Electronic Supplementary Material (ESI) for Polymer Chemistry.


# Supplementary Information

## Length-tuneable biocompatible block copolymer nanorods with a poly(2-methyl-2-oxazine)-corona via heat-induced crystallisation-driven self-assembly

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## Instrumentation

### Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR spectra were attained with the use of a Bruker Avance III 400 MHz spectrometer, using residual protonated solvent as an internal reference. Chemical shifts were reported in parts per million (ppm). Polymer samples were prepared to an approximate concentration of 5 mg $mL^{-1}$ in chloroform-d ($CDCl_3$).

### Size Exclusion Chromatography (SEC)

SEC analyses were conducted using a Shimadzu modular system equipped with a DGU-12A degassing unit, SIL-20 AD automatic injection system, a 5.0 μm bead-size guard column (50 x 7.8 mm) followed by three KF-805 L columns (300 x 8 mm, 10 μm, 5000 Å maximum pore size), SPD-20A ultraviolet and RID-10A refractive index detectors. N,N-dimethylacetamide (DMAc, 0.05 wt% LiBr) was used as eluent. Polymer samples were prepared to a concentration ranging between 2-5 mg $mL^{-1}$ and filtered with the use of 0.45 μm PTFE-based membrane filters prior to analysis. Injection volumes of 100 μL and a flow rate of 1.0 mL $min^{-1}$ were used.

### Ultraviolet-Visible Light Spectrophotometry (UV-VIS)

UV-VIS analyses were conducted using a Shimadzu UV-3600 UV-VIS NIR spectrophotometer equipped with an external temperature control system comprising a S-1700 thermoelectric single cell holder and an NTT-2200PP constant temperature water circulator. Polymer samples were prepared to a concentration of 5 mg $mL^{-1}$ in MilliQ water and filtered through 0.45 μm nylon-based membrane filters prior to measurement. 1 mL of each polymer solution was pipetted into a sealed Shimadzu SUPRASIL® Quartz glass cuvette (10 mm light path), samples were analysed at 700 nm incident light and a 2 °C $min^{-1}$ temperature ramp from 30 to 70 °C with 30 seconds equilibration time.

### Dynamic Light-Scattering (DLS)

DLS analyses were conducted using a Malvern Panalytical Zetasizer Nano-ZS. Polymer solutions were prepared to a concentration of 5 mg $mL^{-1}$ in MilliQ water and filtered through 0.45 μm nylon-based membrane filters prior to measurement. 1 mL of each polymer solution was pipetted into disposable plastic cuvette for analysis then sealed. Thermal analyses were conducted from 30-70 °C using 1 °C increments and 30 seconds of equilibration time. All measurements were recorded in triplicate. Data are reported as number measurements with associated sample polydispersity indices.

### Differential Scanning Calorimetry (DSC)

DSC analyses were conducted using a Perkin-Elmer DSC8500 calorimeter. 2-5 mg of freeze-dried polymer were loaded into 40 μL aluminium pinhole pans then sealed. The pans were prepared for analyses by heating, under nitrogen, to 210 °C before annealing at 130 °C overnight to allow for PiPrOx crystallisation.

Sample analyses were conducted from 30 to 230 °C with a scan rate of 10 °C $min^{-1}$. A baseline scan was conducted using an empty pan in place of a sample pan.

**Transmission Electron Microscopy (TEM)**

TEM imaging was conducted using a FEI Tecnai $G^2$ T-20 microscope operating at 200 kV accelerating voltage from a lanthanum hexaboride ($LaB_6$) thermal emitter. Samples were prepared for TEM analysis by pipetting 2 μL of 0.1 mg $mL^{-1}$ colloidal solution onto the surface of a plasma de-contaminated (XEI Scientific Evactron® 25, 10 seconds) carbon coated copper grid (ProSciTech). After approximately 2 min, excess solution was wicked away and the grid placed onto a drop of UranyLess stain for an additional minute. Excess stain was wicked away and the grids were left to air dry before being returned to the sample box for imaging. Images were captured with the use of a CCD camera and nanorod length was analysed with the use of ImageJ analysis software (v1.53t) (N = 250).

