Supplementary Information

Optimization of nanofiber micelleplexes for DNA delivery

Steven T. G. Street[1,2,3,†] *Hayley C. Parkin,*[2,3] *Lennard Shopperly,* [4,5] *Josie Chrenek,*[4,5] *Keiran Letwin,*[4,5] *Stephanie M. Willerth,*[3,4,5*] *and Ian Manners*[2,3*]

[1] School of Chemistry, University of Bristol, Bristol BS8 1TS, United Kingdom

[2] Department of Chemistry, University of Victoria, Victoria, BC V8W 3V6, Canada

[3] Centre for Advanced Materials and Related Technology (CAMTEC), University of Victoria, 3800 Finnerty Rd, Victoria, BC, V8P 5C2, Canada

[4] Department of Mechanical Engineering, Division of Medical Sciences, University of Victoria, Victoria, BC V8W 2Y2, Canada

[5] School of Biomedical Engineering, University of British Columbia, Vancouver, BC V6T 1Z4, Canada

* Corresponding Author: willerth@uvic.ca and imanners@uvic.ca

Contents

General Experimental Considerations

All reagents and solvents were purchased from Sigma-Aldrich, Acros, Fluka, Fisher Chemical and Alfa Aesar, and used as received unless otherwise noted. All reactions were carried out in an MBraun MB150B-G glove box under nitrogen atmosphere or using standard Schlenk line techniques. Solvents used for self-assembly were HPLC grade and were filtered through PTFE, nylon or cellulose membranes with a pore size of 200 nm before use. Anhydrous solvents were obtained using a modified Grubbs system of alumina columns manufactured by Anhydrous Engineering.[S1] RAFT polymerizations were performed in custom-made Schlenk-vials to fit dry heating blocks. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) was dried over CaH_2, and purified by distillation under reduced pressure. Reactions were monitored by thin layer chromatography (TLC) on Kieselgel 60 F_{254} (Merck). Aromatic compounds were detected with UV light (254 or 365 nm), and amines were detected by staining with ninhydrin. Solvents for TLC are listed in volume:volume percentages. Extracts were concentrated in vacuo using both a Heidolph Hei-VAP Advantage rotary evaporator (bath temperatures up to 50 °C) at a pressure of 15 mmHg (diaphragm pump), and a high vacuum line at room temperature. Reagents used for ROP were dried via vacuum desiccation over phosphorus pentoxide prior to use. DNA concentrations were determined by UV-absorbance using either a NanoDrop 2000 Spectrophotometer from Thermo Scientific, or by using a Cytation 5 plate reader from BioTek, equipped with a take3 microvolume plate. Cell lines were purchased from the American Type Culture Collection (ATCC) through Cedarlane Corporation (Canada). Cell culture media and additives were purchased from Gibco (Thermo Fischer Scientific). The Dulbecco's Minimal Essential Medium (DMEM) formulation contained high glucose (4.5 g/L), Sodium Pyruvate (0.11 g/L), GlutaMAX™, and Phenol Red (15 mg/L), and was missing HEPES (catalogue number: 10569044). The Minimal Essential Medium (MEM) formulation contained GlutaMAX™, and Phenol Red (10 mg/L), and was missing HEPES (catalogue number: 41090101). Phosphate Buffered Saline (PBS) contained NaCl (9 g/L), KH_2PO_4 (144 mg/L) and Na_2HPO_4-7.H2O (795 mg/L, catalogue number: 10010049). TrypLE Express™ was provided with EDTA (458 mg/L) and without Phenol Red (catalogue number: 12604021).

Instrumentation

Preparatory Gel permeation chromatography (Prep GPC)

Preparatory gel permeation chromatography was performed on a Shimadzu Prep GPC equipped with a CBM-20A communications bus module, LC-20AP solvent delivery unit, SIL-10AP autosampler, CTO-40C column oven,SPD-40 UV-Vis detector, RID-20A refractive index detector, and FRC-10A fraction collector. An initial injection of polymer in THF (1 mL, 10 mg/mL) at a flow rate of 3 mL/min using HPLC grade THF as eluent was used to gather the retention times of the species in solution. Using this data, the fraction collector was calibrated to separate the desired peaks into separate vials. Subsequent injections (3 mL, 10 mg/mL, 3 mL/min) were repeated until the desired volume was collected. The resulting solutions were concentrated *in vacuo* to yield the final polymers.

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS measurements were performed using a Bruker Ultraflextreme running in linear mode. MALDI-TOF samples were prepared by depositing approximately 1 μL of the sample (2 mg/mL in THF) onto a stainless-steel sample plate, followed by the deposition of approximately 2 μL of *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile matrix (20 mg/mL in THF), and the sample was allowed to dry in air. If the crystalline matrix could not be observed on the plate, a further aliquot of matrix was added, and the sample dried in air until crystallization was observed. For all samples, a second spot was also prepared with the addition of sodium trifluoroacetate (20 mg/mL in THF) to supress K^+ adducts. The best spectrum was selected for each sample (with/without sodium trifluoroacetate).

Gel permeation chromatography (GPC)

GPC was conducted on a Malvern OMNISEC chromatograph equipped with a refractive index (RI), UV/Vis photodiode detector array, light scattering detector and viscometer. Triethylamine/THF (1% *v/v*) was used as the eluent, with the flow rate set at 1 mL/min. The columns used were T3000, followed by T5000 (Viscotek) at a constant temperature of 35 °C. The calibration (universal) of the RI detector was carried out using polystyrene standards (Viscotek).

Samples were prepared at 1 mg/mL in eluent and filtered through a polytetrafluorethylene membrane filter, pore size = 0.2 µm.

NMR Spectroscopy

^{1}H and ^{13}C NMR spectra were obtained at 25 °C in the solvent specified with Varian or Bruker spectrometers (some equipped with a cryoprobe), operating at the field strengths listed. Chemical shifts are quoted in parts per million with spectra referenced to the residual solvent peak. Multiplicities are abbreviated as: br (broad), s (singlet), d (doublet), t (triplet), q (quartet), p (pentet), m (multiplet) and *app.* (apparent) or combinations thereof. Assignments of ^{1}H-NMR and ^{13}C-NMR signals were made where possible, using COSY, HSQC and HMBC experiments. The DP_n of PFTMC was determined via MALDI-TOF MS, whilst the DP_n of PDMAEMA was determined via 1H-NMR spectrometry by comparing the integration of the PFTMC aromatic protons to the N-CH_3 protons of PDMAEMA.

Ultrasonication

Micelle sonication was carried out using a Hielscher UP100H sonication probe (100W output power) or a Fisherbrand 112xx series advanced ultrasonic cleaner (FB-11203) (37 MHz at 80 % power).

Transfer of samples into water

Samples were transferred into water either through dialysis or through gradual infusion of water and evaporation of residual organic solvent.

<u>For dialysis:</u> Dialysis membranes from Sigma Aldrich were used with a molecular weight cut-off (MWCO) of 12,000 – 14,000 Da. Samples were manually shaken for ~10 s, and then vortex mixed for ~10 s before transfer into the dialysis membrane. Samples were transferred at 2× the desired final concentration, with the volume being corrected gravimetrically post-dialysis. To confirm that no mass loss was occurring during this process, an aliquot of nanofiber solution (700 µL, 1 mg/mL, H_2O) was dried to a solid and weighed. The resulting mass (0.7 mg) confirmed that any mass loss during this process was negligible.

<u>For solvent evaporation:</u> A solution of nanoparticles in self-assembly solvent were added to a pre-weighed vial. A volume of MilliQ water in excess of the self-assembly solvent volume was then added slowly. The vial was left open to air overnight. Subsequently, a gentle stream of air was

used to remove the solvent until the weight equalled the desired mass of water. The vial was then left open to air overnight to ensure any residual organic solvent could evaporate. Finally, the vial was weighed again and the water was added gravimetrically to the desired final volume.

Transmission electron microscopy (TEM)

TEM micrographs were obtained on either a JEOL 1400 microscope with a Gatan Orius SC1000 CCD camera, operated at 120 kV or a JEOL 1011 microscope with an 11 Megapixel CCD camera, operated at 80 kV. Samples were prepared by drop casting 1.5 µL of the micelle solution onto a carbon coated copper grid. Negatively stained samples were additionally drop cast with uranyl acetate in EtOH (8 µL, 3 wt%). Copper grids (400 or 500 mesh) were purchased from Agar Scientific and carbon films (ca. 6 nm) were prepared on mica sheets by carbon sputtering with an Agar TEM Turbo Carbon Coater or a Leica ACE 600 carbon coater. The carbon films were deposited onto the copper grids by floatation on water using the Smith Grid Coating Trough (Ladd Research Industries) and the carbon coated grids were allowed to dry in air.

For micelle contour lengths analysis, a minimum of 200 micelles were traced manually using the Fiji (ImageJ) software package developed at the US National Institute of Health. The number average micelle length (L_n), width (W_n) or diameter (D_n) and weight average micelle length (L_w), width (W_w) or diameter (D_w) were calculated using eq. S1-2 from measurements of the contour lengths/widths (L_i) of individual micelles, where Ni is the number of micelles of length Li, and n is the number of micelles examined in each sample. The distribution of micelle lengths/widths (termed $Đ$) is characterized by both L_w/L_n ($Đ_L$) or W_w/W_n ($Đ_W$) or D_w/D_n ($Đ_D$) and σ (standard deviation, σ_L, σ_W and σ_D).

$$L_n = \frac{\sum_{i=1}^{n} N_i L_i}{\sum_{i=1}^{n} Ni} \qquad L_w = \frac{\sum_{i=1}^{n} N_i L_i^2}{\sum_{i=1}^{n} NiLi} \qquad \text{(eq. S1-2)}$$

Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) experiments were carried out using a Zetasizer Pro (Malvern Panalytical). Samples were prepared at concentrations of 0.1 mg/mL, diluted using filtered solvents (0.45 µm membrane filter). The cuvette used was a low-volume quartz cuvette (ZEN2112, 100 µL volume, 10.0 mm light path). A minimum of five measurements per sample were taken. The correlation function was acquired in real time and analysed with a function capable of modelling multiple exponentials (Cumulant analysis). This process enabled the diffusion

coefficients for the component particles to be extracted, and these were subsequently expressed as effective hydrodynamic radius (R_h) using the Stokes-Einstein relationship for coated nanospheres in H_2O, with core properties of polystyrene latex (RI = 1.590, Absorption = 0.010, dispersant RI = 1.33, dispersant viscosity = 0.887, dispersant dielectric constant 78.5). As these measurements assume that the particles are spherical, measurements of nanofiber size via DLS are not absolute, but still provide a useful method for monitoring the colloidal stability of the samples.

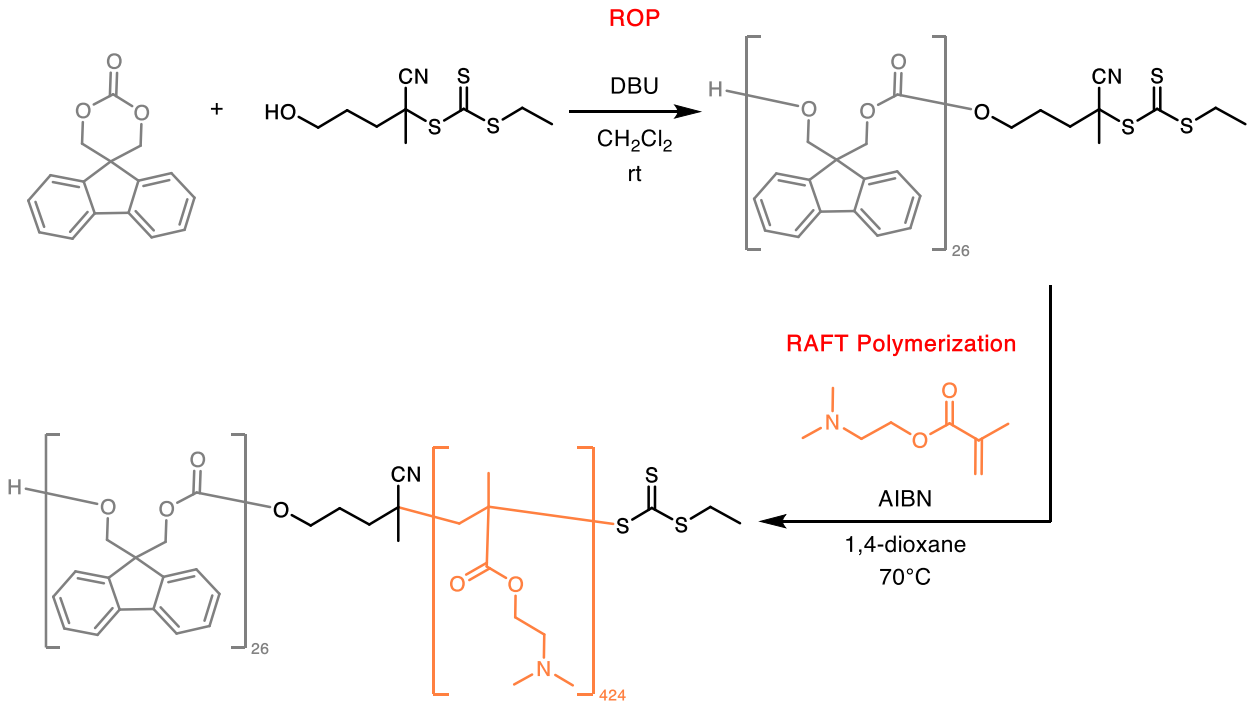
ζ-Potential Measurements

ζ-potential measurements were recorded on a Zetasizer Pro (Malvern Panalytical), following the Smoluchowski approximation at 25 °C. Samples were diluted to 100 μg/mL in 5 mM NaCl buffer, with each cuvette containing 700 μL of micelle solution. A minimum of five measurements per sample were taken, each consisting of between 10 and 100 cycles per run. The average ζ-potential was calculated from the individual measurements taken, with error represented as σ.

