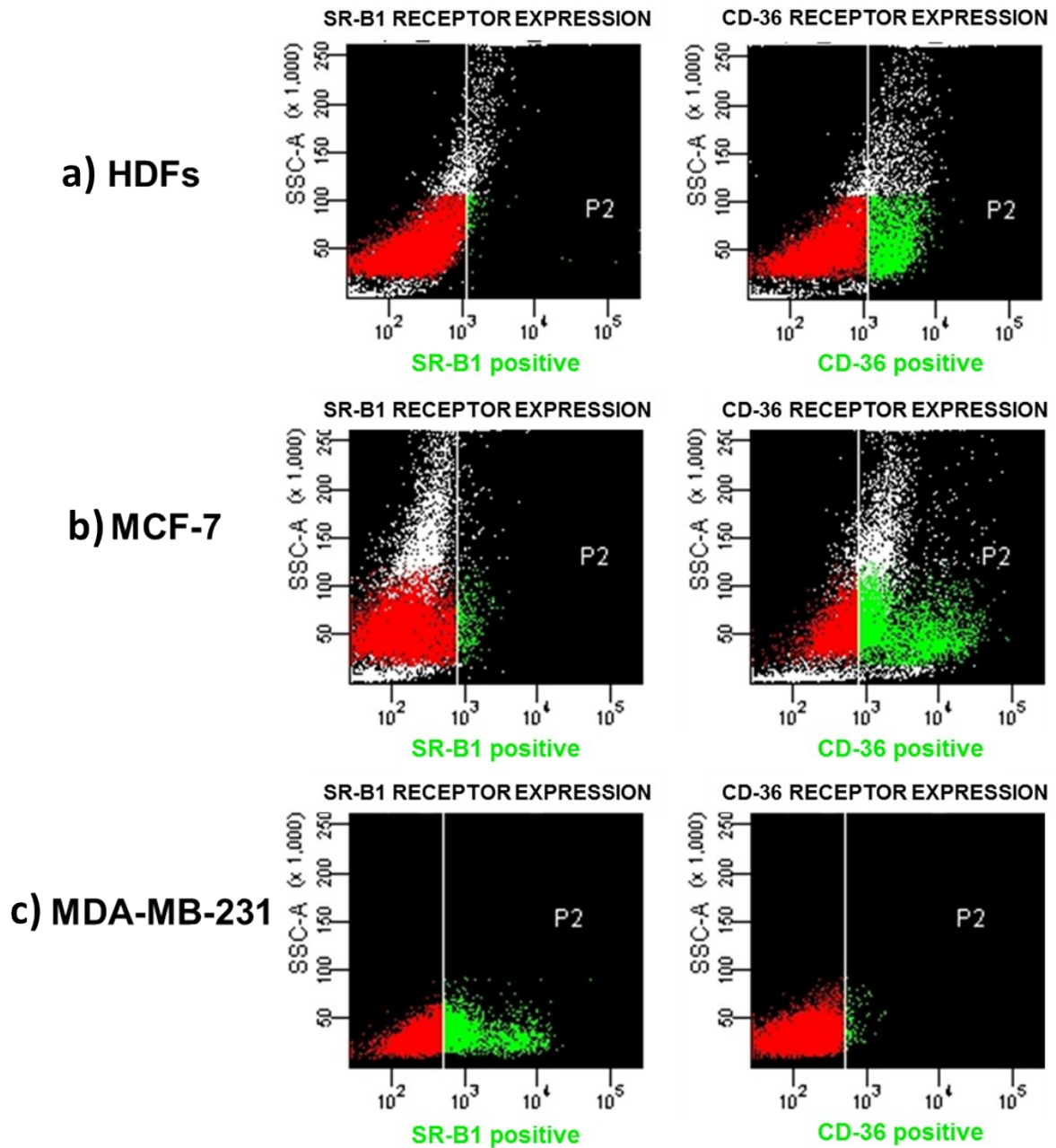
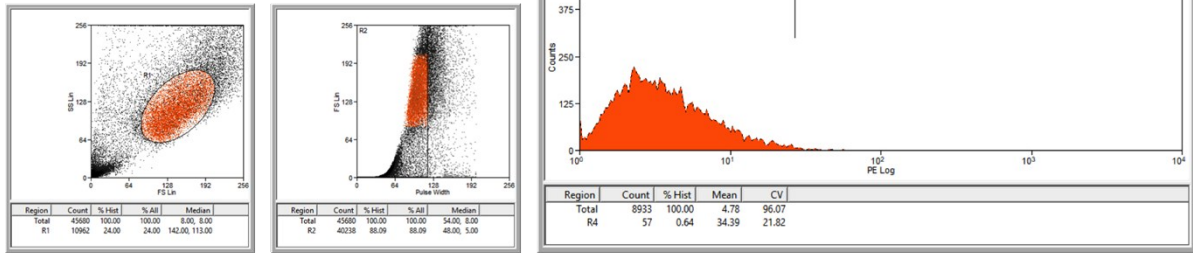
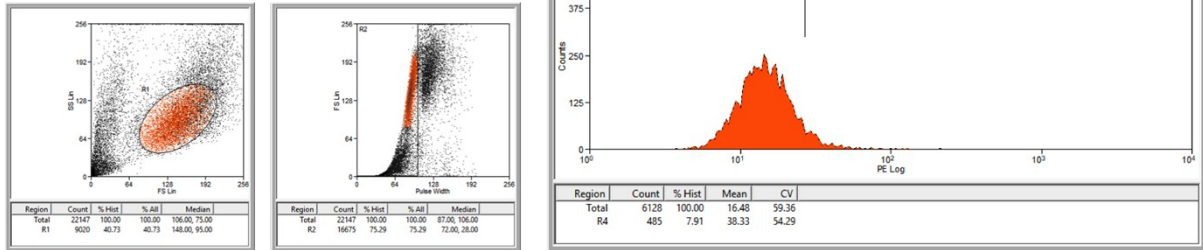