**Atomic Force Microscopy (AFM)**

AFM imaging and measurements were conducted using a Cypher ES Atomic Force Microscope (Oxford Instrument, Asylum Research, Santa Barbara, CA, USA) at room temperature (25 °C). 0.5 mg $mL^{-1}$ aqueous sample solutions were drop-cast and left to settle for 20 min before being dried under pure nitrogen gas. Images were collected using amplitude-modulated-AFM (AM-AFM). Young's modulus (elasticity) was measured using contact mode where force vs. distance data (force-curve measurements) were converted to indentation data. An OMCL-240TS cantilever (Olympus) with a nominal spring constant (ks¬) of 2 nN $nm^{-1}$ was used for each measurement. Each cantilever was calibrated using the thermal spectrum method, while the lever sensitivity was determined using force spectroscopy. The spring constant was resolved via the inverse optical lever sensitivity (InVOLS) using force curve measurements of the underlying mica surface.

**Flow Cytometry**

Flow cytometry was conducted using a BD FACS Canto II flow cytometer. Solid state (L1, 488 nm) and HeNe (L2, 633 nm) excitation lasers were used in the detection of propidium iodide (PI, ex. 535 nm, em. 617 nm) and cyanine-5 (Cy5, ex. 651 nm, em. 670 nm) fluorophores respectively. NIH/3T3 murine fibroblasts suspensions were incubated at room temperature with PI for 30 min to establish live/dead populations. Stained cells were pipetted into 12 x 75 mm tubes for analysis by flow cytometry. Samples were run at a high flow rate (120 μL $min^{-1}$) and 10,000 events were recorded per sample (triplicate, N = 2 biological replicates). Samples were analysed using FlowJo v10.8.1 and gated on live populations and % Cy5 positivity.

**Confocal Imaging**

Cells were treated with 500 μg $mL^{-1}$ concentration of Cy5 labelled nanoparticles (Ex. 639. Em. 779) and imaged over time. Cell lysosomes and nuclei were stained using Lysotracker Green (Ex. 497. Em. 618) and Hoechst 33362 (Ex. 410. Em. 496) respectively. Live cell images were obtained using a Leica TCS SP8 Laser-scanning confocal microscope with HCX PL APO 63x (NA 1.40) oil objective. 405 Diode (L1), Argon (L2), and HeNe 633 (L3) lasers were used in the detection of the fluorophores. Images were analysed with FIJI and deconvoluted with LAS X version 4.3.0.24308. Processing was performed using lightning deconvolution with Digital Signal Enhancement (DSE) of 3 frames with a weight 0.2 and periodicity of 1. Images are brightness and contrast-adjusted. Imaging is representative of three or more experiments.

## Supplementary Figures and Tables

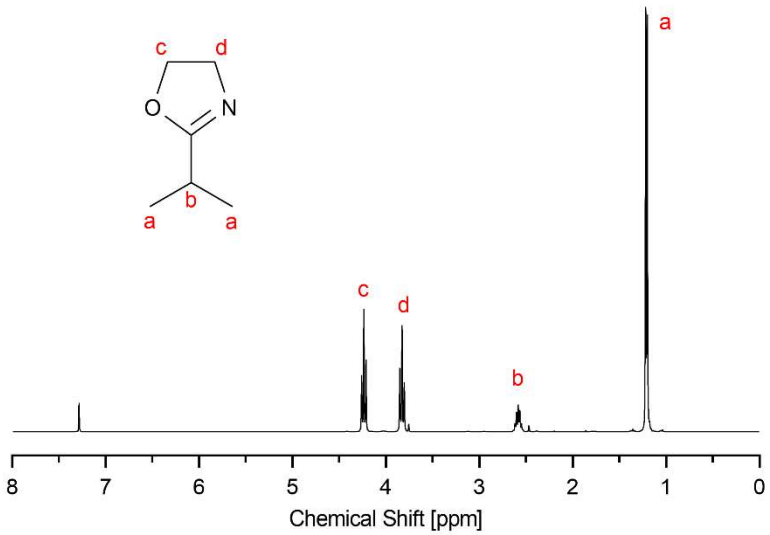


**Figure S1.** $^{1}H$ NMR ($CDCl_3$, 400 MHz) spectrum of iPrOx monomer. δ: 4.23 (t, $CH_2$, 2H, J = 9.4 Hz), 3.81 (t, $CH_2$, 2H, J = 9.4 Hz), 2.57 (h, CH, 1H, J = 6.7 Hz), 1.20 (d, $CH_3$, 6H, J = 6.7 Hz).

