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SUPPORTING INFORMATION

Nitroxide-functional PEGylated nanostars arrest cellular oxidative stress and exhibit preferential accumulation in co-cultured breast cancer cells

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Synthetic method to prepare PFP polymers (P4, P5) and control polymer (C1, C2)

Linear polymers containing PFP moieties (**P4**, **P5**) were prepared using RAFT polymerisation. Briefly, deinhibited $OEGA_{480}$, deinhibited pentafluorophenyl acrylate (PFPA), AIBN, -(((benzylthio)carbonothioyl)thio)propanoic acid (BSPA, RAFT agent), and anhydrous toluene were introduced into a 6 ml vial charged with a magnetic stirrer, with stoichiometry $OEGA_{480}$:PFPA:BSPA:AIBN was used as 38:2:1:0.1 (to make polymer **P4**) and 16:24:1:0.1 (to make polymer **P5**). The mixture was then purged with nitrogen for 30 minutes followed by immersion in an oil bath preheated at 70 °C. After 5h, the mixture was cooled down to stop the polymerisation. ^{1}H NMR spectra of crude mixtures were used to determine conversions of each monomer. The products were purified by precipitation in an excess of petroleum benzine to remove any impurities and unreacted reagents, and subsequently dried overnight under vacuum. The two polymers were denoted as P-($OEGA_{38}$-*co*-$PFPA_2$), **P4** and P-($OEGA_{14}$-*co*-$PFPA_{21}$), **P5**.

PFP linear polymers (**P4**, **P5**) were further functionalised with Cy5 and TEMPO moieties using the same chemistry as employed for preparing stars **S1**-**S7**. Specifically, Cy5 amine was introduced into a solution of PFP polymer in $CDCl_3$ in the presence of TEA, and the reaction was left at ambient temperature for 24h before 4-amino TEMPO was introduced. The mole ratio of Cy5 amine and TEMPO amine in the reaction mixtures were calculated to assure the incorporation of similar or closely similar equivalents of Cy5 and TEMPO in star **S4**. For reaction mixture containing polymer **P4**, after 72h of adding Cy5 amine, three equivalents of ethanol amine was introduced. The reaction was conducted in $CDCl_3$ in an amber NMR tube and monitored using ^{19}F NMR.

Characterisation

^{1}H-NMR and ^{19}F-NMR

Spectra were recorded on a Bruker UltraShield (400 MHz for ^{1}H NMR and 376 MHz for ^{19}F NMR) spectrometer at 25 °C running Bruker Topspin Software, using $CDCl_3$ or $(CD_3)_2SO$ as solvent. ^{1}H chemical shifts and ^{19}F chemical shifts were reported as parts per million (ppm) and were referenced to the residual un-deuterated solvent (^{1}H NMR, $CDCl_3$, δ 7.26 ppm, or $(CD_3)_2SO$, δ 2.50 ppm).

Gel permeation chromatography (GPC)

GPC analyses of polymer samples were performed in *N*,*N*-dimethylacetamide (DMAc) with 0.03% w/v LiBr and 0.05% 2,6-dibutyl-4-methylphenol (BHT) using a Shimadzu modular system comprising a DGU-12A degasser, an SIL-10AD automatic injector, and a 5.0 µm bead-size guard column (50 × 7.8 mm) followed by four 300 × 7.8 mm linear Phenogel columns (bead size: a 5.0 µm; pore sizes: 105, 104, 103, and 500 Å) and an RID-10A differential refractive-index detector. The temperature of the columns was maintained at 50 °C using a CTO-10A oven, and the flow rate was kept at 1 mL min^{-1} using an LC-10AT pump. A molecular weight calibration curve was produced using commercial narrow molecular weight distribution polystyrene standards with molecular weights ranging from 500 to 10^6 g mol^{-1}. Polymer solutions were prepared in the eluent and filtered through 0.45 µm PTFE filters prior to injection.