Figure S5. Flow cytometry experiments reveal the expression of SR-B1 and CD-36 receptors by control cells and target breast cancer cells. (a) Human dermal fibroblast (**HDF**) cells are normal (non-cancerous) cells. They do not express the SR-B1 receptor but test positive for the CD-36 receptor. (b) **MCF-7** is a breast cancer cell line that exhibits very low expression of SR-B1 but overexpresses CD-36. (c) **MDA-MB-231** are triple negative breast cancer cells that overexpress SR-B1 but express negligible levels of the CD-36 receptor.

HDFs



MCF7



MDMB231

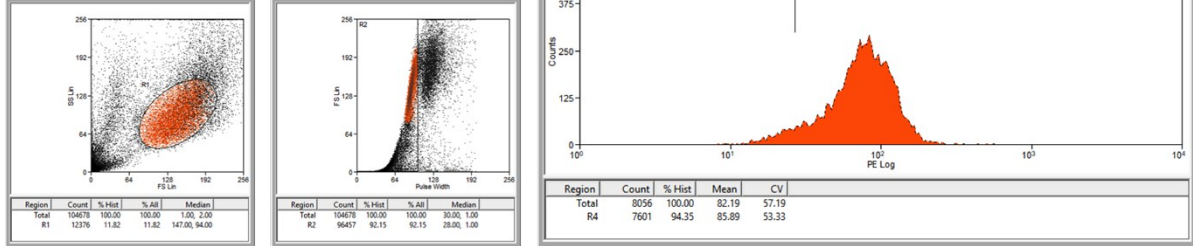


Figure S6. Uptake of $(97\text{ G}_{58} + 3\text{ M}_{60})\text{-(H}_{300}\text{-stat-ERh}_1\text{)-D}_{52}$ framboidal vesicles by HDF cells (top), MCF-7 cells (middle) and MDA-MB-23 cells (bottom) monitored by flow cytometry. Far left graphs indicate FS vs. SS, middle graphs indicate pulse width vs. SS and far right graphs indicate the fluorescence intensity of the rhodamine-labeled framboidal vesicles taken up by the three types of cells analyzed using an Orange FL3 equipped with Ex613/Em20 for detection of rhodamine. R4 denotes the limit that denotes positive uptake with respect to the negative control.