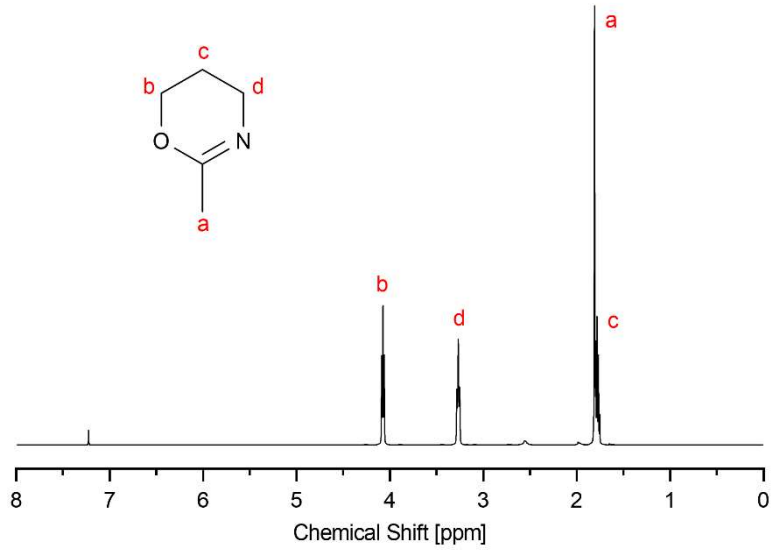


**Figure S2.** $^{1}$H NMR ($CDCl_3$, 400 MHz) spectrum of MeOz monomer. δ: 4.07 (t, $CH_2$, 2H, J = 5.5 Hz), 3.27 (t, $CH_2$, 2H, J = 5.6 Hz), 1.81 (s, $CH_3$, 3H), 1.78 (q, $CH_2$, 2H, J = 5.3 Hz).

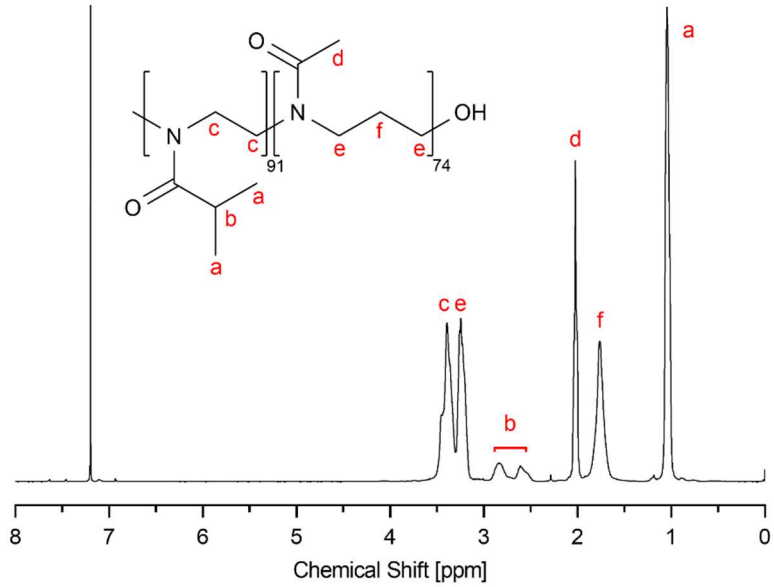


**Figure S3.** $^{1}$H NMR ($CDCl_3$, 400 MHz) spectrum of $PiPrOx_{91}$-b-$PMeOz_{74}$ block copolymer. δ: 3.39 ($2CH_2$, 364H), 3.24 ($2CH_2$, 296H), 3.01 ($CH_3$, 3H), 2.84-2.61 (CH, 91H), 2.02 ($CH_3$, 222H), 1.76 ($CH_2$, 148H), 1.04 ($2CH_3$, 546H).