Transfection Experiments

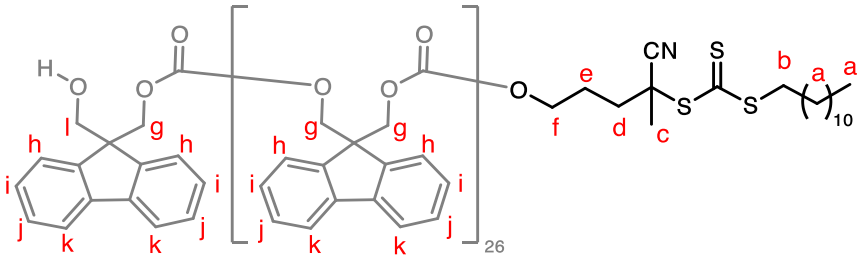
Transfection experiments were analyzed using a combined ONE-Glo™ and CellTiter-Fluor™ assay (E7110, Promega corporation), according to the procedure provided. Cells were cultured in black-walled 96-well plates, with clear glass bottoms (Corning, part no: 3603). CellTiter-Fluor™ fluorescence was measured at 30 °C using either a Cytation 5 Fluorescence Imaging Plate Reader (BioTek) (for Figure 4, Figure 6, Figure S10-S11, and Figure S13-S17) using a 390/20 nm excitation and 505/20 emission filter set, or using a Tecan infinite M200 Pro (Tecan Trading AG) (Figure 5) using a 390/9 nm excitation and 505/20 emission filter set. After fluorescence measurements were taken, samples were further processed according to the procedure outlined and transferred into opaque white 96-well plates (Thermo Scientific part no: 236108). Luminescence (Luciferase ONE-Glo™) was then measured at 30 °C using either a Cytation 5 Fluorescence Imaging Plate Reader (BioTek) (for Figure 4, Figure 6, Figure S10-S11, and Figure S13-S17) or using a Tecan infinite M200 Pro (Tecan Trading AG) (Figure 5). Data analysis was carried out in GraphPad Prism 9 (GraphPad Software), with significance determined via 2-way ANOVA analysis with multiple comparisons (Tukey or Šídák's correction, as specified). For further specific details see the protocol on page S16.

Synthetic Procedures



Scheme S1. Synthesis of PFTMC$_{26}$-*b*-PDMAEMA$_{424}$ (**P2**) via sequential ring opening polymerization (ROP) and reversible addition-fragmentation chain-transfer (RAFT) polymerization.

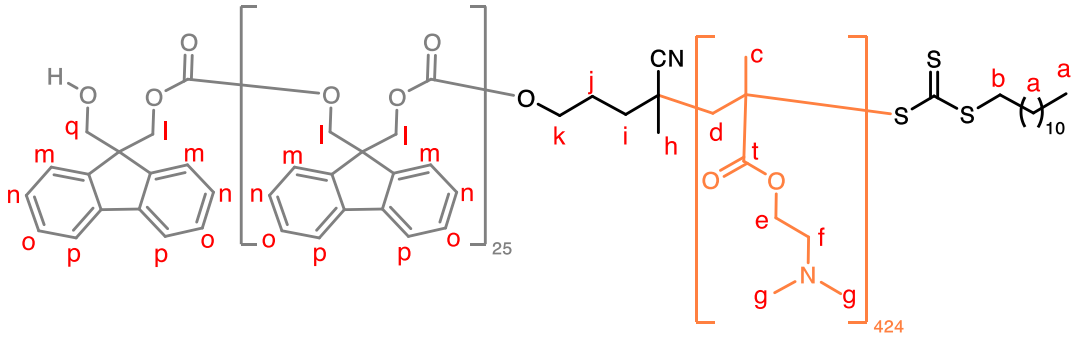
PFTMC$_{26}$-CTA



PFTMC$_{26}$-CTA was synthesized according to the procedure outlined by Street et. al.[S2] A flask was charged with DBU (15.4 μL, 0.8 eq), 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl] pentanol (RAFT-CTA) (50 mg, 250 μL of 250 μg/mL, 1.0 eq), and 250 μL of CH_2Cl_2 and allowed to stir for 15 mins. In a separate flask, spiro[fluorene-9,5′-[1,3]-dioxan]-2′-one (FTMC)[S2] (646 mg, 0.128 mmol, 20 eq) was dissolved in 4 mL CH_2Cl_2. The FTMC solution was then added to the flask containing the DBU/RAFT-CTA solution, and allowed to stir at 22 °C for 30 mins. The crude

reaction mixture was quenched by addition of benzoic acid and purified by precipitation into ice cold Et_2O (50 mL). The solution was decanted and the polymer was collected, redissolved in CH_2Cl_2, and precipitated into ice cold Et_2O. The solid was collected by centrifugation at 5000 RPM for 5 mins. This process was repeated 3× yielding PFTMC$_{26}$-CTA as a pale-yellow solid after drying *in vacuo*. **^{1}H-NMR** (300 MHz, CD_2Cl_2) 7.72 (52H, d, J = 7.6 Hz, Hh) 7.49 (52H, d, *J* = 7.5 Hz, Hk), 7.37 (52H, t, *J* = 7.3 Hz, Hi), 7.24 (52H, t, *J* = 7.5 Hz, Hj), 4.60 – 4.15 (102H, m, Hg), 4.08 (2H, t, *J* = 6.6 Hz, Hf), 3.70 (2H, d, *J* = 6.4 Hz, Hl), 3.31 (2H, t, *J* = 7.4 Hz, Hb), 2.33 – 2.22 (1H, m, Hd), 2.10 – 1.94 (1H, m, Hd), 1.90 – 1.83 (2H, m, He) 1.81 (3H, s, Hc), 1.26 (23H, s, Ha), DP_n = 26; **MALDI-TOF MS** for $C_{435}H_{347}NNaO_{79}S_3$ $[M_{26} + Na]^+$, calculated: 6971.2; found: 6,960.4, DP_n = 26, $Đ_M$ = 1.04; **GPC** (*n*-Bu_4NBr/THF, PS standard): M_n = 8,100 g/mol, $Đ_M$ = 1.09).

PFTMC$_{26}$-*b*-PDMAEMA$_{424}$ (P2)



To a solution of PFTMC$_{26}$-CTA (65 mg, 0.009 mmol, 1.0 eq) and 2-(dimethylamino)ethyl methacrylate (DMAEMA, 473 µL, 2.81 mmol, 300 eq) in 1,4-dioxane (5 mL), a solution of AIBN in 1,4-dioxane was added (0.46 mg, 0.003 mmol, 0.3 eq in 4.6 µL). The reaction mixture was stirred until homogenous before undergoing three freeze-pump-thaw cycles, and then heated to 70 °C for 24 h. The reaction mixture was quenched by submersion in liquid nitrogen and exposure to air, and purified by precipitation into hexanes. The solution was centrifuged at 5000 RPM for 5 mins. The supernatant was decanted off, the polymer dried, and precipitated from CH_2Cl_2 into hexanes twice more. PFTMC homopolymer was removed by preparative GPC using selective fractionation with THF as the eluent and a flow rate of 3 mL/min. The supernatant was removed, and the polymer was dried *in vacuo* to yield PFTMC$_{26}$-*b*-PDMAEMA$_{424}$ as a colourless solid. **^{1}H-NMR** (500 MHz, CD_2Cl_2) 7.79 – 7.68 (52H, m, Hm), 7.49 (52H, d, *J* = 7.6 Hz, Hp), 7.37 (52H, t, *J* = 7.6 Hz, Hn), 7.24 (52H, t, *J* = 7.4 Hz, Ho), 4.48 – 4.26 (102H, m, Hl), 4.03 (848H, s, He),

3.79 (2H, t, J = 5.8 Hz, Hq), 3.30 (2H, t, J = 10.7 Hz, Hb), 2.53 (853H, s, Hf), 2.25 (2549H, s, Hg), 1.90 (289H, s, Hd [*rm*]), 1.81 (486H, s, Hd [*rr*]), 1.43 (88H, dd, J = 14.5, 8.3 Hz, Hd [*mm*]), 1.32 – 1.19 (105H, m, Ha & Hc [*mm*]), 1.03 (405H, s, Hc [*rm*]), 0.95 – 0.77 (847H, m, Hc [*rr*]), DP_n PFTMC = 26, DP_n PDMAEMA = 424; **GPC** (*n*-Bu_4NBr/THF, PS standard): M_n = 87,900 g/mol, $Đ_M$ = 1.16).

Self-assembly procedures

General procedure for the self-assembly of P1 and P2

Unimer solutions (either 20 mg/mL or 200 mg/mL) of either **P1** or **P2** were prepared by dissolution in common solvent (THF). An aliquot of this solution was then further diluted in an amount of common solvent appropriate to the final concentration of polymer and solvent composition. To this solution, selective solvent (**P1** = MeOH, **P2** = EtOH) was added slowly, and the vial was sealed, manually shaken for 10 s and then vortexed mixed for a further 10 s.

For samples annealed at 60 °C (**P2**): Where indicated, the sample was then annealed at 60 °C for 1 h and allowed to cool in the heating block until it reached rt (23°C), before being aged for 24 h.

For samples aged at 23 °C (**P1**): Where indicated, the sample was then aged at 23°C for 24 h.

Example procedure for the self-assembly of $PFTMC_{16}$-*b*-$PDMAEMA_{131}$ (P1)

An aliquot of **P1** unimer solution (50 µL, 200 mg/mL in THF) was diluted in THF (50 µL). To this solution, MeOH was then slowly added (900 µL), and the sample was manually shaken for 10 s, then vortex mixed for 10 s, and aged at 23 °C for 24 h. The resulting disperse nanofibers were then imaged via TEM.

Example procedure for the self-assembly of $PFTMC_{26}$-*b*-$PDMAEMA_{424}$ (P2)

An aliquot of **P2** unimer solution (50 µL, 20 mg/mL in THF) was diluted in THF (150 µL). To this solution, EtOH was then slowly added (800 µL), and the sample was manually shaken for 10 s, then vortex mixed for 10 s, and annealed at 60 °C for 24 h. The resulting disperse nanofibers were then imaged via TEM.

General Procedure for the preparation of seed nanofibers from disperse nanofibers

Disperse nanofibers (ranging from 1 mg/mL to 10 mg/mL) were sonicated for at least 3 h using a Hielscher UP100H sonication probe according to the setup outlined by Street et. al[S2] or

Fisherbrand 112xx series advanced ultrasonic cleaner (FB-11203), at 37 MHz and 80 % power. The temperature was kept between 0 °C and 23°C using an ice bath. The resulting seed nanofibers were then imaged via TEM.

Example procedure for the preparation of seed nanofibers from disperse nanofibers (P1)

Disperse **P1** nanofibers (1 mL, 10 mg/mL) were sonicated for 3 h using a Hielscher UP100H sonication probe, with the temperature kept between 0 °C and 23°C using an ice bath. The resulting seed nanofibers were then imaged via TEM. It was observed that the solution of nanofibers became noticeably less viscous and more transparent after sonication.

Example procedure for the preparation of seed nanofibers from disperse nanofibers (P2)

Disperse **P2** nanofibers (1 mL, 1 mg/mL) were sonicated for 3 h using a Fisherbrand 112xx series advanced ultrasonic cleaner (FB-11203) operated in sweep mode at 37 MHz and 80 % power. The temperature was kept between 0 °C and 20°C using an ice bath. The resulting seed nanofibers were then imaged via TEM.

General procedure for the preparation of low dispersity nanofibers from seed nanofibers using the seeded-growth method (living CDSA)

A solution of seed **P1** nanofibers (between 0.1 mg/mL and 1 mg/mL) were diluted in a volume of selective solvent appropriate to the final concentration of polymer and solvent composition. To this solution, an aliquot of unimer solution in common solvent (THF, 20 mg/mL) appropriate to the desired m_{unimer}:m_{seed} ratio was added, the sample was manually shaken for 10 s, then vortex mixed for 10 s, and aged at 23°C for 24 h. The resulting low dispersity nanofibers were then imaged via TEM.

Samples with an m_{unimer}:m_{seed} ratio of above 10 were prepared via iterative addition of aliquots of no more than 10 equivalents of unimer, followed by ageing for 24h between the addition of each aliquot.

Example procedure for the preparation of low dispersity nanofibers from seed nanofibers

34 nm **P1** seeds ($Đ_L$ = 1.10, σ = 11 nm, 100 µL, 10 mg/mL) were diluted in MeOH (900 µL). To this solution, **P1** unimer solution (15 µL, 200 mg/mL, m_{unimer}:m_{seed} = 3) was added, and the sample was manually shaken for 10 s, then vortex mixed for 10 s, and aged at 23°C for 24 h. The resulting low dispersity **P1** nanofibers were characterized via TEM (L_n = 140 nm, $Đ_L$ = 1.05, σ_L = 32 nm, W_n = 14 nm, $Đ_W$ = 1.09, σ_W = 4 nm) and DLS (R_h = 49 nm ± 1 nm, 0.1 mg/mL, diluted in MeOH).

Example procedure for the transfer of low dispersity nanofibers into water via dialysis

140 nm **P1** nanofibers ($Đ_L$ = 1.05, σ = 32 nm, 1 mL, 2 mg/mL) were placed inside a dialysis membrane (Sigma Aldrich, MWCO = 12,000 – 14,000 Da), sealed with clips (Spectrum Chemical), and dialyzed into deionized water (500 mL) for 24 h with a minimum of three dialysate changes. The dialysis membrane was opened, and the nanofiber solution was transferred to a vial. The solution was weighed, and filtered, deionized water was added to make the sample up to 1 mg/mL gravimetrically (2 g). The resulting low dispersity **P1** nanofibers were characterized via TEM (L_n = 137 nm, $Đ_L$ = 1.05, σ_L = 30 nm, W_n = 13 nm, $Đ_W$ = 1.04, σ_W = 3 nm), DLS (R_h = 39 ± 1 nm in H_2O, 41 nm ± 1 nm in 5 mM NaCl, 0.1 mg/mL), and ζ-potential (*app.* ζ-potential = +17.6 ± 0.6 mV).\

Example procedure for the transfer of low dispersity nanofibers into water via syringe-pump infusion

25 nm **P2** nanofibers ($Đ_L$ = 1.10, σ = 8 nm, 100 µL, 1 mg/mL) were transferred to a pre-weighed vial (2.865 g). 200 µL of MilliQ water was added slowly via micropipette. The solvent was left to evaporate overnight. Using a gentle flow of air, the solvent was removed until the vial weighed 2.960 g. The solvents were again left open to air overnight. Subsequently, the vial was weighed again (2.931 g) and thus 34 µL of water was added to reach the desired final concentration and volume (100 µL, 1 mg/mL, vial weight = 2.965 g). ^{1}H NMR (diluted in DMSO-d_6) showed no residual organic solvent.

General procedure for the preparation of nanospheres via dialysis

Unimer solutions (20 mg/mL) of **P1** or **P2** were prepared by dissolution in common solvent (either THF or DMSO as indicated). Aliquots of these unimer solutions appropriate to the desired composition and concentration of the resulting nanospheres were combined in a vial, and further diluted in an amount of common solvent appropriate to the final concentration of polymer. The sample was manually shaken for 10 s, then vortex mixed for 10 s. This solution (500 µL – 5 mL, 1 – 5 mg/mL) was then placed inside a dialysis membrane (Sigma Aldrich, MWCO = 12,000 – 14,000 Da), sealed with clips (Spectrum Chemical), and dialyzed into deionized water (500 mL) for 24 h with a minimum of three dialysate changes. The dialysis membrane was opened, and the nanosphere solution was transferred to a vial. The solution was weighed, and filtered deionized water was added to make the sample up to 1 mg/mL gravimetrically. The resulting nanospheres were characterized via TEM, DLS, and ζ-potential.