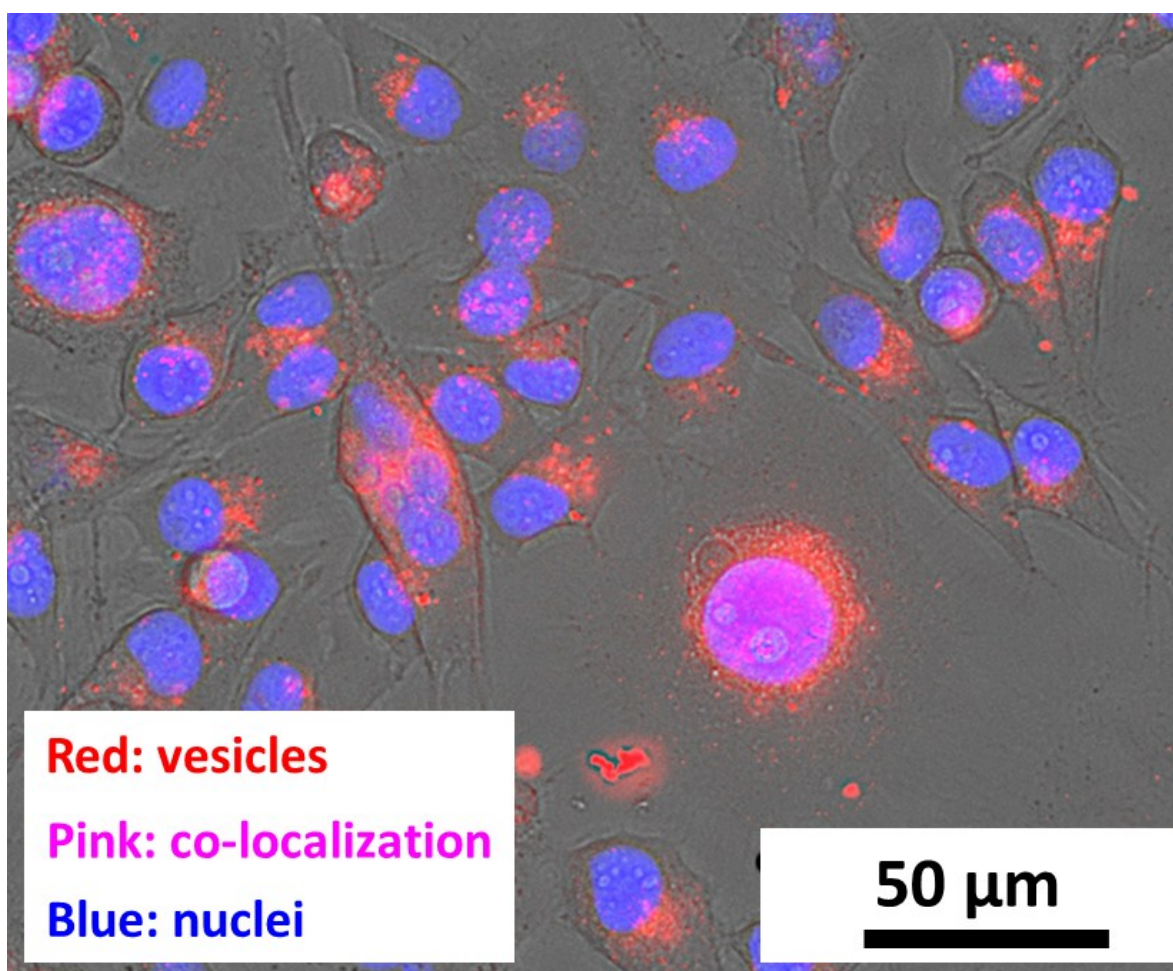


Figure S7. Uptake of rhodamine-labeled $(97 \text{ G}_{58} + 3 \text{ M}_{60})\text{-(H}_{300}\text{-stat-ERh}_1\text{)-D}_{52}$ framboidal vesicles by MDA-MB 231 cells for 16 h. The fluorescence micrograph indicates the rhodamine-labeled nanoparticles (red) that are internalized by these cancer cells. Nuclear staining was performed using Hoechst 33342 dye (blue). Pink regions indicate co-localization of the fluorescently-labeled nanoparticles and DNA.