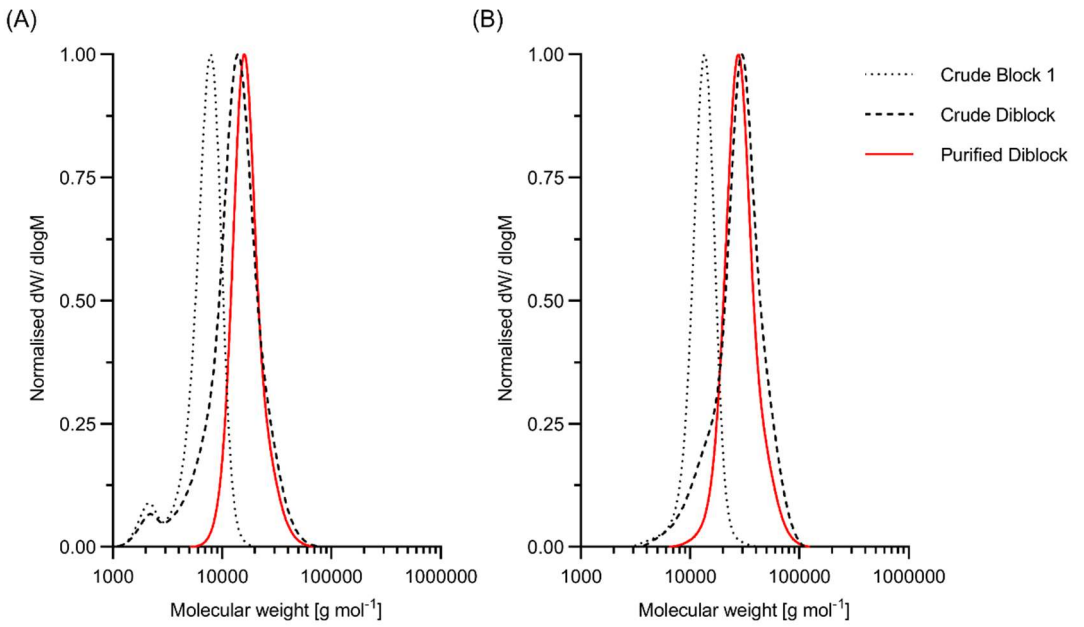


**Figure S4.** Normalised size-exclusion chromatograms of (A) PiPrOx$_{45}$-b-PMeOz$_{47}$ and (B) PiPrOx$_{91}$-b-PMeOz$_{74}$ (dashed).

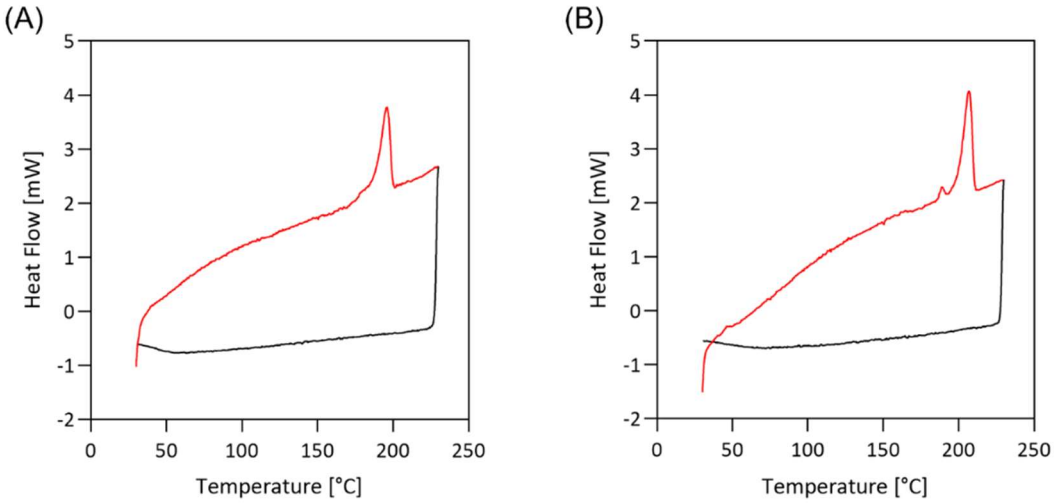


**Figure S5.** Baseline-subtracted DSC analysis of (A) PiPrOx$_{45}$-b-PMeOz$_{47}$ and (B) PiPrOx$_{91}$-b-PMeOz$_{74}$. The BCPs were heated from 30 °C to 230 °C with a scan rate of 10 °C min$^{-1}$. Heating scans (red) and cooling scans (black) are shown. Polymer samples were heated to 210 °C for 10 min, then annealed at 130 °C overnight prior to analysis to erase thermal history and allow PiPrOx to crystallise.