Dynamic Light Scattering (DLS)

Samples were run at 25 °C on a Malvern Zetasizer ZS series equipped with a 4 mW laser at λ = 633 nm and a detector angle 173° running DTS software. Viscosity and refractive index were assumed to be 0.899 cP and 1.338 as for PBS and PEG particles, which were used to convert intensity-average hydrodynamic diameter to number and volume-based measurement. Z-average and the polydispersity index (PDI) illustrating the average diameters and size distribution were determined via a cumulants analysis of the measured intensity autocorrelation function by the DTS software. Samples were filtered using 0.45 µM Nylon syringe filters to remove all the contaminants and dust prior to measurement.

Fluorescence

Fluorescence spectra were acquired on a Shimadzu RF-5301 fluorescence spectrometer in quartz cuvettes of 10 mm path length.

Table S1. Polymerisation characteristics of the PFP stars.

Stars	**Ratio** [Arm]:[PFPA]:[MBAA]:[AIBN]	**Arm incorporation**[a]	M_n (kDa)[b]	$Đ$[b]	**PFPA loaded in star** (µmol/mg)[c]
P1	1 : 10 : 8 : 0.3	82.2%	95.6	1.15	0.60
P2	1 : 20 : 8 : 0.3	85.4%	100.5	1.21	1.01
P3	1 : 40 : 8 : 0.3	87.8%	96.5	1.22	2.87

[a] Data obtained from GPC deconvolution of the arm and star peaks of crude mixtures; [b] obtained from GPC traces of purified products. Polystyrene was used as standard for GPC analysis. [c] acquired from ^{19}F NMR using trifluoroacetic as an external standard, see experimental section for details.

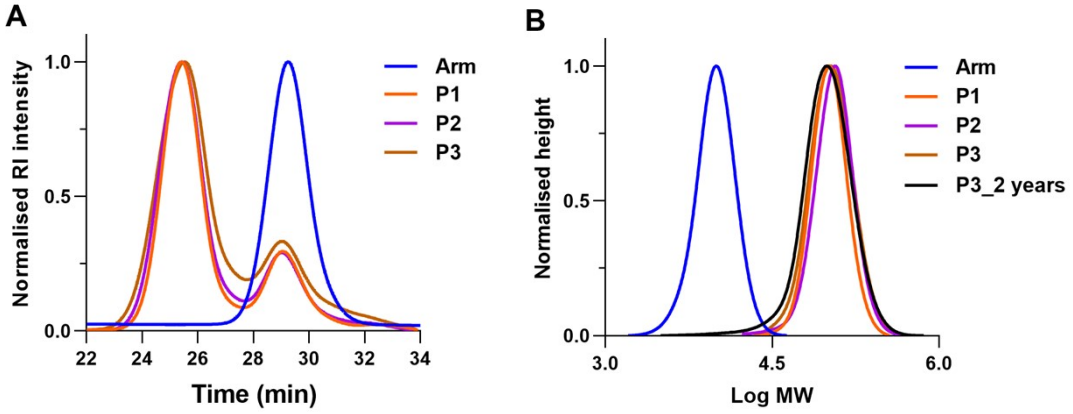


Figure S1. A. GPC traces of POEGA arm and unpurified PFP stars (**P1**-**P3**). B. Molecular weight distribution of POEGA arm, three purified PFP stars (**P1**-**P3**) and star **P3** after two years stored at -20 °C as determined by GPC.