Example procedure for the preparation of P1 nanospheres via dialysis

An aliquot of **P1** unimer solution (250 µL, 20 mg/mL in DMSO) was diluted in DMSO (750 µL). The sample was manually shaken for 10 s, then vortex mixed for 10 s, before being placed inside a dialysis membrane (Sigma Aldrich, MWCO = 12,000 – 14,000 Da), sealed with clips (Spectrum Chemical), and dialyzed into deionized water (500 mL) for 24 h with a minimum of three dialysate changes. The dialysis membrane was opened, and the nanosphere solution was transferred to a vial. The solution was weighed, and filtered deionized water was added to make the sample up to 1 mg/mL gravimetrically (5 g). The resulting nanospheres were characterized via TEM (D_n = 14 nm, $Đ_D$ = 1.05, σ = 3 nm), DLS (R_h = 68 ± 4 nm in H_2O, 62 nm ± 2 nm in 5 mM NaCl, 0.1 mg/mL), and ζ-potential (*app.* ζ-potential = +25.5 ± 0.4 mV).

Characterization data for novel nanofibers and spheres reported in this work

Nanofibers **F4**: TEM (THF/EtOH, 20:80 *v/v*): Figure S7A: L_n = 25 nm, $Đ_L$ = 1.10, σ_L = 8 nm; (H_2O): Figure S7B, L_n = 27 nm, $Đ_L$ = 1.05, σ_L = 6 nm, W_n = 12 nm, $Đ_W$ = 1.04, σ_W = 3 nm; DLS: R_h = 39 ± 1 nm in 5 mM NaCl, 0.1 mg/mL; and ζ-potential: *app.* ζ-potential = +15.6 ± 1.8 mV.
Nanospheres **S3**: TEM (H_2O): Figure S7C-D, D_n = 16 nm, $Đ_D$ = 1.03, σ = 3 nm; DLS: R_h = 24 ± 1 nm in 5 mM NaCl, 0.1 mg/mL; and ζ-potential: *app.* ζ-potential = +12.1 ± 2.2 mV.

Formation of polymer:DNA complexes

Micelleplex Formation

Optimized general procedure: As previously reported,[S3] an aqueous solution of nanofibers or nanospheres (20 μg/mL for *N*/*P* = 2 and 5, or 200 μg/mL for *N*/*P* = 10, 20, 30 and 60, pH 7.4) was diluted in an appropriate amount of water and added to HEPES (40 mM) + glucose (10 wt%), pH 7.4 in a 1:1 ratio, yielding an aqueous solution of nanofibers/nanospheres (2 μg/mL – 74 μg/mL) in HEPES (20 mM) + glucose (5 wt%), pH 7.4 (HBG). In a separate vial, pDNA (ca. 200-700 μg/mL) was diluted in an appropriate amount of water and added to HEPES (40 mM) + glucose (10 wt%), pH 7.4 in a 1:1 ratio, yielding an aqueous solution of pDNA (2 μg/mL) in HBG. To the nanofiber solution, the pDNA solution was added in a 1:1 ratio, yielding a solution of nanofibers (1 μg/mL – 37 μg/mL) + pDNA (1 μg/mL) in HBG. This solution was manually shaken for 10 s, and then vortex mixed for a further 10 s. Micelleplexes form instantaneously, but were aged for 24 h before use to allow for any particle aggregation to subside. *Note:* It is important that pDNA is added to nanofibers/nanospheres and not vice-versa, otherwise particle precipitation will occur.

Polyplex Formation

An aqueous solution of $PDMAEMA_{249}$ (20 μg/mL for *N*/*P* = 2 and 5, or 200 μg/mL for *N*/*P* = 10, 20, 30 and 60, pH 7.4) was diluted in an appropriate amount of water and added to HEPES (40 mM) + glucose (10 wt%), pH 7.4 in a 1:1 ratio, yielding an aqueous solution of $PMAEMA_{249}$ (2 μg/mL – 74 μg/mL) in HBG. In a separate vial, pDNA (ca. 200-700 μg/mL) was diluted in an appropriate amount of water and added to HEPES (40 mM) + glucose (10 wt%), pH 7.4 in a 1:1 ratio, yielding an aqueous solution of pDNA (2 μg/mL) in HBG. To the pDNA solution, the $PMAEMA_{249}$ solution was added in a 1:1 ratio, yielding a solution of $PMAEMA_{249}$ (1 μg/mL – 37 μg/mL) + pDNA (1 μg/mL) in HBG. This solution was manually shaken for 10 s, and then vortex mixed for a further 10 s. Polyplexes were aged for 24 h before use to facilitate accurate comparisons with the equivalent micelleplexes.

Lipofectamine™ 2000 lipoplex preparation

Lipofectamine™ 2000 lipoplexes were prepared according to the manufacturers protocol (Thermo Fisher), using growth media without serum as the diluent (MEM). Lipoplexes were prepared

immediately prior to transfection experiments as per the manufacturers protocol. Lipofectamine™ 2000 was used as the positive control for transfection to quantify the effectiveness of the other delivery systems and provide comparisons of micelleplexes and polyplexes with a commonly used lipoplex formulation.

Cell culture protocols

U-87 MG human GBM cells (CRL-1573, ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM, 11960044, Thermo Fisher) with 10% fetal bovine serum (FBS, 12483020, Thermo Fisher) in a humidified 5% CO_2 incubator at 37°C. Media changes were performed every two days. Once the cells reached approximately 80% confluency, they were detached from the culture surface using 0.25% trypsin-EDTA (15400054, Thermo Fisher) diluted in Dulbecco's phosphate-buffered saline (DPBS, 14190144, Thermo Fisher) and were cryopreserved at a density of 1×10^6 cells/mL in CryoStor® CS10 cell freezing medium (07930, STEMCELL Technologies). Cryopreservation was performed using a slow rate-controlled cooling protocol in which the cells were stored at -80°C overnight before transferring to -135°C liquid nitrogen.

HEK293 human embryonic kidney cells (HTB-14, ATCC) were cultured in Eagle's Minimum Essential Medium (MEM, 41090101, Thermo Fisher) with 10% fetal bovine serum (FBS, 12483020, Thermo Fisher) in a humidified 5% CO_2 incubator at 37°C. Media changes were performed every two days. Once the cells reached approximately 80% confluency, they were detached from the culture surface using 0.25% trypsin-EDTA (15400054, Thermo Fisher) diluted in Dulbecco's phosphate-buffered saline (DPBS, 14190144, Thermo Fisher) and were cryopreserved at a density of 1×10^6 cells/mL in CryoStor® CS10 cell freezing medium (07930, STEMCELL Technologies). Cryopreservation was performed using a slow rate-controlled cooling protocol in which the cells were stored at -80°C overnight before transferring to -135°C liquid nitrogen.

Plasmid DNA amplification

Plasmid amplification was performed using standard techniques, using DH5α *E. coli*. pDNA was extracted and purified using either a QIAprep Spin Miniprep Kit or QIAGEN Plasmid Maxi Kit (Qiagen) using the procedure provided by Qiagen. The resulting pDNA was sequenced before use (Eurofins) to confirm its identity.

Transfection Studies

Micelleplexes and polyplexes were aged for 24 h prior to addition to cells. Lipofectamine™ 2000 lipoplexes were prepared immediately prior to addition to cells. The optimum lipofectamine™ 2000:pDNA ratio was found to be 4:1 for the original studies in U-87 MG,[S3] so this was the concentration used for all transfection experiments. All transfection experiments utilized the pGL4.51 [luc2/CMV/Neo] plasmid (6,358 bp, Promega corporation, product number E1320, Figure S9). This plasmid was used to transfect cells with the reporter gene *luc2*, thereby facilitating the quantitation of transfection efficiency through luminescence measurements of luciferase in the presence of luciferin. To also probe cell viability within the same sample, a CellTiter-Fluor™ cell viability assay was conducted concurrently in a combined 'ONE-Glo™ + Tox Luciferase Reporter and Cell Viability Assay' (E7110, Promega corporation).

Optimized Transfection Procedure: U-87 MG or HEK293 cells were cultured and transferred to a black-walled, clear-bottom 96-well plate at a density of 2×10^4 - 6×10^4 cells/well (2×10^5 - 6×10^5 cells/mL) for cell density experiments, and 4×10^4 cells/well for all other experiments in growth media supplemented with 10 % FBS (100 µL) at 37 °C (micelleplexes and polyplexes are also prepared at this time). After 24 h incubation, the growth media was removed and fresh media without FBS was added (50 µL/well). To each well, either transfection solution (micelleplex, polyplex, lipoplex) or control (buffer, pDNA, polymer control etc) was added as appropriate (100 µL/well) for a total volume of 150 µL per well. The cells were incubated with the transfection solution for 4 h, after which the transfection solution was removed via aspiration and fresh growth media with FBS was added (100 µL, DMEM + 10 % FBS). Note: all samples were removed from cells and replaced with fresh media after 4 h incubation, including Lipofectamine™ 2000 and the other controls. The cells were then incubated for a further 20 h.

24 h after the transfection complexes were added to the cells, 50 µL of growth media was removed (leaving 50 µL remaining), and the 'ONE-Glo™ + Tox Luciferase Reporter and Cell Viability Assay' was performed according to the protocol. Briefly, CellTiter-Fluor™ solution (50 µL/well, 2×) was added. After 30 min incubation, the fluorescence was read on a fluorescence plate reader (λ_{ex} = 390/20, λ_{em} = 505/20). After this, One-Glo™ reagent (100 µL/well) was added, and the cells were incubated at 25 °C for 3 minutes (total volume of 200 µL/well), which lysed the cells and released the luciferase protein into the supernatant. The supernatant was then transferred to an opaque, white 96-well plate, and the luminescence was read on a luminescence plate reader.

The luciferase expression quantifies transfection efficiency, whilst the CellTiter-Fluor™ fluorescence quantifies cell viability. The results were expressed in terms of the % Lipofectamine™ 2000 positive control (or in RLU) for luminescence measurements, and in terms of % control cells for cell viability measurements.

Supplementary Figures

Table S1. Molecular weight, composition, and characterization of the polymers studied in this work. Data for **P1** and $PDMAEMA_{249}$ is reproduced with permission from the Royal Society of Chemistry.[S2]

Name	Polymer	M_n (g/mol) via GPC	M_w (g/mol) via GPC	$Đ_M$ via GPC	M_n (g/mol) via NMR	PFTMC DP_n [a]	PDMAEMA DP_n [b]
P1	$PFTMC_{16}$-*b*-$PDMAEMA_{131}$	9,700	15,000	1.55	24,900	16	131
P2	$PFTMC_{26}$-*b*-$PDMAEMA_{424}$	87,900	102,100	1.16	73,500	26	424
	$PDMAEMA_{249}$	63,100	73,900	1.17	39,400	-	249

[a] calculated via MALDI-TOF.
[b] calculated via NMR.
$Đ_M$ is the molar mass dispersity, M_w / M_n

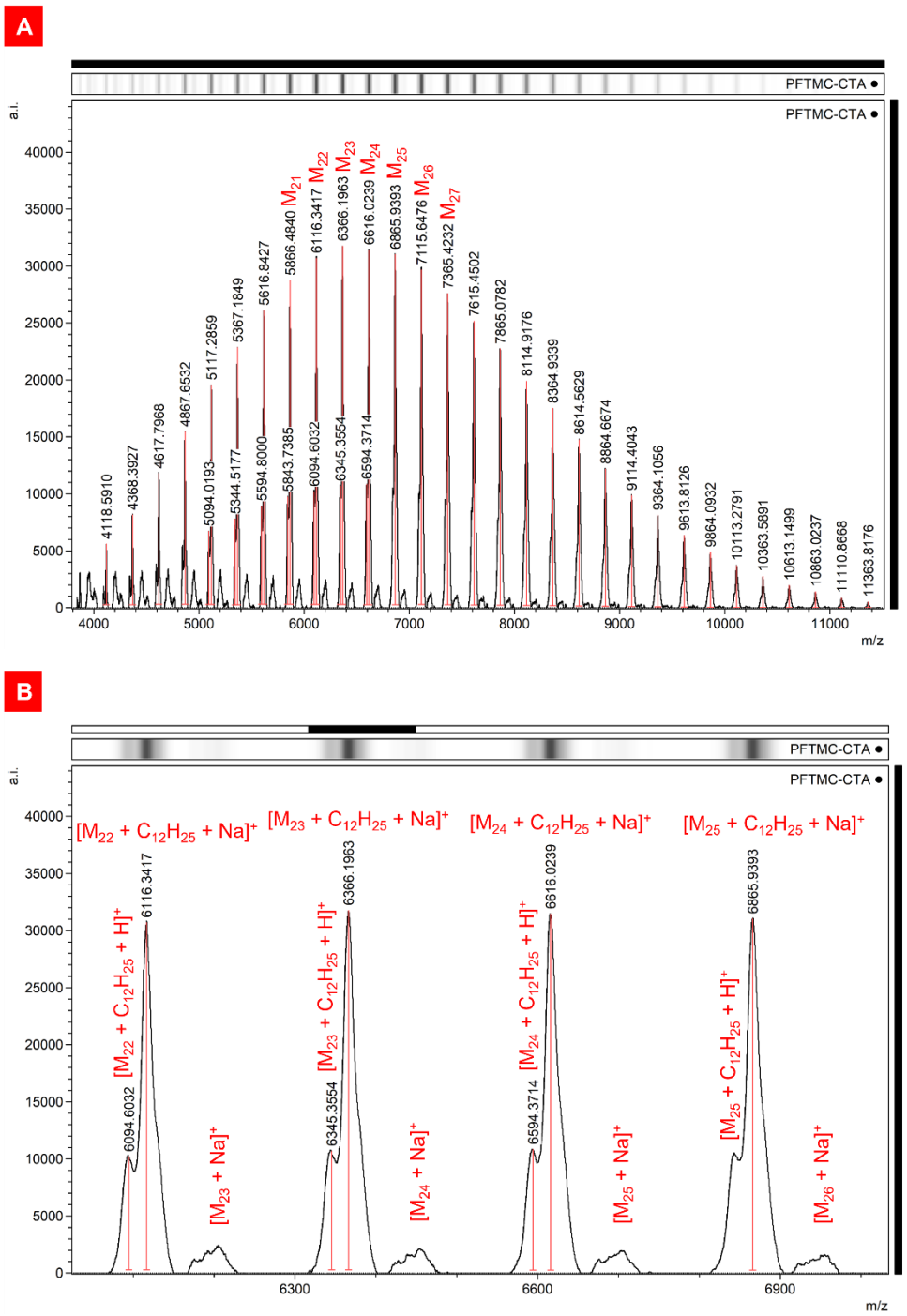


Figure S1. MALDI-TOF mass spectrum of PFTMC$_{26}$-CTA. (A) HRMS for $C_{435}H_{347}NNaO_{79}S_3$ (DP of 26), $[M + C_{12}H_{25} + Na]^+$, calculated: 6971.2; found: 6,960.4. The major peak observed corresponds to $[M + C_{12}H_{25} + Na]^+$; (B) Magnification of MALDI-TOF mass spectrum of PFTMC$_{26}$-CTA, with the various adducts labelled.