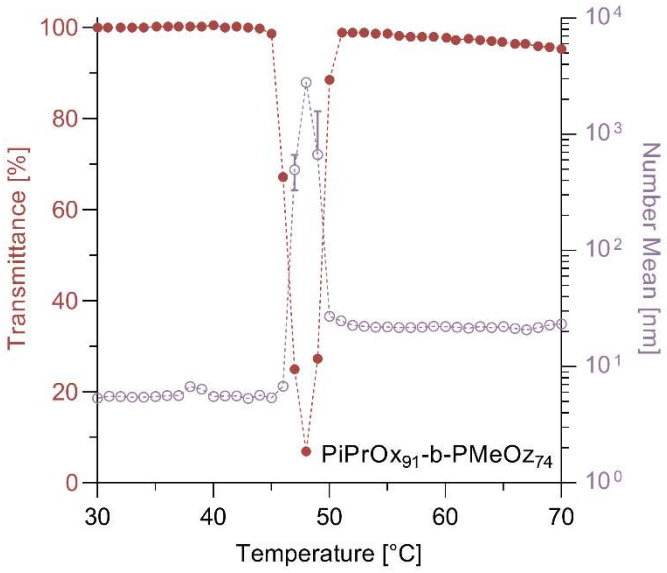


**Figure S6.** Cloud point temperature (TCP) determination of $PiPrOx_{91}$-b-$PMeOz_{74}$ (5 mg $mL^{-1}$ in MilliQ water). UV-VIS turbidimetry measurements were conducted using 600 nm incident light. DLS measurements were conducted in triplicate; error bars correspond to standard deviation.

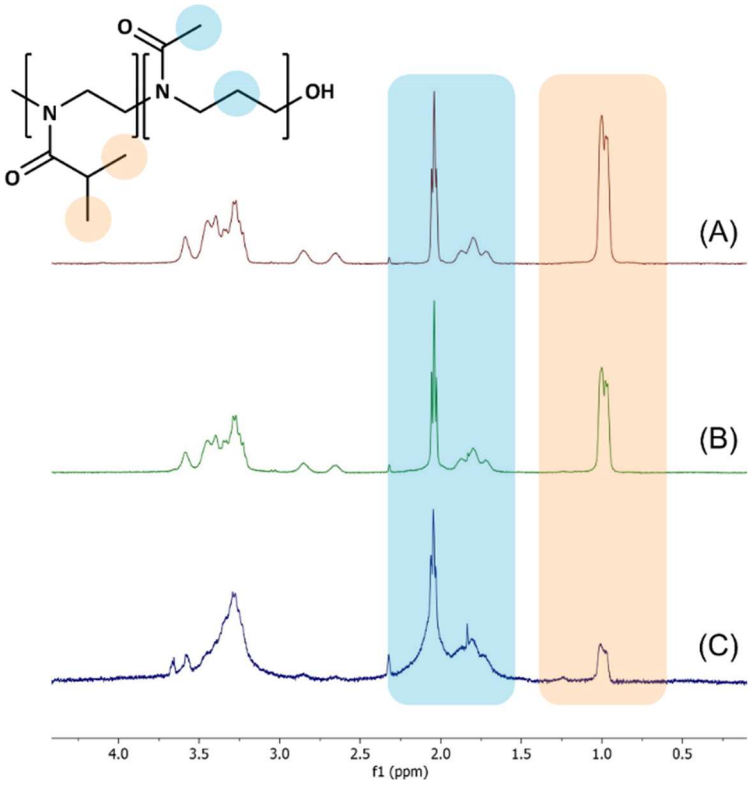


**Figure S7.** Changes to the $^{1}H$ NMR ($D_2O$, 400 MHz) spectrum of a 1 mg $mL^{-1}$ solution of $PiPrOx_{45}$-b-$PMeOz_{47}$ as it is annealed at 70 °C for (A) 0, (B) 6, and (C) 24 hours.