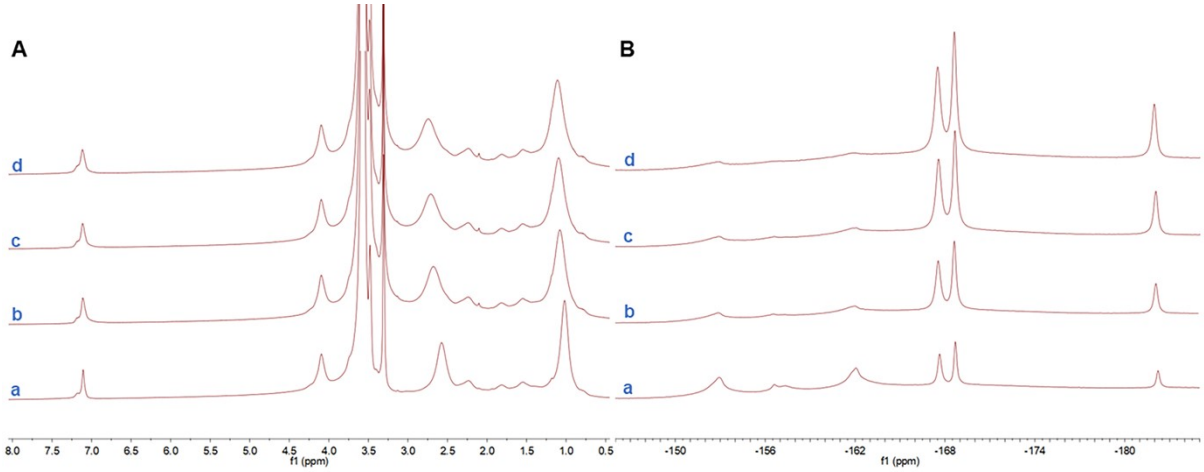


Figure S2. ^{1}H-NMR (A) and ^{19}F-NMR (B) spectra of the reaction mixture of star **P2** and amino-TEMPO at different time points: 15 minutes (a), 6 hours (b), 12 hours (c) and 24h (d). Solvent: $CDCl_3$.