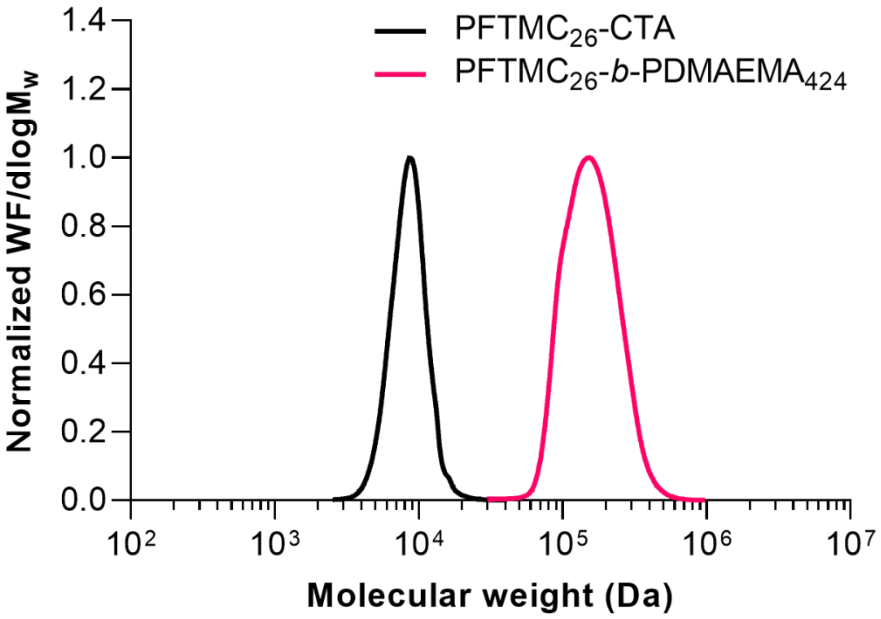


Figure S2. GPC Chromatograms (refractive index detection) in n-Bu_4NBr/THF of $PFTMC_{26}$-CTA (black trace) and $PFTMC_{26}$-*b*-$PDMAEMA_{424}$ (**P2**, pink trace).

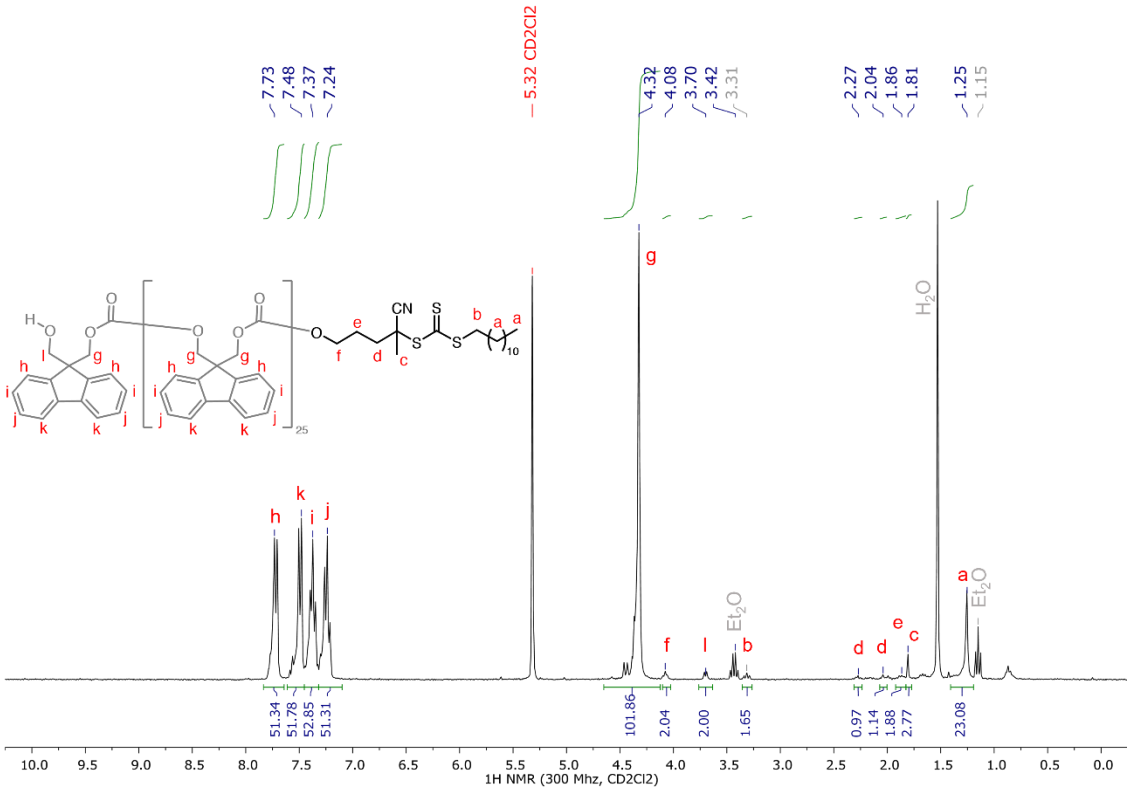


Figure S3. ^{1}H-NMR spectrum of PFTMC$_{26}$-CTA in CD_2Cl_2 (300 MHz).

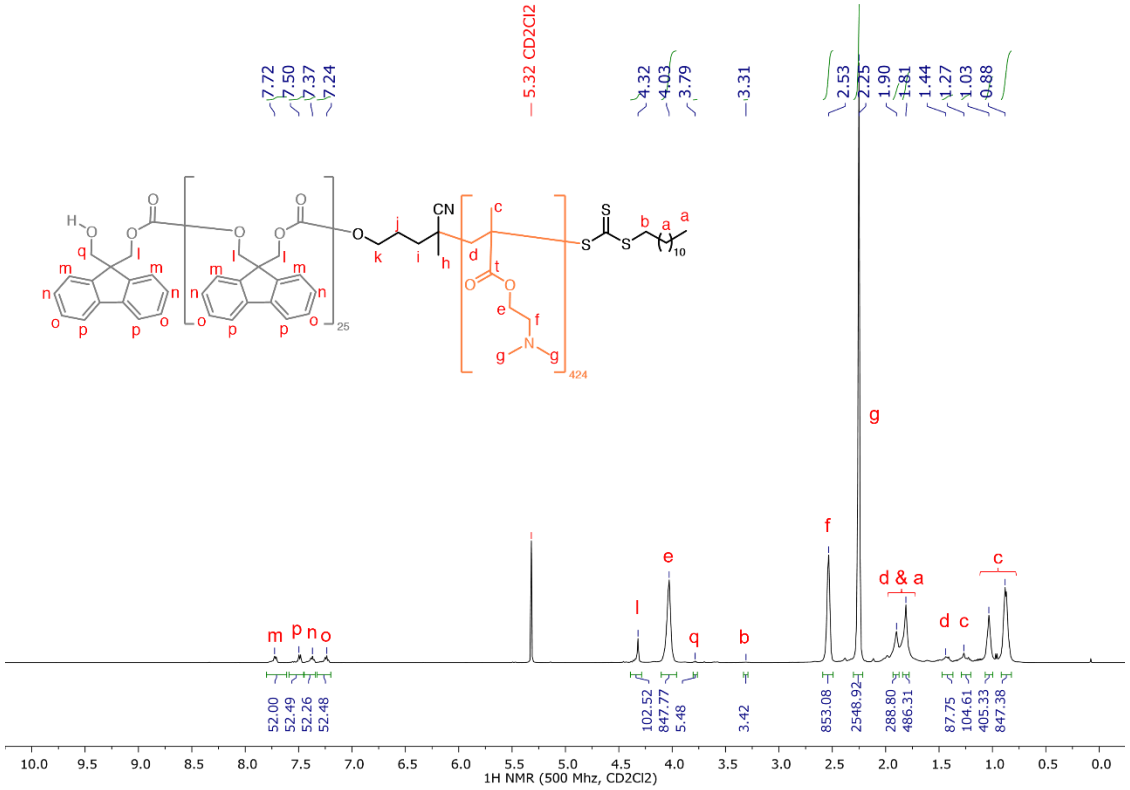


Figure S4. ^{1}H-NMR spectrum of PFTMC$_{26}$-b-PDMAEMA$_{424}$ in CD_2Cl_2 (500 MHz).

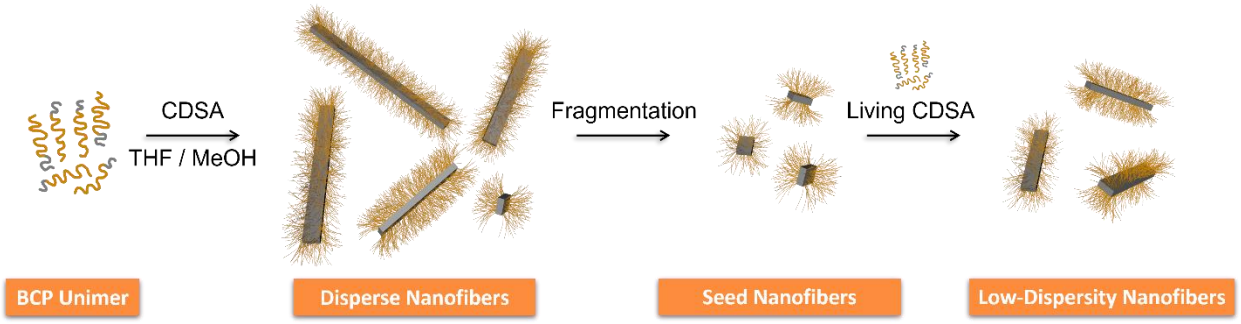


Figure S5. Overview of the process for producing length-controlled nanofibers via living crystallization-driven self-assembly (CDSA). Unimeric block copolymer (BCP) in a common solvent for both blocks is added to a selective solvent that induces crystallization of the core-forming block, yielding length-disperse nanofibers. Sonication-induced fragmentation of these disperse nanofibers yields low-dispersity seed nanofibers. Further addition of unimer to the seed nanofibers induces epitaxial growth, and low-dispersity length-controlled nanofibers are formed. Subsequent transfer into water (e.g. through dialysis) yields length-controlled nanofibers in water. Reproduced with permission from the American Chemical Society.[S3]

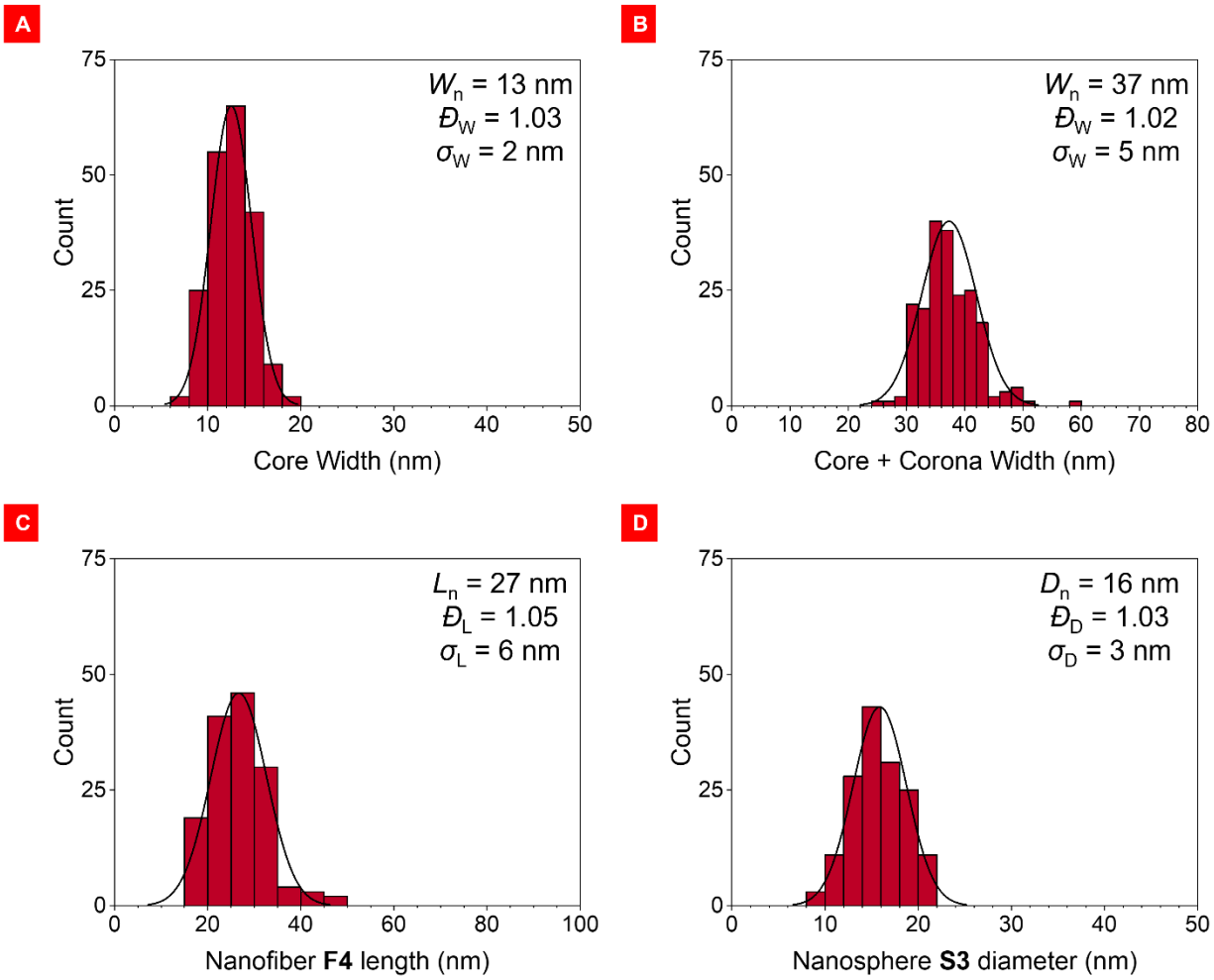


Figure S6. (A-B) Width histograms for **P2** nanofibers **F4** (L_n = 27 nm, $Đ_L$ = 1.05, σ_L = 6 nm) as measured via TEM. (A) The width of the nanofiber core. (B) The overall width of the nanofiber, which is the core + corona (in the dry state). The nanofiber corona was visualized via negative staining with uranyl acetate solution (UA, 3 wt% in EtOH). (C) Length histogram for **P2** nanofibers **F4**. (D) Diameter histogram for **P2** nanospheres **S3**.

Table S2. Summary of the characterization data for the nanofibers and nanospheres studied in this work. Data for **F2-3**, **F5** and **S2**[S2] as well as **F1**[S3] and **S2**[S4] have been previously reported and are reproduced with permission from the Royal Society of Chemistry and the American Chemical Society. **F4** and **S3** are new to this work. THF/MeOH (2:8 *v*/*v*) was used for the self-assembly of **P1** nanofibers, whilst THF/EtOH (2:8 *v*/*v*) was used for the self-assembly of **P2** nanofibers.