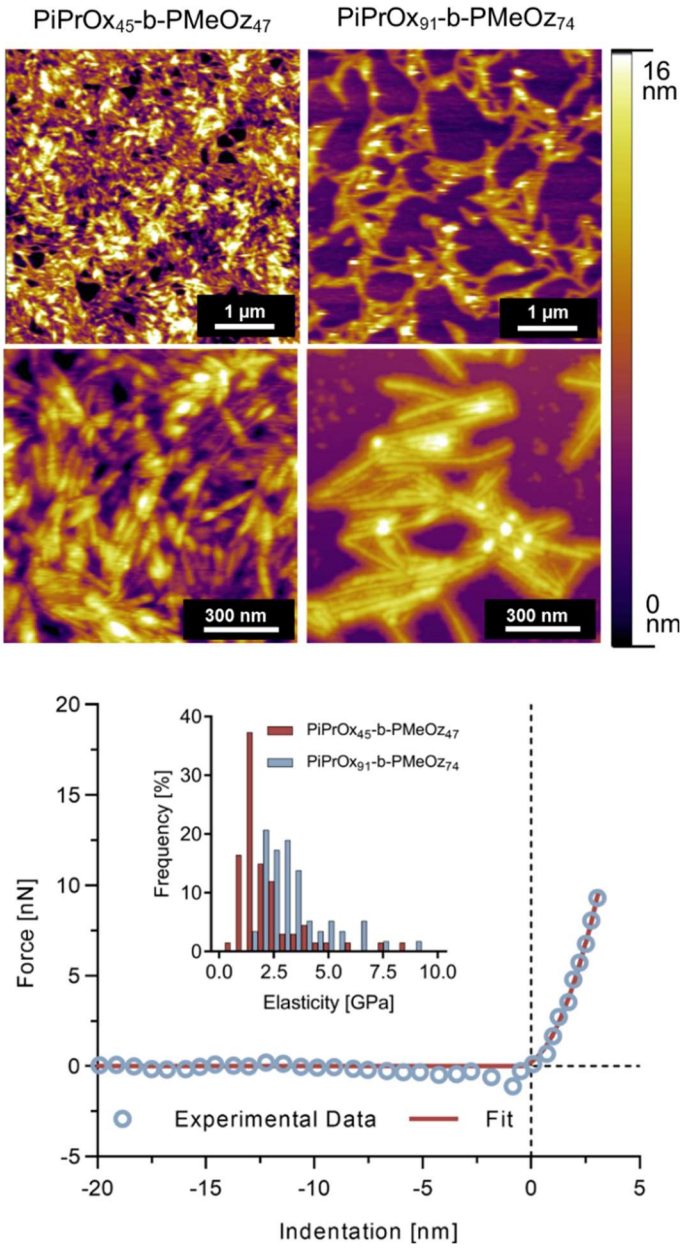


**Figure S8.** AFM height and indentation data for nanoparticles prepared form PiPrOx$_{45}$-b-PMeOz$_{47}$ and PiPrOx$_{91}$-b-PMeOz$_{74}$ BCPs.