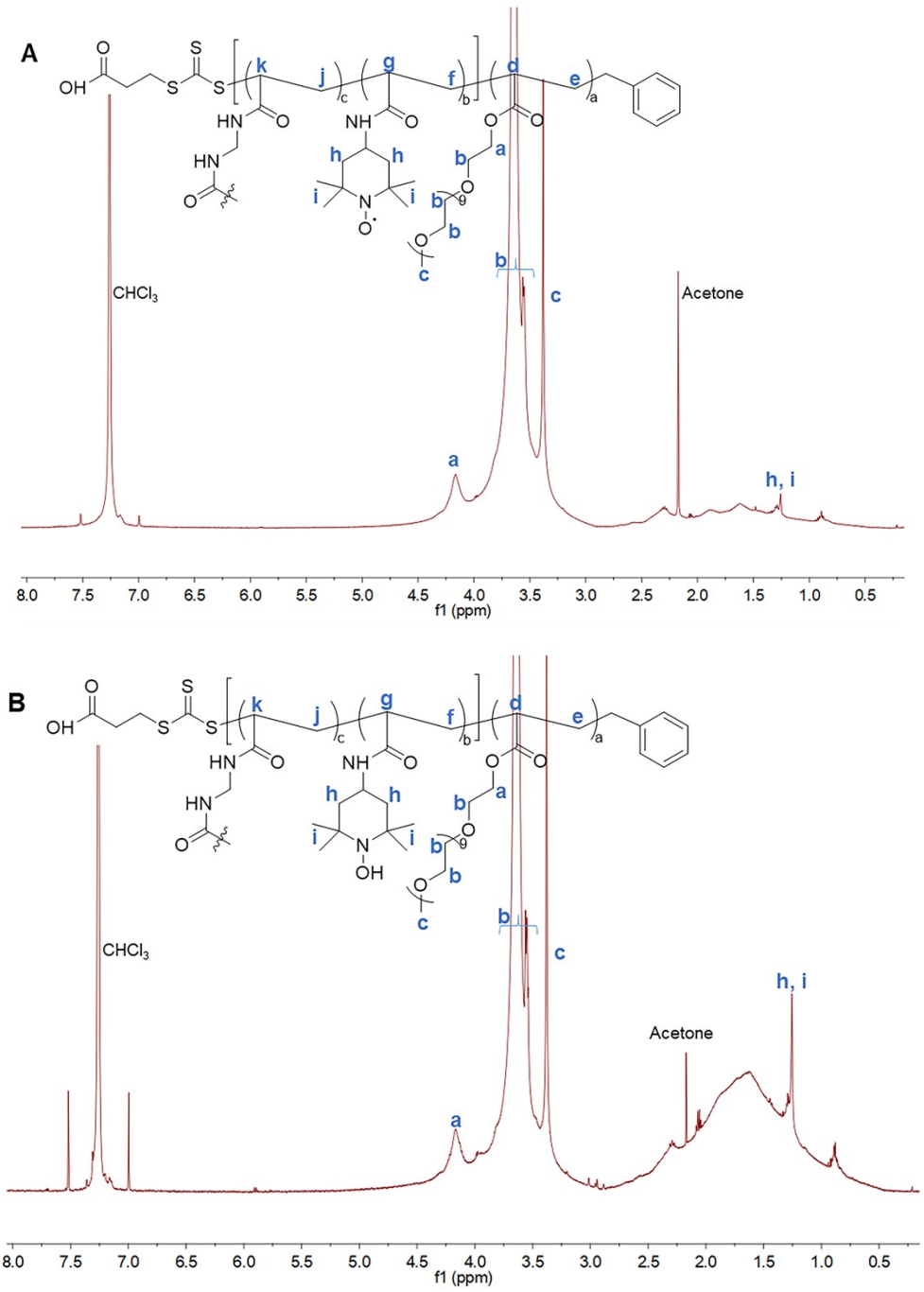


Figure S3. ^{1}H-NMR in $CDCl_3$ of **S2** (A) and after reduction by ascorbic acid (B), with main peak assignments.

Table S2. Stoichiometry in preparation of TEMPO based NanoStars and their characteristics

PFP stars (starting materials)		**TEMPO-amine** (mg)	**Cy5-amine** (mg)	M_n (kDa)[a]	$Đ$[a]	**Name of TEMPO stars**[b] (products)
Name	Weight (mg)					
P1	50	5.7	-	94.1	1.26	**S1**
P2	50	9.5	-	125.4	1.47	**S2**
P3	50	27.1	-	108.4	1.31	**S3**
P3	20	10.8	0.9	103.8	1.60	**S4**
-	-	-	-	130.8	2.02	**S5**[c]

[a] obtained from GPC traces of purified products; [b] these stars were prepared based on the PFP stars **P1**-**P3** as stated; [c] **S3** was reduced by ascorbic acid to form hydroxyl amine functionalised polymer.

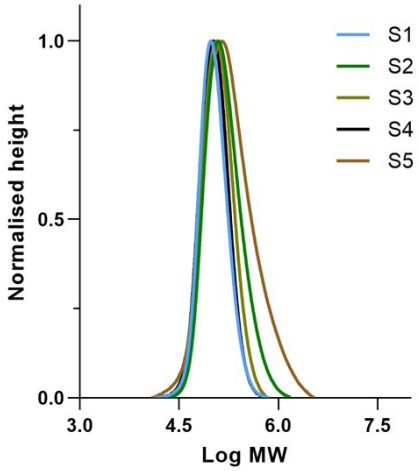


Figure S4. Molecular weight distribution of TEMPO based stars as determined by GPC.

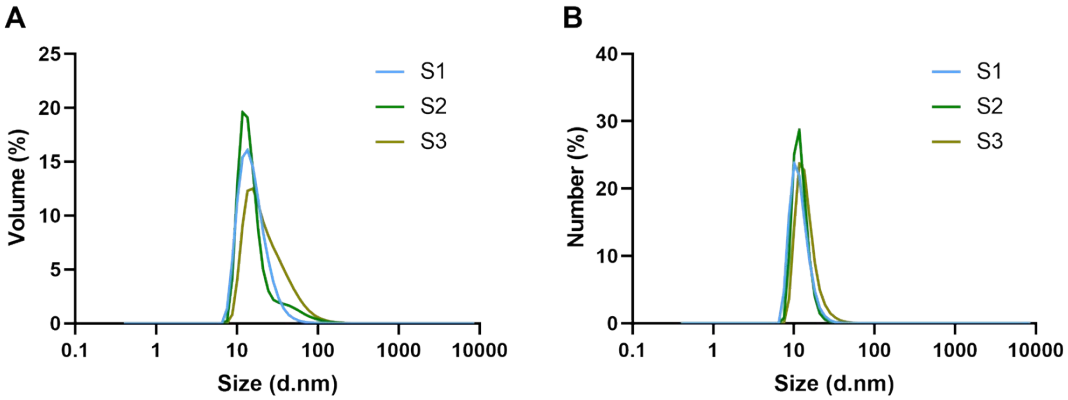


Figure S5. Size distribution by volume (A) and by number (B) for stars **S1**-**S3** in water at 1.0 mg/ml. Data were obtained via dynamic light scattering.