Sample	Polymer	Morphology	Length in self-assembly solvent (nm)[a]	$Đ_L$ in self-assembly solvent	Length/ Diameter in Water (nm)[a]	$Đ_L/Đ_D$ in Water	R_h in 5 mM NaCl (nm)[b]	ζ-potential in 5 mM NaCl (mv)
F1	**P1**	Nanofiber	72 ± 15	1.04	71 ± 13	1.03	31 ± 1	+12.3 ± 0.6
F2	**P1**	Nanofiber	140 ± 32	1.05	137 ± 30	1.05	41 ± 1	+17.6 ± 0.6
F3	**P1**	Nanofiber	93 ± 22	1.05	103 ± 27	1.07	70 ± 2	26.4 ± 1.1
F4	**P2**	Nanofiber	25 ± 8	1.10	27 ± 6	1.05	39 ± 1	+15.6 ± 1.8
F5	**P1**	Nanofiber	28 ± 12	1.20	27 ± 9	1.12	26 ± 1	+18.6 ± 0.3
S1	**P1**	Nanosphere	-	-	15 ± 3	1.05	65 ± 1	+7.4 ± 0.2
S2	**P1**	Nanosphere	-	-	14 ± 3	1.05	62 ± 2	+25.5 ± 0.4
S3	**P2**	Nanosphere	-	-	16 ± 3	1.03	24 ± 1	+12.1 ± 2.2

[a] Recorded via TEM, this represents the core dimensions, not including the corona.
[b] Recorded via DLS, this represents the core + corona dimensions of an equivalent sphere.
$Đ_L$ is the nanofiber length dispersity, L_w / L_n. $Đ_D$ is the nanosphere diameter dispersity, D_w / D_n.

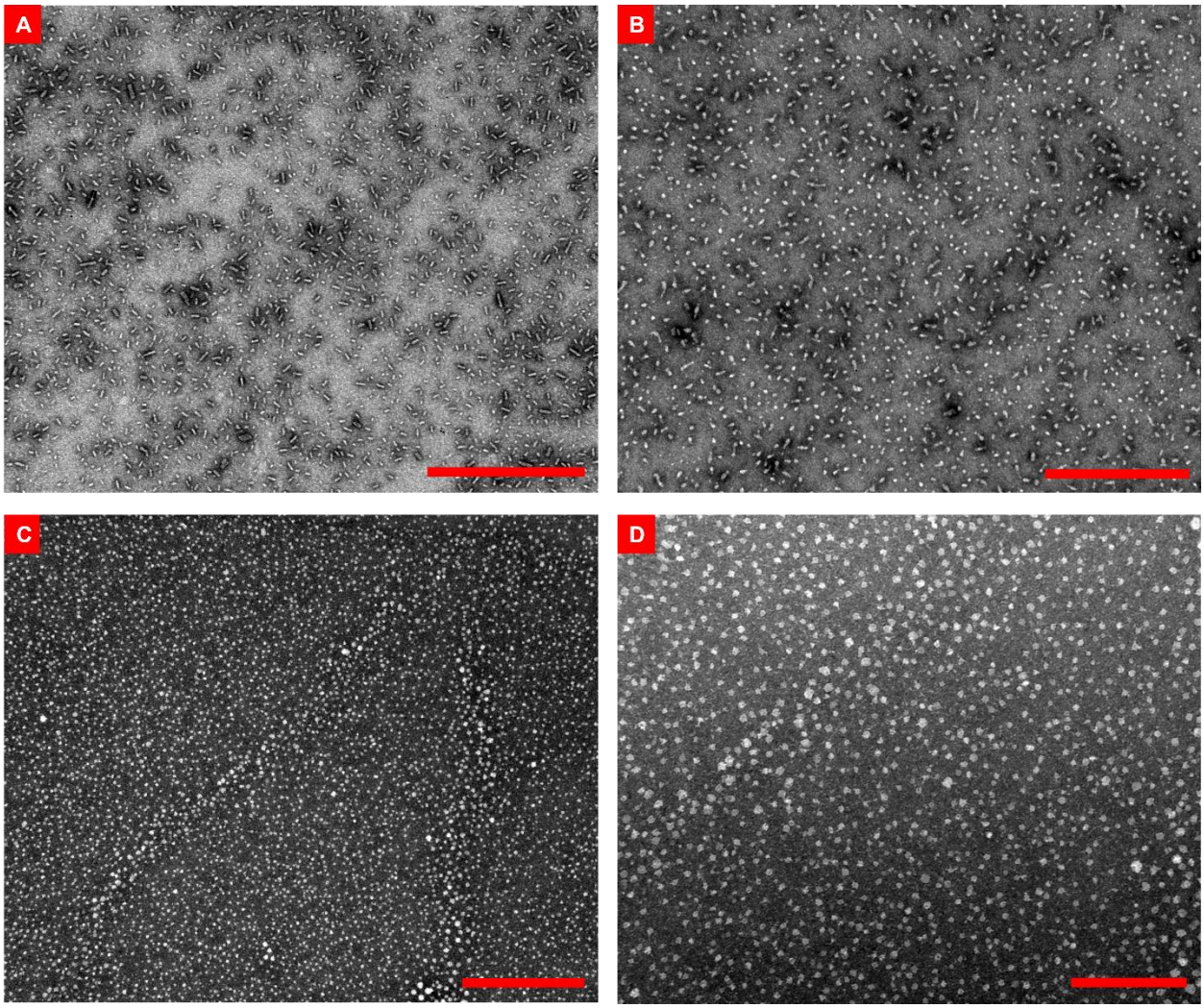


Figure S7. TEM Micrographs of **P2** nanofibers **F4** and nanospheres **S3** (D_n = 16 nm, $Đ_D$ = 1.03) used in this work. (A-B) Nanofibers **F4** (A) in THF/EtOH (2:8 *v*/*v*) after preparation (L_n = 25 nm, $Đ_L$ = 1.10) and (B) after transfer into water (L_n = 27 nm, $Đ_L$ = 1.05). (C-D) nanospheres **S3** in water. All samples were stained using UA (3 wt%). Scale bars in A-C are 1000 nm, and 500 nm in D.

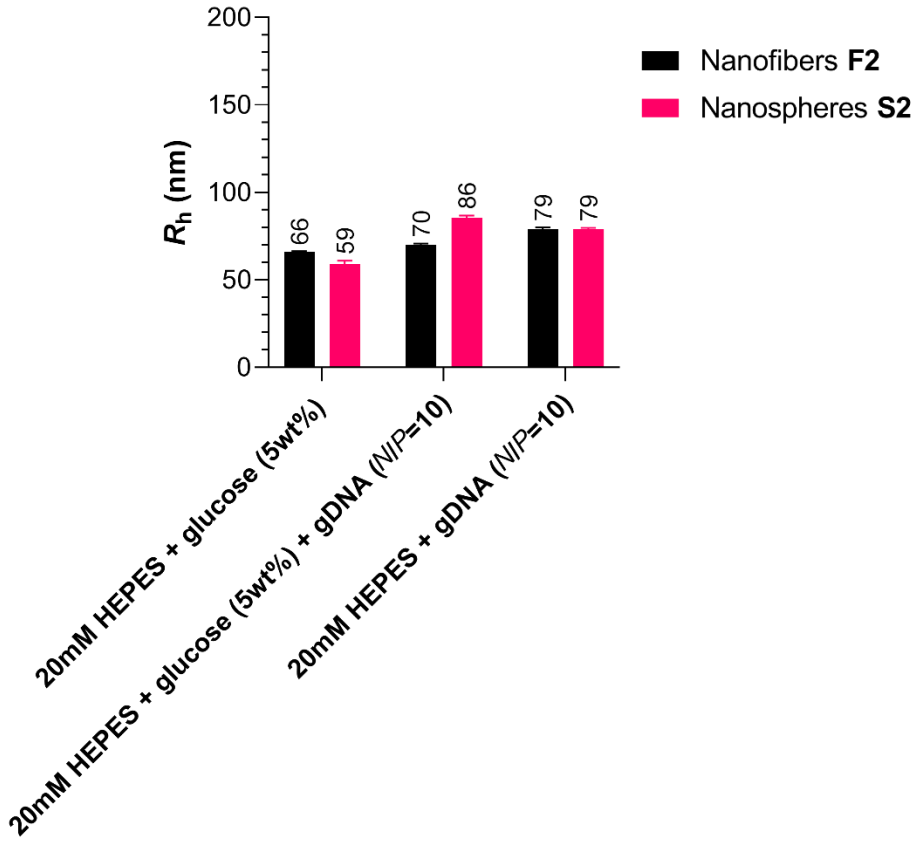


Figure S8. Examination of the effects of glucose upon the colloidal stability of micelleplexes as measured via the R_h of nanofibers **F2** (L_n = 137 nm, $Đ_L$ = 1.05, σ = 30 nm) and nanospheres **S2** (D_n = 14 nm, $Đ_D$ = 1.05, σ = 3 nm) complexed to gDNA (*N/P* = 10) after 24 h aging. Samples were dissolved in 20 mM HEPES either with or without 5wt% glucose before gDNA was added and the samples aged for 24 h. In all cases, the observed R_h was consistent with that expected for individual nanofibers, indicating that aggregation was supressed.

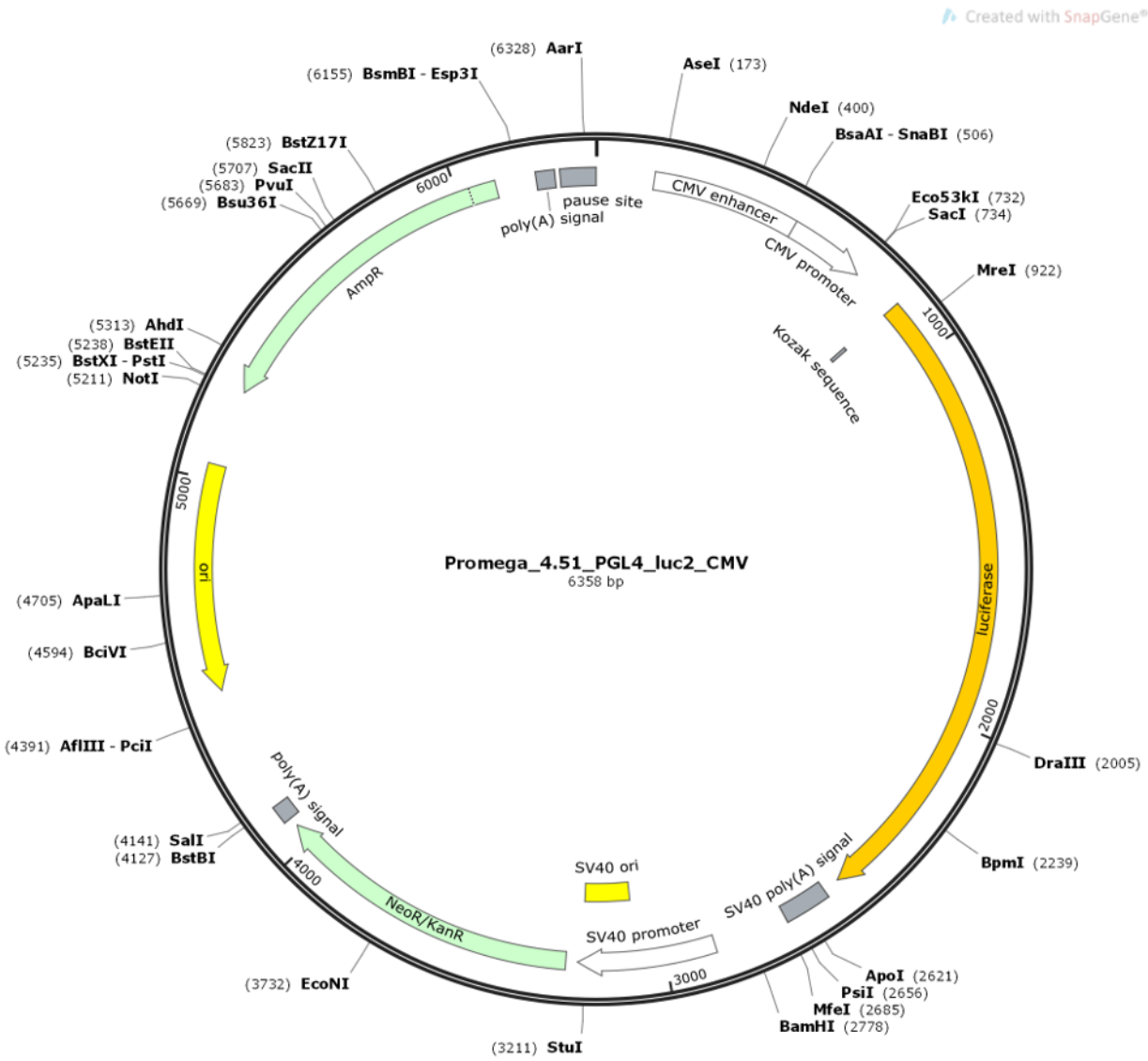


Figure S9. Plasmid map for the pGL4.51[luc2/CMV/Neo] plasmid used to transfect cells with *luc2* (6,358 bp, Promega corporation, product number E1320). Produced with SnapGene Viewer (Dotmatics).