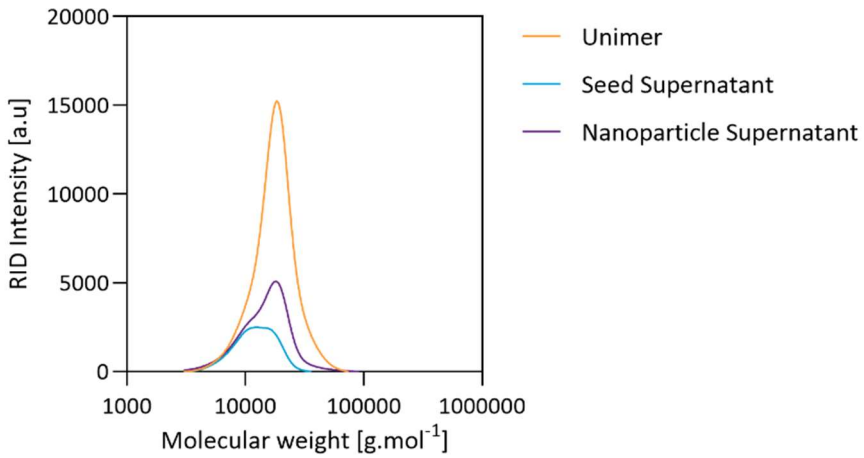


**Figure S9.** Size-exclusion chromatograms. Orange: $PiPrOx_{45}$-b-$MeOz_{47}$ unimer at 2.5 mg $mL^{-1}$. Blue: polymer retrieved from the supernatant of centrifugally filtered seeds. Purple: polymer retrieved from the supernatant of centrifugally filtered (Vivaspin 100kDa MWCO) 100 nm nanoparticles after being stored at 0.03125 mg $mL^{-1}$ for 24 h at 37 °C.