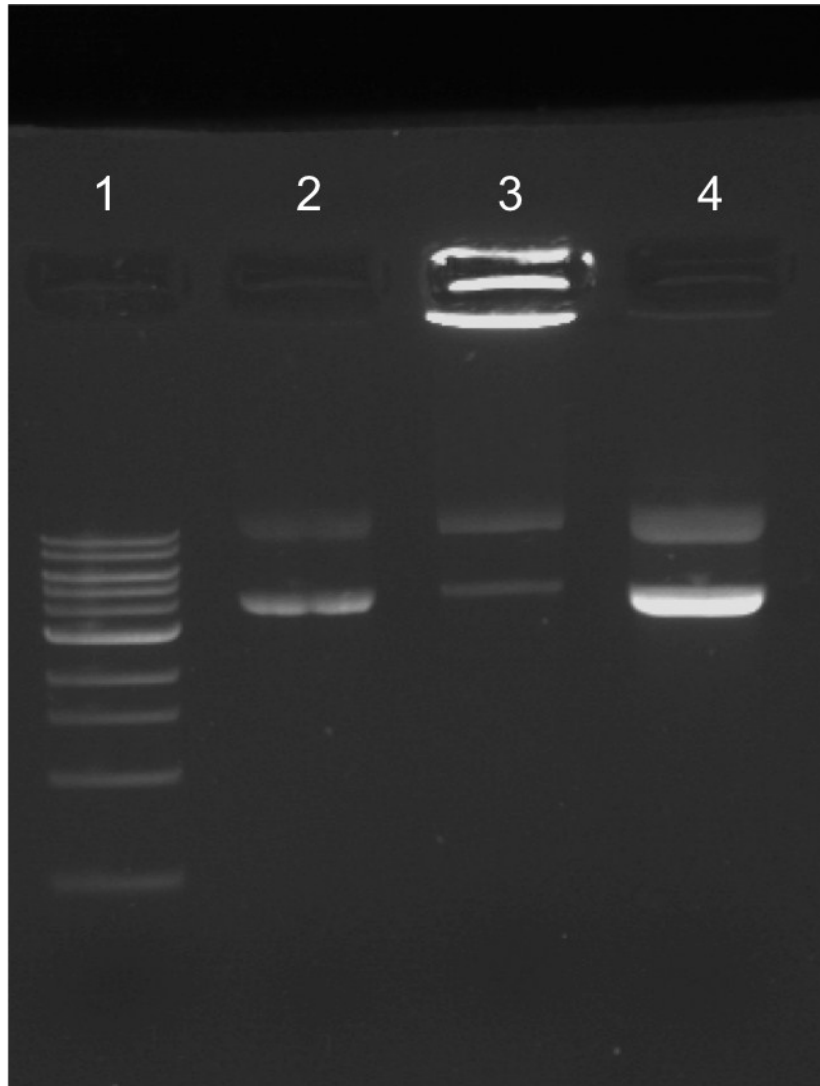


Figure S8. Gel shift assay using a 0.8% TBE agarose gel. The gel was run in TBE buffer for approximately 30 min at an applied voltage of 100 V. Lane 1: 1kb DNA ladder (0.50 µg). Lane 2: pEGFP uncut plasmid (50 ng). Lane 3: 5.0 µl of (97 G₅₈ + 3 M₆₀)-(H₃₀₀-stat-ERh₁)-D₅₂ framboidal vesicles after electroporation in the presence of pEGFP, Lane 4: 5.0 µl of the same binary mixture of (97 G₅₈ + 3 M₆₀)-(H₃₀₀-stat-ERh₁)-D₅₂ framboidal vesicles + pEGFP without any electroporation, for which there is no shift with respect to the control shown in Lane 2. Note that most of the DNA in the Lane 3 sample has remained at the top of the well, showing that the encapsulated plasmid DNA has shifted relative to the non-electroporated control (Lane 4) and the free plasmid DNA (Lane 2). In Lane 3, there are also bands corresponding to free plasmid DNA (compared to Lane 2), suggesting that the vesicles do not hinder the migration of this component. It is also worth emphasizing that most of the supercoiled band has disappeared in the free plasmid DNA (compare Lane 3 to Lane 4), indicating that this relatively compact conformation is much more likely to be encapsulated within the vesicles compared to the corresponding relaxed conformation. Indeed, ImageJ software analysis indicates that approximately 87% of the supercoiled pEGFP becomes encapsulated within the vesicles during electroporation, which corresponds to almost 70% of the total plasmid DNA.