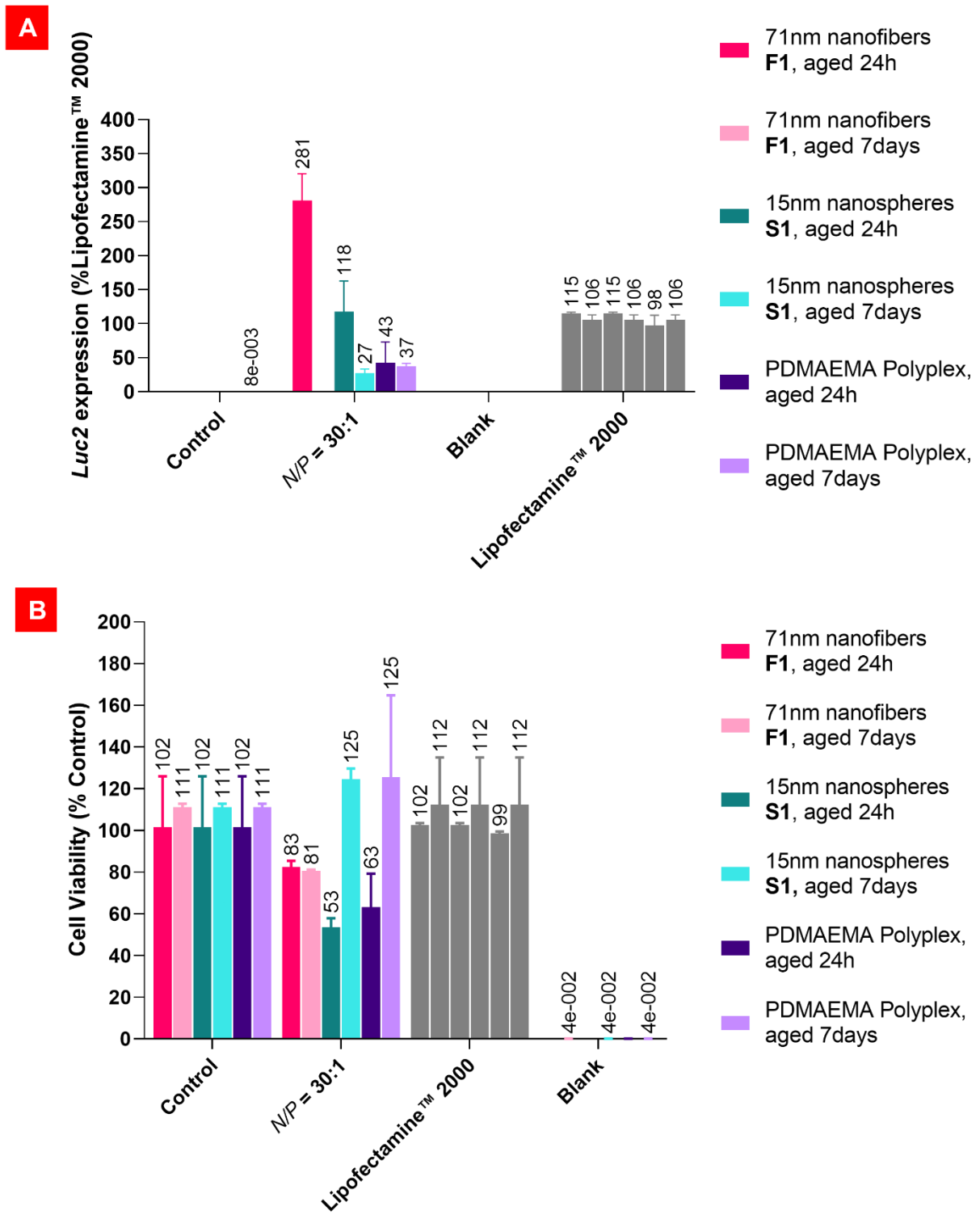


Figure S10. Examination of the effects of polymer/pDNA complex temporal stability upon (A) transfection efficiency and (B) cell viability in U-87 MG glioblastoma cells transfected with a *luc2* plasmid (6,358 bp). The transfection efficiency and cell viability of 71 nm nanofiber **F1**

micelleplexes ($Đ_L$ = 1.03, σ = 13 nm), 15 nm nanosphere **S1** micelleplexes ($Đ_D$ = 1.05, σ = 3 nm) and PDMAEMA$_{249}$ polyplexes (D_n = 66 nm, $Đ_D$ = 1.08, σ = 19 nm for *N*/*P* = 60)[S3] was examined after 24 h aging and 7 days aging. After this time, cells were incubated with pDNA complexes (*N*/*P* = 30, 1µg/mL pDNA) for 4 h, and the *luc2* expression and cell viability quantified after 24 h using a combined ONE-Glo™ and CellTiter-Fluor™ assay. Results for (A) were expressed as % of the lipofectamine2000 control, and for (B) as % of control cells. Each value represents the median, with the 95% confidence interval (CI) as error (n = 3). Data for 24 h ageing has been previously published, and is reproduced with permission by the American Chemical Society.[S3]

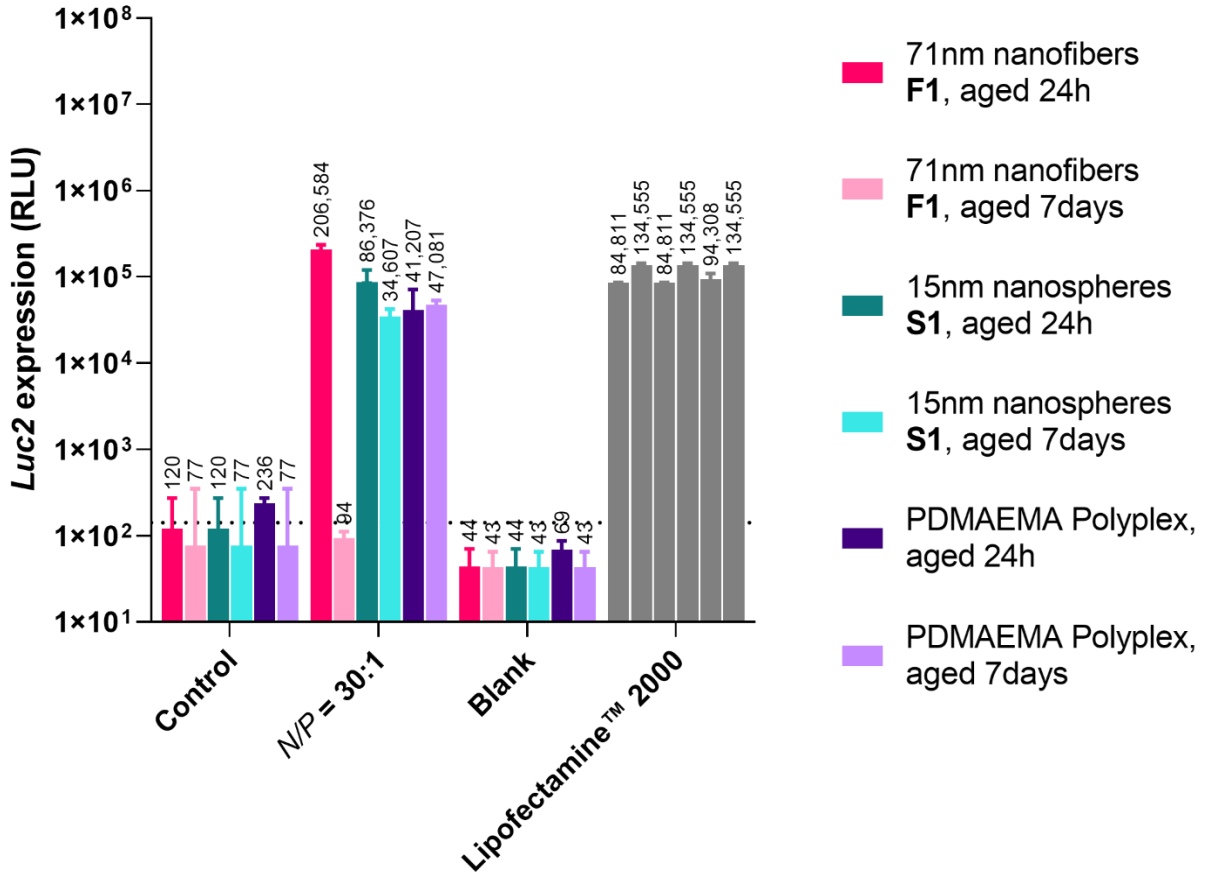


Figure S11. Examination of the effects of polymer/pDNA complex temporal stability upon transfection efficiency in U-87 MG glioblastoma cells transfected with a *luc2* plasmid (6,358 bp). Results are expressed as raw *luc2* expression (in RLU) on a logarithmic axis. The transfection efficiency and cell viability of 71 nm nanofiber **F1** micelleplexes ($Đ_L$ = 1.03, σ = 13 nm), 15 nm nanosphere **S1** micelleplexes ($Đ_D$ = 1.05, σ = 3 nm) and PDMAEMA$_{249}$ polyplexes (D_n = 66 nm,

$Đ_D$ = 1.08, σ = 19 nm for *N*/*P* = 60)[S3] was examined after 24 h aging and 7 days aging. After this time, cells were incubated with pDNA complexes (*N*/*P* = 30, 1µg/mL pDNA) for 4 h, and the *luc2* expression and cell viability quantified after 24 h using a combined ONE-Glo™ and CellTiter-Fluor™ assay. Each value represents the median, with the 95% confidence interval (CI) as error (n = 3).

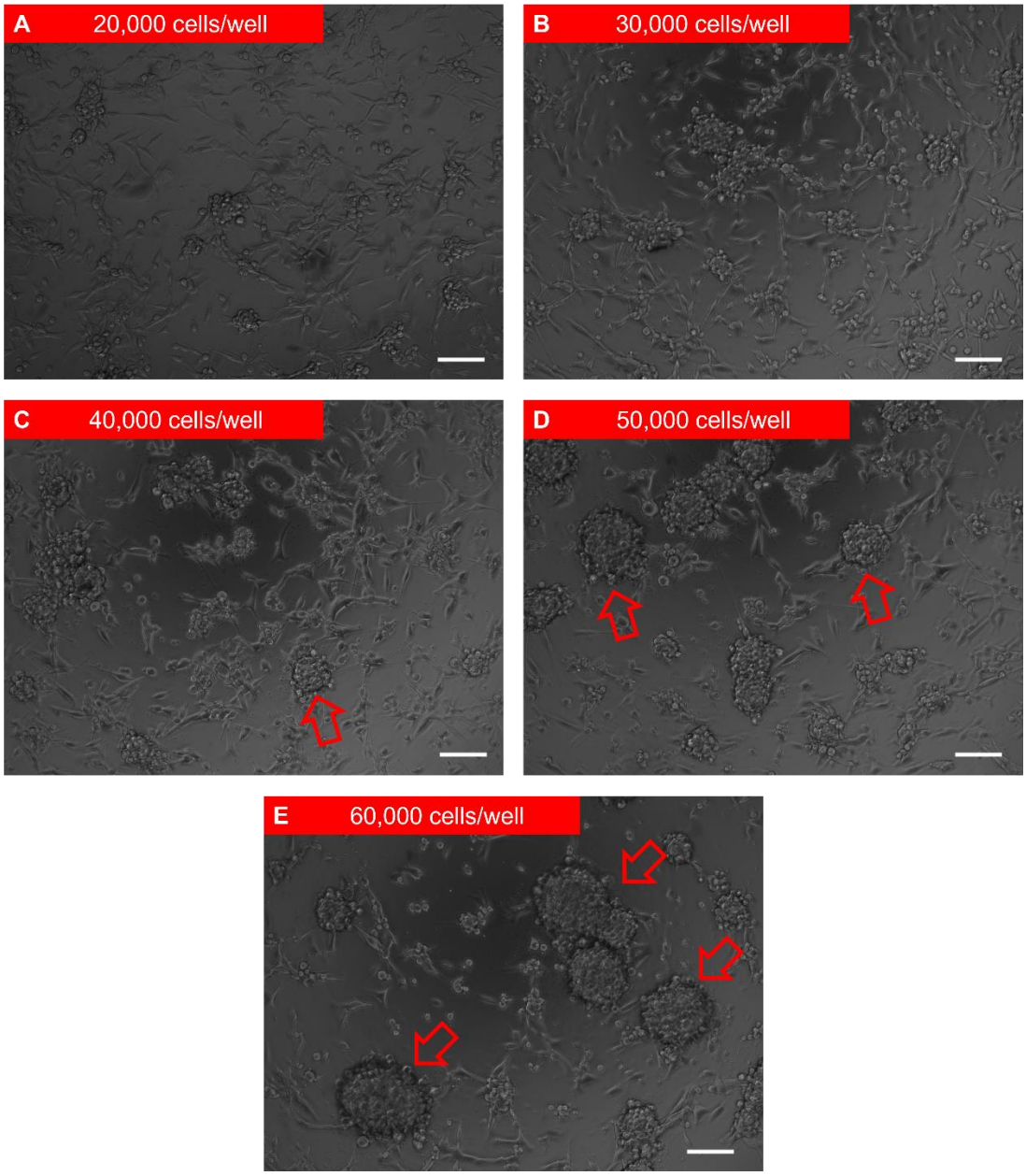


Figure S12. Brightfield microscopy images of U-87 MG cells plated at different densities, demonstrating the effects upon cell morphology. Examples of cancer stem cells (CSCs) are indicated with the red arrows. Scale = 100 µm.

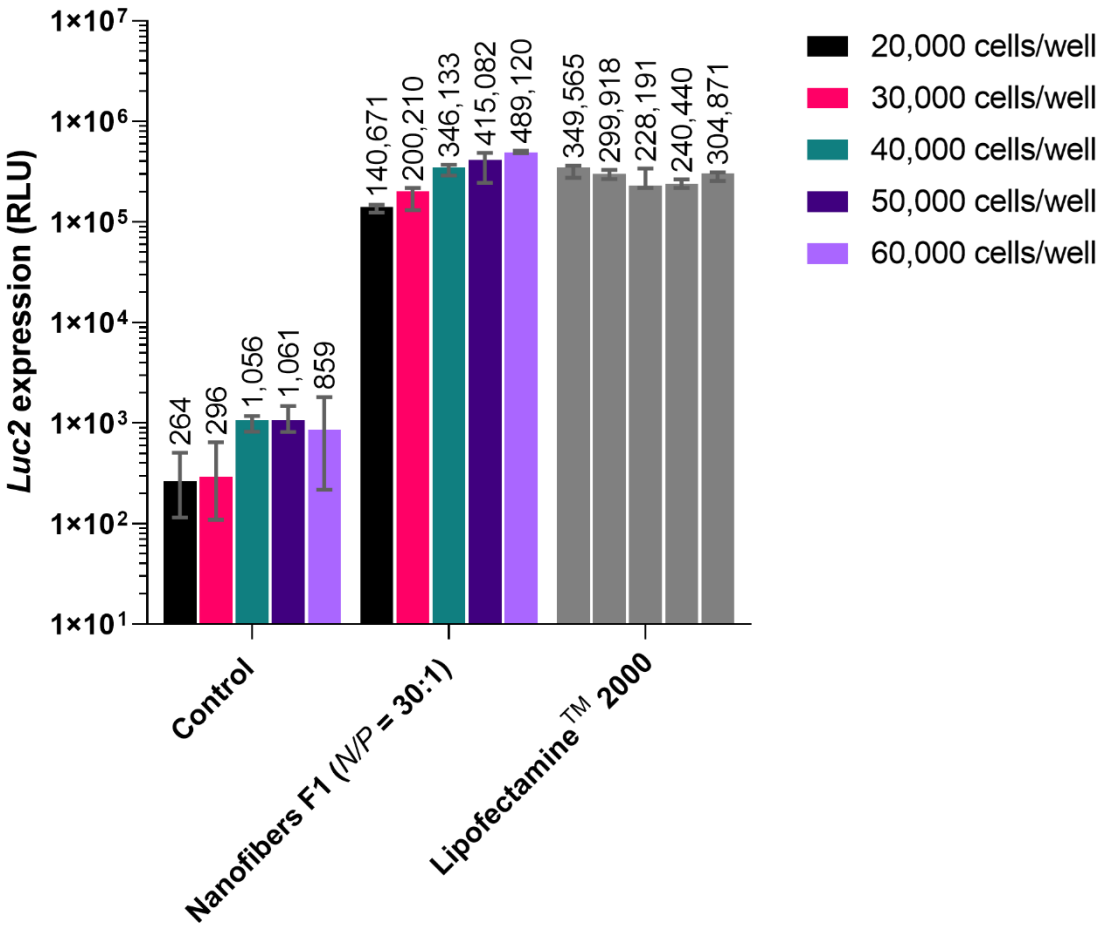


Figure S13. Examination of the effects of cell density upon the transfection efficiency of U-87 MG glioblastoma cells transfected with a *luc2* plasmid (6,358 bp) using nanofiber **F1** micelleplexes (L_n = 71 nm, $Đ_L$ = 1.03, σ = 13 nm) and lipofectamine™ 2000. Cells were incubated with pDNA complexes (1µg/mL pDNA) for 4 h at densities ranging from 20,000 cells/well to 60,000 cells/well in 96-well plates, with the *luc2* expression and cell viability quantified after 24 h using a combined ONE-Glo™ and CellTiter-Fluor™ assay. Lipofectamine™ 2000 was prepared and used immediately according to the instructions, whilst nanofiber **F1** micelleplexes were aged for 24 h prior to use. Results are expressed as raw *luc2* expression (in RLU) on a logarithmic axis. Each value represents the median, with the 95% confidence interval (CI) as error (n = 3).