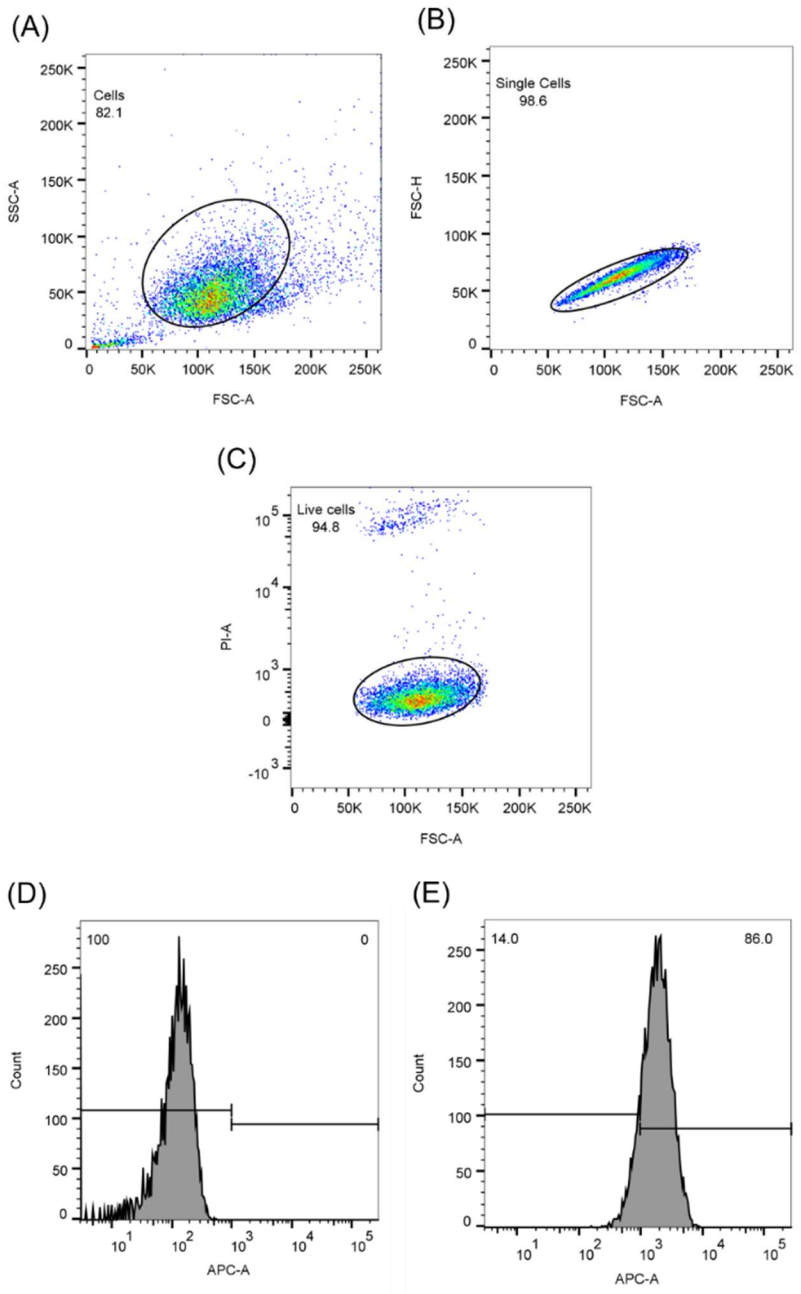


**Figure S10.** Representative gating strategy of NIH/3T3 murine fibroblast association with Cy5-labelled nanoparticles via flow cytometry. (A) Debris and other contaminants were excluded from the cell population. (B) Of the cells, single cells were selected. (C) Dead cells were identified with the use of propidium iodide staining and excluded. (D) Fluorescence histogram of a representative negative control. (E) Non-normalised fluorescence histogram after incubation with Cy5-labelled PiPrOx$_{45}$-b-PMeOz$_{47}$ nanoparticles for 240 min at 37 °C.

**Table S1.** PiPrOx$_{91}$-b-PMeOz$_{74}$ nanoparticle length dispersities as determined by TEM imaging (N = 250 per sample).

| **PiPrOx$_{91}$-b-PMeOz$_{74}$** | **50 °C – 3 h** | | | **70 °C – 1 h** | | |
|---|---|---|---|---|---|---|
| | **$L_n$ [nm]** | **SD** | **$L_w/L_n$** | **$L_n$ [nm]** | **SD** | **$L_w/L_n$** |
| Seeds | 55 | 17 | 1.10 | - | - | - |
| 1 | 106 | 29 | 1.07 | 105 | 24 | 1.05 |
| 2 | 151 | 38 | 1.06 | 123 | 35 | 1.08 |
| 3 | 180 | 47 | 1.07 | 156 | 36 | 1.05 |
| 4 | 240 | 69 | 1.09 | 171 | 62 | 1.13 |

**Table S2.** PiPrOx$_{45}$-b-PMeOz$_{47}$ nanoparticle length dispersities as determined by TEM imaging (N = 250 per sample).

| **PiPrOx$_{45}$-b-PMeOz$_{47}$** | **50 °C – 24 h** | | | **70 °C – 6 h** | | |
|---|---|---|---|---|---|---|
| | **$L_n$ [nm]** | **SD** | **$L_w/L_n$** | **$L_n$ [nm]** | **SD** | **$L_w/L_n$** |
| Seeds | 49 | 14 | 1.08 | - | - | - |
| 1 | 94 | 28 | 1.09 | 91 | 32 | 1.12 |
| 2 | 154 | 47 | 1.09 | 143 | 44 | 1.10 |
| 3 | 210 | 73 | 1.12 | 195 | 63 | 1.10 |
| 4 | 211 | 85 | 1.15 | 258 | 95 | 1.13 |

**Table S3.** Normalised Cyanine-5 MFI of gated samples analysed via flow cytometry.

| Polymer | Incubation time [min][a] | MFI [a.u.][b] | SD |
|---|---|---|---|
| $PiPrOx_{45}$-b-$PMeOz_{47}$ | 240 | 2932 | 417 |
| | 120 | 2123 | 126 |
| | 60 | 1162 | 47 |
| | 60 (4 °C) | 311 | 50 |
| | 30 | 880 | 164 |
| | 10 | 424 | 92 |
| $PiPrOx_{91}$-b-$PMeOz_{74}$ | 240 | 3173 | 1053 |
| | 120 | 2107 | 548 |
| | 60 | 1371 | 332 |
| | 60 (4 °C) | 495 | 88 |
| | 30 | 1056 | 335 |
| | 10 | 647 | 166 |
| $PMeOx_{48}$-b-$PiPrOx_{47}$ | 240 | 1109 | 255 |
| | 120 | 799 | 173 |
| | 60 | 479 | 85 |
| | 60 (4 °C) | 206 | 34 |
| | 30 | 357 | 94 |
| | 10 | 246 | 54 |
| **Negative Control** | - | 151 | 19 |

[a] Unless otherwise stated samples were incubated at 37 °C

[b] Cy5 MFI of the nanoparticles was normalised against a 0.03 mg $mL^{-1}$ $PiPrOx_{40}$-Cy5 homopolymer in D-PBS solution.