Table S3. Size distribution by volume and by number of **S1**-**S3** in water at 1.0 mg/ml. Data were obtained using DLS technique.

Star	Z-average (d.nm)	PDI	Volume mean (d.nm)	Number mean (d.nm)
S1	35	0.43	16	11
S2	57	0.30	18	10
S3	56	0.24	27	17

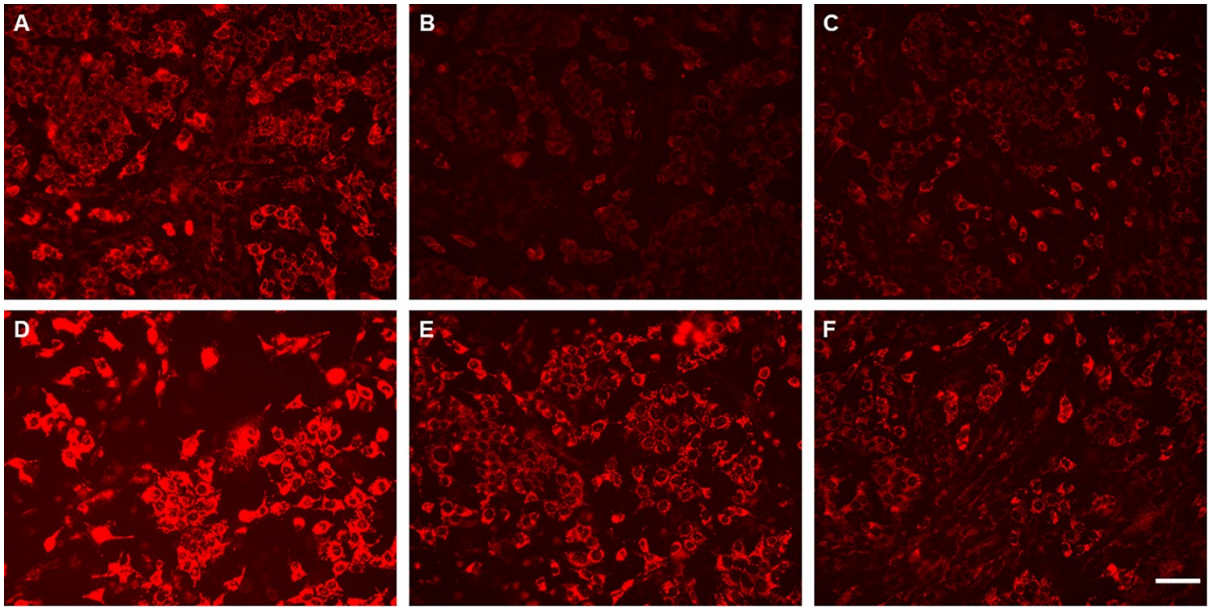


Figure S6. CellROX Deep Red channels for ROS detection in co-cultured breast cancer cells and fibroblasts when challenged with TBHP: cells were treated with **S1**, **S2**, **S3** (0.125 mg/mL, D, E, C) or TEMPO (0.38 mM, F) for one hour followed by exposure to TBHP (200 µM for 20 hours) before ROS staining. Control cells without treatment (A) or treated with **S3** only (B) are also presented. Scale bar: 100 µm. Images were obtained from an Operetta High content imaging system with a 20X magnification objective.