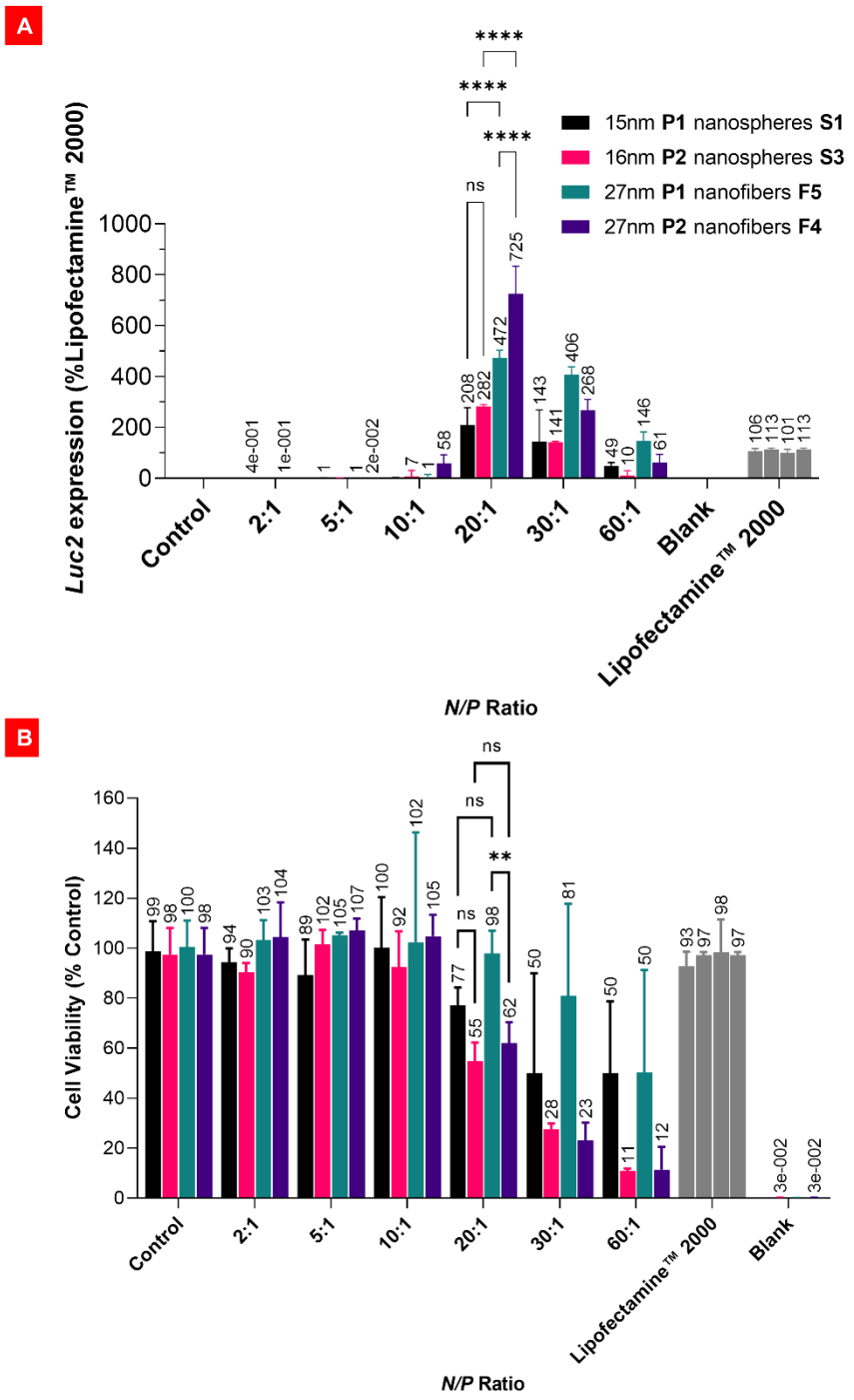


Figure S14. Examination of the effects of polymer DP_n upon (A) the transfection efficiency and (B) the cell viability of U-87 MG glioblastoma cells transfected with a *luc2* plasmid. This figure contains the full dataset, including the control and *N*/*P* ratios of 2:1 to 5:1. 15 nm **P1** nanospheres **S1** ($Đ_D$ = 1.05, σ = 3 nm) were compared to 27 nm **P1** nanofibers **F5** ($Đ_L$ = 1.12, σ = 9 nm), 16 nm **P2** nanospheres **S3** ($Đ_D$ = 1.03, σ = 3 nm) and 27 nm **P2** nanofibers **F4** ($Đ_L$ = 1.05, σ = 6 nm). Cells

were incubated with pDNA complexes (1μg/mL pDNA) for 4 h, with the *luc2* expression and cell viability quantified after 24 h using a combined ONE-Glo™ and CellTiter-Fluor™ assay. Lipofectamine™ 2000 was prepared and used immediately according to the instructions, whilst all other polymeric complexes were aged for 24 h prior to use. Results for A were expressed as % of the lipofectamine2000 control, and for B as % of control cells. The median value is plotted, with the 95% confidence interval (CI) as error (n = ≥3). ****, ** and ns indicate significance of p<0.0001. p<0.01 and no significance as determined by 2-way ANOVA analysis with multiple comparisons (Tukey correction). Data for **P1** nanofibers **F5** and nanospheres **S1** has been previously reported and is reproduced with permission from the American Chemical Society.[S3]

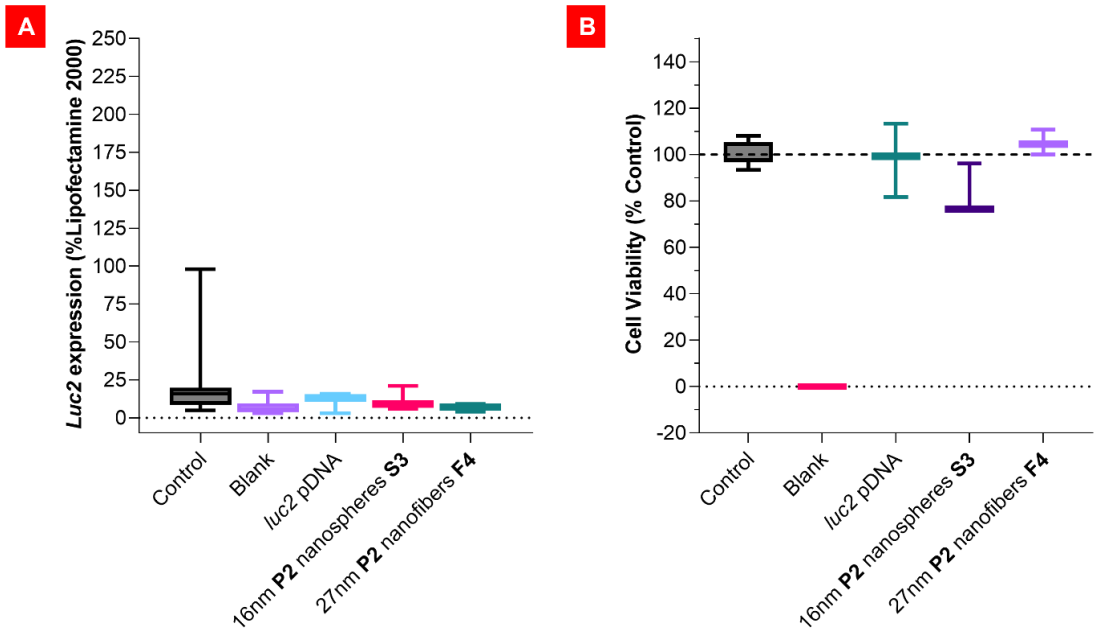


Figure S15. Examination of the effects of polymer DP_n upon (A) the transfection efficiency and (B) the cell viability of U-87 MG glioblastoma cells transfected with a *luc2* plasmid (6,358 bp) using nanofiber **F4** (L_n = 27 nm, $Đ_L$ = 1.05, σ = 6 nm) and 16 nm nanosphere **S3** micelleplexes ($Đ_D$ = 1.03, σ = 3 nm). This figure contains control samples. Cells were incubated with blank media, pDNA (1μg/mL) or polymer samples without pDNA (concentration equivalent to N/P = 10) for 4 h, with the *luc2* expression and cell viability quantified after 24 h using a combined ONE-Glo™ and CellTiter-Fluor™ assay. Lipofectamine™ 2000 was prepared and used immediately according to the instructions, whilst all other samples were aged for 24 h prior to use. Results for (A) were expressed as % of the lipofectamine2000 control, and for (B) as % of control cells. The median value is plotted, with the 95% confidence interval (CI) as error (n = ≥3).

Table S3. Tabulated statistical significance of the luciferase expression in Figure S14A.

Tukey's multiple comparisons test	Predicted (LS) mean diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
Control					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	0.000	-26.51 to 26.51	No	ns	>0.9999
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	0.000	-23.29 to 23.29	No	ns	>0.9999
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	0.000	-26.51 to 26.51	No	ns	>0.9999
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	0.000	-25.35 to 25.35	No	ns	>0.9999
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	0.000	-28.34 to 28.34	No	ns	>0.9999
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	0.000	-25.35 to 25.35	No	ns	>0.9999
2:1					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	0.4891	-48.60 to 49.58	No	ns	>0.9999
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	0.4165	-48.68 to 49.51	No	ns	>0.9999
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	0.5659	-48.53 to 49.66	No	ns	>0.9999
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	-0.07262	-49.17 to 49.02	No	ns	>0.9999
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	0.07671	-49.02 to 49.17	No	ns	>0.9999
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	0.1493	-48.94 to 49.24	No	ns	>0.9999
5:1					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	0.8289	-48.26 to 49.92	No	ns	>0.9999
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	-0.03669	-49.13 to 49.06	No	ns	>0.9999
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	1.079	-48.01 to 50.17	No	ns	>0.9999
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	-0.8656	-49.96 to 48.23	No	ns	>0.9999
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	0.2499	-48.84 to 49.34	No	ns	>0.9999
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	1.116	-47.98 to 50.21	No	ns	>0.9999
10:1					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	-12.38	-54.89 to 30.14	No	ns	0.8739
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	-2.981	-37.69 to 31.73	No	ns	0.9961
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	-62.14	-104.7 to -19.63	Yes	**	0.0012
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	9.397	-33.12 to 51.91	No	ns	0.9397
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	-49.76	-98.86 to -0.6716	Yes	*	0.0456
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	-59.16	-101.7 to -16.65	Yes	**	0.0023
20:1					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	-46.43	-95.52 to 2.665	No	ns	0.0711
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	-259.7	-308.8 to -210.6	Yes	****	<0.0001
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	-528.1	-577.2 to -479.0	Yes	****	<0.0001
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	-213.3	-262.4 to -164.2	Yes	****	<0.0001
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	-481.7	-530.8 to -432.6	Yes	****	<0.0001
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	-268.4	-317.5 to -219.3	Yes	****	<0.0001
30:1					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	36.42	-6.092 to 78.94	No	ns	0.1210
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	-221.3	-256.0 to -186.6	Yes	****	<0.0001
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	-99.04	-141.6 to -56.52	Yes	****	<0.0001
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	-257.7	-300.2 to -215.2	Yes	****	<0.0001
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	-135.5	-184.6 to -86.37	Yes	****	<0.0001
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	122.3	79.76 to 164.8	Yes	****	<0.0001

60:1					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	34.53	-7.984 to 77.05	No	ns	0.1546
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	-97.78	-132.5 to -63.06	Yes	****	<0.0001
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	-12.29	-54.80 to 30.23	No	ns	0.8763
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	-132.3	-174.8 to -89.79	Yes	****	<0.0001
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	-46.82	-95.91 to 2.275	No	ns	0.0676
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	85.49	42.98 to 128.0	Yes	****	<0.0001
Blank					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	-0.3360	-30.40 to 29.73	No	ns	>0.9999
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	0.0001390	-24.55 to 24.55	No	ns	>0.9999
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	-0.3360	-30.40 to 29.73	No	ns	>0.9999
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	0.3362	-29.73 to 30.40	No	ns	>0.9999
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	5.684e-014	-34.71 to 34.71	No	ns	>0.9999
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	-0.3362	-30.40 to 29.73	No	ns	>0.9999
Lipofectamine 2000 (0.4uL)					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	-1.137e-013	-42.52 to 42.52	No	ns	>0.9999
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	0.000	-34.71 to 34.71	No	ns	>0.9999
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	-1.137e-013	-42.52 to 42.52	No	ns	>0.9999
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	1.137e-013	-42.52 to 42.52	No	ns	>0.9999
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	0.000	-49.09 to 49.09	No	ns	>0.9999
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	-1.137e-013	-42.52 to 42.52	No	ns	>0.9999

Table S4. Tabulated statistical significance of the cell viability in Figure S14B.