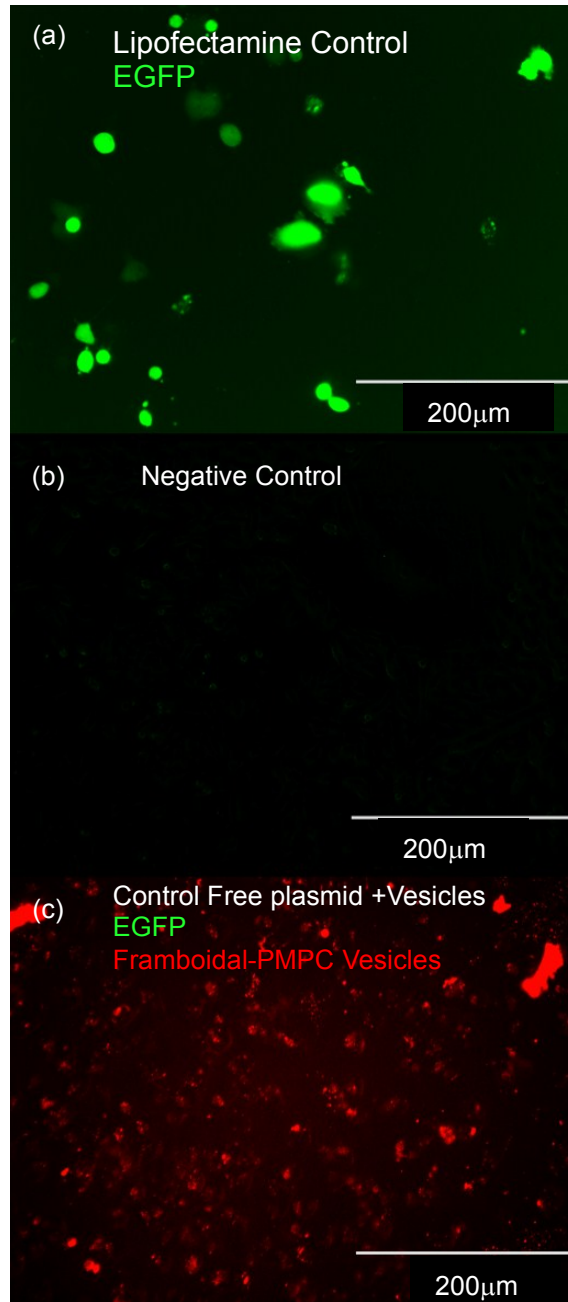


Figure S9. Controls for the intracellular delivery of EGFP plasmid DNA within MDA-MB-231 cells using $(97 G_{58} + 3 M_{60})-(H_{300}-stat-ERh_1)-D_{52}$ framboydal vesicles. (a) Positive control: Lipofectamine transfected MDA-MB-231 cells shown in green. (b) Negative control: no transfection agents or plasmid DNA or vesicles (just MDA-MB-231 cells). (c) Negative control: uptake of rhodamine-labeled $(97 G_{58} + 3 M_{60})-(H_{300}-stat-ERh_1)-D_{52}$ framboydal vesicles by MDA-MB 231 cells. The red fluorescence indicates rhodamine-labeled framboydal vesicles that are internalized by these cancer cells. In this case there is no expression of EGFP plasmid DNA because this component was not encapsulated within the vesicles but instead merely present as free chains in solution.

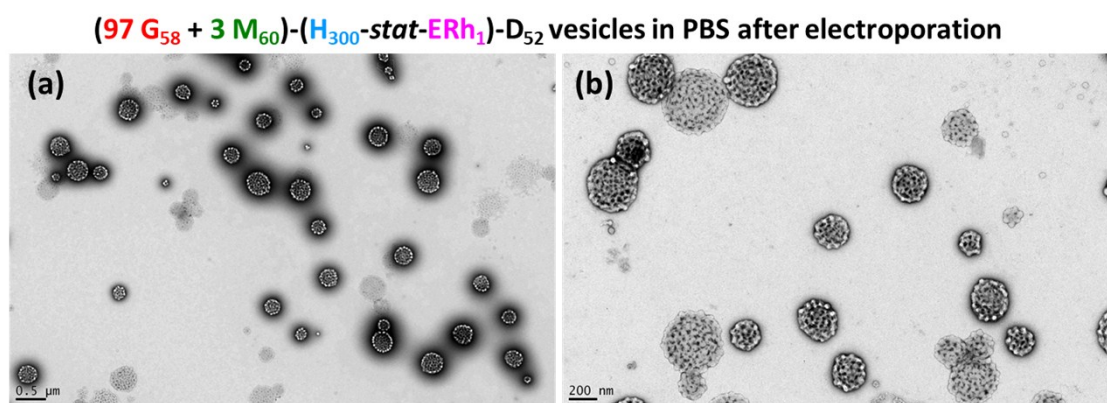


Figure S10. Representative TEM images obtained for (97 G₅₈ + 3 M₆₀)-(H₃₀₀-stat-ERh₁)-D₅₂ framboidal vesicles after electroporation to encapsulate plasmid DNA (pEGFP). (a) Low magnification TEM image and (b) high magnification TEM image. These images confirm that the original vesicle morphology remains intact after electroporation.