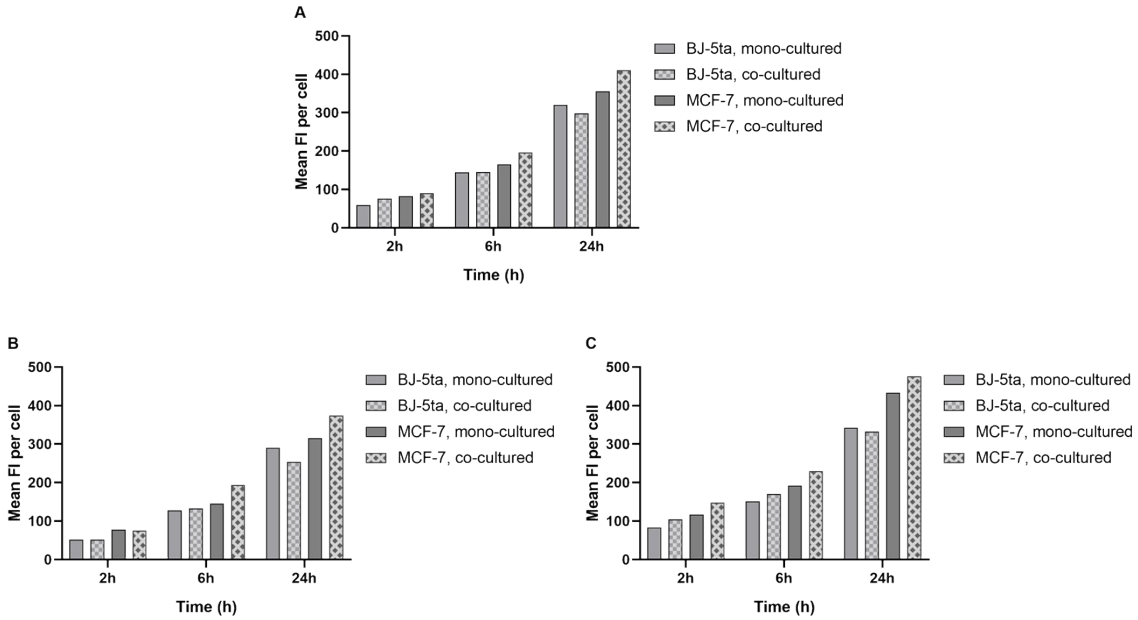


Figure S7. Mean fluorescence intensity per cell (MFI) when TEMPO-Cy5 star **S4** was introduced to mono-cultured or co-cultured cells of BJ-5ta and MCF-7 for 2 hours, 6 hours and 24 hours. Each graph represents data from one experiment from three technical replicates.

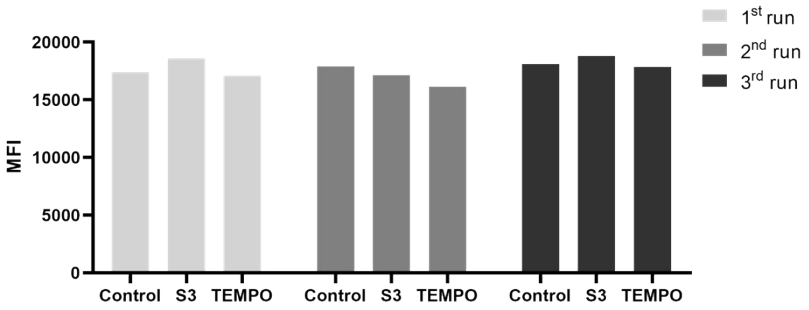


Figure S8. Fluorescence intensity from cell media containing AlamarBlue with added **S3** (0.125 mg/mL) or free TEMPO (0.38 mM). Media (Alamar Blue diluted at 10% in DMEM - 10% FBS - 1% pen/strep) were collected after introducing for two hours to co-cultured wells without any polymer treatment and were analysed using a Clariostar plate reader. For each run, media were collected from different wells.

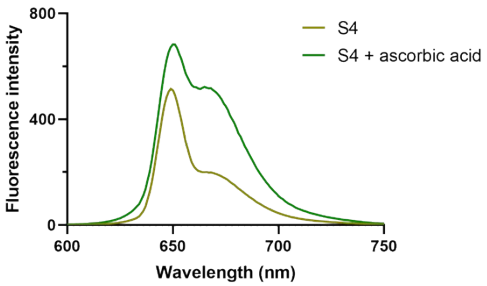


Figure S9. Fluorescence spectrum of **S4** when excited by a wavelength 650 nm, with and without the presence of ascorbic acid. [**S4**] = 0.25 mg/mL; [ascorbic acid] = 0.5 mM (normal tissue concentration of ascorbic acid is 0.03-10 mM).[1, 2]

Table S4. Characteristics of PFP linear polymers (**P4**, **P5**) and their functionalised derivatives.