Tukey's multiple comparisons test	Predicted (LS) mean diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
Control					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	0.000	-13.61 to 13.61	No	ns	>0.9999
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	0.000	-11.95 to 11.95	No	ns	>0.9999
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	0.000	-13.61 to 13.61	No	ns	>0.9999
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	0.000	-13.01 to 13.01	No	ns	>0.9999
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	0.000	-14.55 to 14.55	No	ns	>0.9999
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	0.000	-13.01 to 13.01	No	ns	>0.9999
2:1					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	7.620	-17.57 to 32.81	No	ns	0.8609
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	-2.089	-27.28 to 23.11	No	ns	0.9965
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	-7.546	-32.74 to 17.65	No	ns	0.8643
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	-9.709	-34.90 to 15.49	No	ns	0.7490
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	-15.17	-40.36 to 10.03	No	ns	0.4025
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	-5.457	-30.65 to 19.74	No	ns	0.9429
5:1					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	-4.668	-29.86 to 20.53	No	ns	0.9631
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	-11.17	-36.36 to 14.02	No	ns	0.6581
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	-12.60	-37.79 to 12.59	No	ns	0.5649
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	-6.503	-31.70 to 18.69	No	ns	0.9081
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	-7.932	-33.13 to 17.26	No	ns	0.8459
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	-1.430	-26.62 to 23.76	No	ns	0.9989

10:1					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	9.545	-12.27 to 31.36	No	ns	0.6677
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	-4.443	-22.26 to 13.37	No	ns	0.9161
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	-0.3919	-22.21 to 21.43	No	ns	>0.9999
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	-13.99	-35.81 to 7.830	No	ns	0.3456
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	-9.937	-35.13 to 15.26	No	ns	0.7353
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	4.052	-17.77 to 25.87	No	ns	0.9629
20:1					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	21.40	-3.798 to 46.59	No	ns	0.1262
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	-18.79	-43.98 to 6.408	No	ns	0.2171
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	13.66	-11.53 to 38.86	No	ns	0.4960
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	-40.18	-65.38 to -14.99	Yes	***	0.0003
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	-7.734	-32.93 to 17.46	No	ns	0.8555
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	32.45	7.253 to 57.64	Yes	**	0.0057
30:1					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	30.68	8.863 to 52.50	Yes	**	0.0020
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	-28.13	-45.95 to -10.32	Yes	***	0.0004
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	31.60	9.780 to 53.42	Yes	**	0.0014
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	-58.82	-80.63 to -37.00	Yes	****	<0.0001
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	0.9166	-24.28 to 26.11	No	ns	0.9997
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	59.73	37.91 to 81.55	Yes	****	<0.0001
60:1					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	41.17	19.35 to 62.98	Yes	****	<0.0001
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	1.031	-16.78 to 18.85	No	ns	0.9988
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	37.82	16.00 to 59.64	Yes	****	<0.0001
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	-40.13	-61.95 to -18.32	Yes	****	<0.0001
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	-3.348	-28.54 to 21.85	No	ns	0.9858
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	36.79	14.97 to 58.61	Yes	***	0.0001
Lipofectamine 2000 (0.4uL)					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	-3.521	-25.34 to 18.30	No	ns	0.9751
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	-4.976	-22.79 to 12.84	No	ns	0.8867
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	-3.521	-25.34 to 18.30	No	ns	0.9751
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	-1.455	-23.27 to 20.36	No	ns	0.9981
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	0.000	-25.19 to 25.19	No	ns	>0.9999
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	1.455	-20.36 to 23.27	No	ns	0.9981
Blank					
15nm **P1** nanospheres **S1** vs. 16nm **P2** nanospheres **S3**	0.000	-15.43 to 15.43	No	ns	>0.9999
15nm **P1** nanospheres **S1** vs. 27nm **P1** nanofibers **F5**	0.000	-12.88 to 12.88	No	ns	>0.9999
15nm **P1** nanospheres **S1** vs. 27nm **P2** nanofibers **F4**	0.000	-15.43 to 15.43	No	ns	>0.9999
16nm **P2** nanospheres **S3** vs. 27nm **P1** nanofibers **F5**	0.000	-15.66 to 15.66	No	ns	>0.9999
16nm **P2** nanospheres **S3** vs. 27nm **P2** nanofibers **F4**	0.000	-17.81 to 17.81	No	ns	>0.9999
27nm **P1** nanofibers **F5** vs. 27nm **P2** nanofibers **F4**	0.000	-15.66 to 15.66	No	ns	>0.9999

Table S5. Tabulated statistical significance of the luciferase expression in Figure 5A.

Šídák's multiple comparisons test	Predicted (LS) mean diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
DMEM - DMEM + 10% FBS					
Lipofectamine™ 2000	59.58	28.78 to 90.39	Yes	***	0.0001
71nm **P1** nanofibers **F1**	97.16	70.49 to 123.8	Yes	****	<0.0001
27nm **P2** nanofibers **F4**	96.36	69.68 to 123.0	Yes	****	<0.0001
16nm **P2** nanospheres **S3**	98.29	71.61 to 125.0	Yes	****	<0.0001

Table S6. Tabulated statistical significance of the cell viability in Figure 5B.

Šídák's multiple comparisons test	Predicted (LS) mean diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
DMEM - DMEM + 10% FBS					
Lipofectamine™ 2000	-1.839	-15.75 to 12.08	No	ns	0.9942
71nm **P1** nanofibers **F1**	-19.50	-31.55 to -7.454	Yes	***	0.0009
27nm **P2** nanofibers **F4**	-21.79	-33.84 to -9.741	Yes	***	0.0003
16nm **P2** nanospheres **S3**	-18.18	-30.23 to -6.133	Yes	**	0.0019

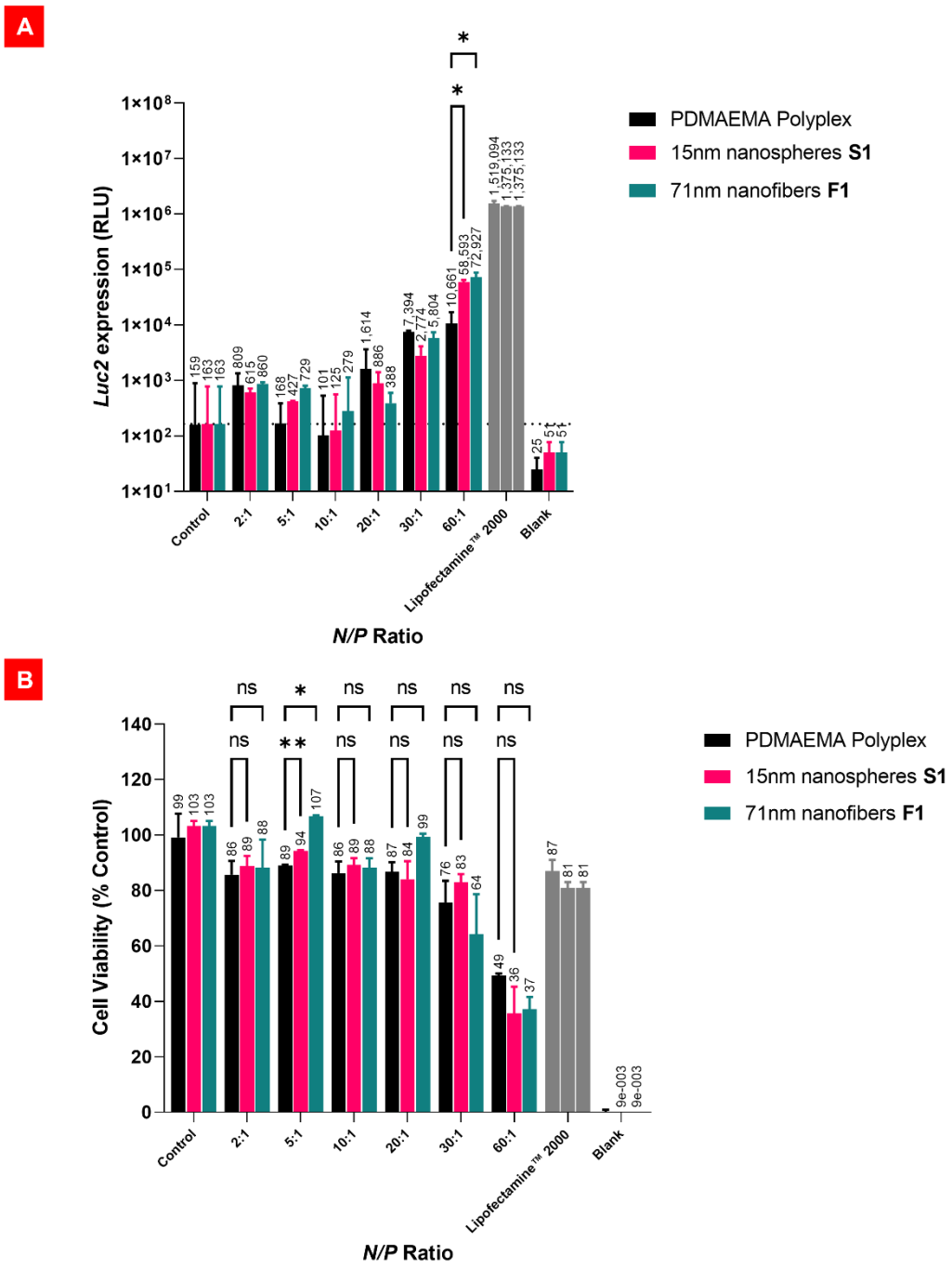


Figure S16. Examination of the effects of particle morphology upon (A) the transfection efficiency and (B) the cell viability of HEK293 cells transfected with a *luc2* plasmid (6,358 bp). The transfection efficiency and cell viability of 71 nm nanofiber **F1** micelleplexes ($Đ_L$ = 1.03, σ = 13 nm) was compared to 15 nm nanosphere **S1** micelleplexes ($Đ_D$ = 1.05, σ = 3 nm) and $PDMAEMA_{249}$ polyplexes (D_n = 66 nm, $Đ_D$ = 1.08, σ = 19 nm for N/P = 60).[S3] Cells were incubated with pDNA complexes (N/P = 60, 1µg/mL pDNA) for 4 h, with the *luc2* expression and

cell viability quantified after 24 h using a combined ONE-Glo™ and CellTiter-Fluor™ assay. Lipofectamine™ 2000 was prepared and used immediately according to the instructions, whilst all other polymeric complexes were aged for 24 h prior to use. Results for (A) were expressed as the relative light units (RLU), and for (B) as % of control cells. The median value is plotted, with the 95% confidence interval (CI) as error ($n = \geq 3$). P values are listed as 'ns' (no significance), '*' ($P < 0.05$) or '**' ($P < 0.01$) as determined by 2way ANOVA analysis with multiple comparisons (Tukey correction).

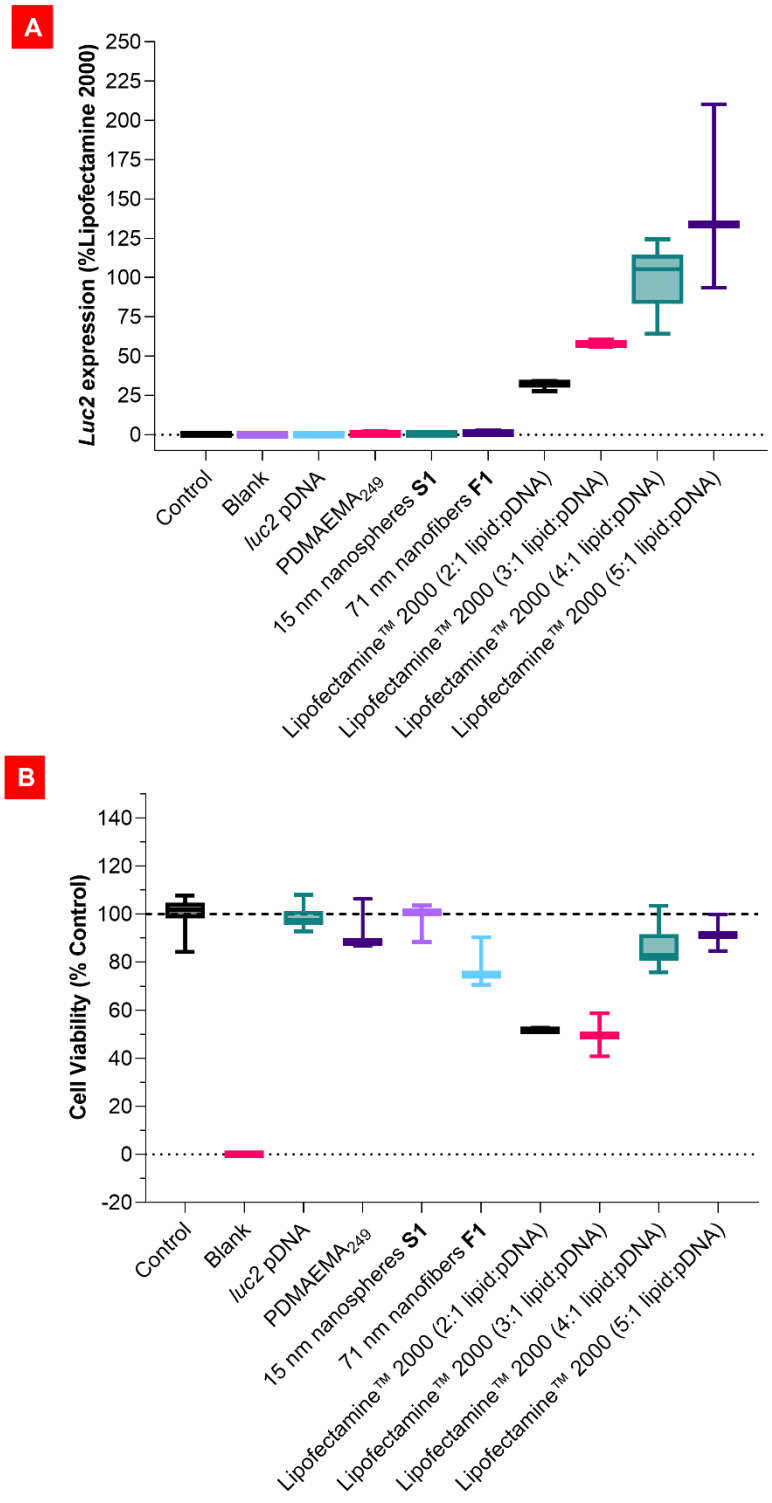


Figure S17. Examination of the effects of particle morphology upon (A) the transfection efficiency and (B) the cell viability of HEK293 cells transfected with a *luc2* plasmid (6,358 bp). This figure contains control samples, including the optimization of the lipofectamine 2000 positive control. Cells were incubated with blank media, pDNA, polymer samples without pDNA, or

lipofectamine™ 2000 at varied lipid:pDNA ratios (1μg/mL pDNA) for 4 h, before the supernatant was removed and replaced with fresh media. After 24 h, the *luc2* expression and cell viability were quantified using a combined ONE-Glo™ and CellTiter-Fluor™ assay. Results for (A) were expressed as % of the lipofectamine2000 control (4:1 lipid:pDNA), and for (B) as % of control cells. The median value is plotted, with the 95% confidence interval (CI) as error (n = ≥3).

References

S1 A. B. Pangborn, M. A. Giardello, R. H. Grubbs, R. K. Rosen and F. J. Timmers, *Organometallics*, 1996, **15**, 1518–1520.

S2 S. T. G. Street, Y. He, R. L. Harniman, J. D. Garcia-Hernandez and I. Manners, *Polym. Chem.*, 2022, **13**, 3009–3025.

S3 S. T. G. Street, J. Chrenek, R. L. Harniman, K. Letwin, J. M. Mantell, U. Borucu, S. M. Willerth and I. Manners, *J. Am. Chem. Soc.*, 2022, **144**, 19799–19812.

S4 H. C. Parkin, J. D. Garcia-Hernandez, S. T. G. Street, R. Hof and I. Manners, *Polym. Chem.*, 2022, **13**, 2941–2949.