Table S1. SAXS structural parameters obtained for the morphologies formed by the (97 G₅₈ + 3 M₆₀)-(H₃₀₀-*stat*-ERh₁)-D₅₂ triblock copolymer in aqueous solution at various pH. The volume and scattering length density of the brush/corona block (V_{brush} and ξ_{brush} , respectively) and the core block (V_{core} and ξ_{core} , respectively) were calculated from the copolymer composition and mass density of the blocks, and used as fixed parameters for the fitting. Representative parameters for population 1 corresponding to vesicles: R_{mc} is the radius from the centre of the vesicle to the centre of the membrane and $\sigma_{R_{\text{mc}}}$ is the associated standard deviation, T_{mc} is the thickness of the hydrophobic component of the membrane and $\sigma_{T_{\text{mc}}}$ is the associated standard deviation, D_v is the mean vesicle

diameter such that $D_v = 2(R_{\text{mc}} + \frac{1}{2}T_{\text{mc}} + 2R_g)$, where the R_g is the radius of gyration of the brush/corona block. Representative parameters for population 2 corresponding to weakly-interacting cationic spherical micelles: R_s is the core radius, σ_{R_s} is the standard deviation of the core radius, R_{PY} is the Percus-Yevick correlation radius of densely-packed spherical micelles and F_{PY} is the Percus-Yevick effective volume fraction of the packed micelles. c_2/c_1 is the ratio of the volume fraction of the copolymer that forms spherical micelles (population 2) to that forming vesicles (population 1).

Copolymer Composition	pH	V_{brush}^b /nm ³	V_{core}^c /nm ³	ξ_{brush}^b $\times 10^{10}$ /cm ⁻²	ξ_{core}^c $\times 10^{10}$ /cm ⁻²	Population 1 – Vesicles ^a			c_2/c_1	Population 2 – Spherical Micelles ^a			Population 3 – Mass Fractals
						R_{mc} ($\sigma_{R_{\text{mc}}}$) /nm	T_{mc} ($\sigma_{T_{\text{mc}}}$) /nm	D_v / nm		R_s (σ_{R_s}) /nm	R_{PY} /nm	F_{PY} /nm	d
(97 G ₅₈ + 3 M ₆₀)-(H ₃₀₀ - <i>stat</i> -ERh ₁)-D ₅₂	7.4	12.1	77.7	11.96	10.80	131 (32)	22.1 (5.0)	294	0.149	9.01 (1.7)	12.5	0.48	
	5.5	12.1	77.7	11.96	10.80	137 (32)	21.5 (5.0)	305	0.137	8.12 (1.6)	12.7	0.48	
	3.0	29.6	60.2	10.73	11.06					7.15 (4.2)			1.76

^a Fixed fitting parameters: $R_g = 2.4$ nm and $x_{\text{sol}} = 0.4$.

^b At pH 7.4 and 5.5, $V_{\text{brush}} = \frac{M_{n, \text{PGMA}}}{N_A \rho_{\text{PGMA}}}$ and $\xi_{\text{brush}} = \xi_{\text{PGMA}}$. At pH 3.0, $V_{\text{brush}} = \frac{M_{n, \text{PGMA}}}{N_A \rho_{\text{PGMA}}} + \frac{M_{n, \text{PDPA}}}{N_A \rho_{\text{PDPA}}}$ and $\xi_{\text{brush}} = \varphi_{\text{PGMA}} \xi_{\text{PGMA}} + (1 - \varphi_{\text{PGMA}}) \xi_{\text{PDPA}}$ where φ_{PGMA} is the volume fraction of PGMA in the corona.

At pH 7.4 and 5.5, $V_{\text{core}} = \frac{M_{n,PHPMA}}{N_A \rho_{PHPMA}} + \frac{M_{n,PDPA}}{N_A \rho_{PDPA}}$ and $\xi_{\text{core}} = \varphi_{PHPMA} \xi_{PHPMA} + (1 - \varphi_{PHPMA}) \xi_{PDPA}$ where φ_{PHPMA} is the volume fraction of PHPMA in the core-forming block. At pH 3.0, $V_{\text{core}} = \frac{M_{n,PHPMA}}{N_A \rho_{PHPMA}}$ and $\xi_{\text{core}} = \xi_{PHPMA}$.

References

- (1) Ilavsky, J.; Jemian, P. R. *J. Appl. Crystallogr.* **2009**, 42, 347.