PFP polymers				Modified polymers		
Name	M_n (kDa)[a]	M_n (kDa)[b]	$Đ$[b]	Name	Cy5 loaded (nmol/mg polymer)	TEMPO loaded (nmol/mg polymer)
P-(OEGA$_{38}$-*co*-PFPA$_2$), **P4**	19.0	16.3	1.45	**C1**	0.05	0.00
P-(OEGA$_{14}$-*co*-PFPA$_{21}$), **P5**	12.0	17.9	1.32	**C2**	0.16	1.43

[a] obtained from conversion by ^{1}H NMR analysis; [b] obtained from GPC traces of purified products. Polystyrene was used as standard for GPC analysis.

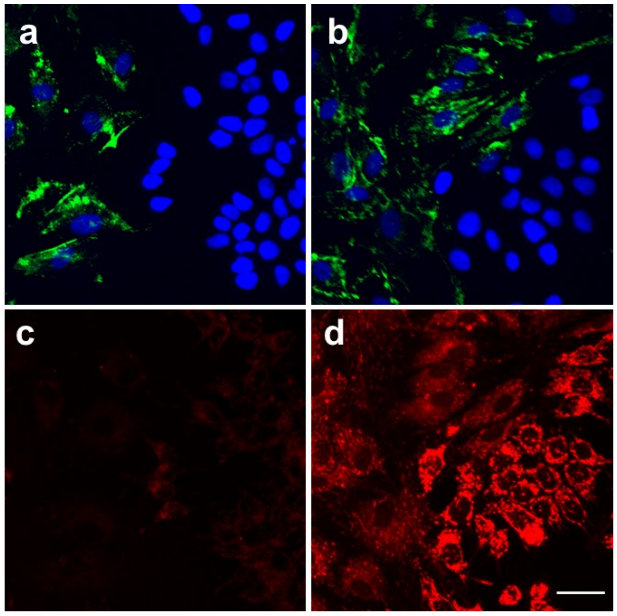


Figure S10. Merged channels of Hoechst 33342 (blue) and caveolin-1 (green) (a, b) and the corresponding Cy5 channels (c, d) of co-cultured BJ-5ta and MCF-7 cells when treated with polymer **C1** (Cy5-labelled linear polymer, a, c) or polymer **C2** (Cy5-TEMPO linear polymer, b, d) at 0.125 mg/mL. Cy5 images (c, d) were captured before cells were fixed and stained for visualisation of caveolin-1 (a fibroblast marker[3]) and nuclei, and the images were captured at the same position (a, b). Images were obtained from an Operetta High content imaging system with a 20X magnification objective. Scale bar 50 µm.

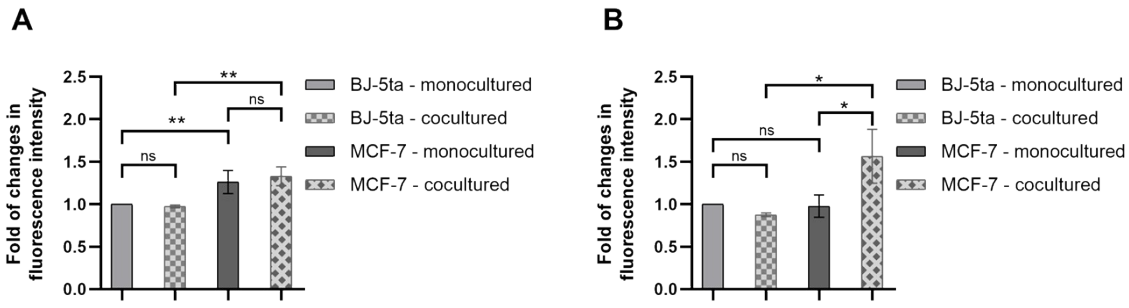


Figure S11. Fold of change in mean fluorescence intensity per cell (MFI) when Cy5 polymer **C1** (A) or Cy5-TEMPO-polymer **C2** (B) was introduced to mono-cultured or co-cultured cells of BJ-5ta and MCF-7 for 24 hours. In each graph, data from each group of the same experiment were normalised to the value of the mono-cultured fibroblasts treated with labelled polymers. Matched two-way ANOVA with Sidak's multiple comparisons - post hoc test was used for statistically different analysis among MFI from different populations, (*), $p < 0.05$; (**), $p < 0.01$; ns: not significant; error bar: S.E.M.

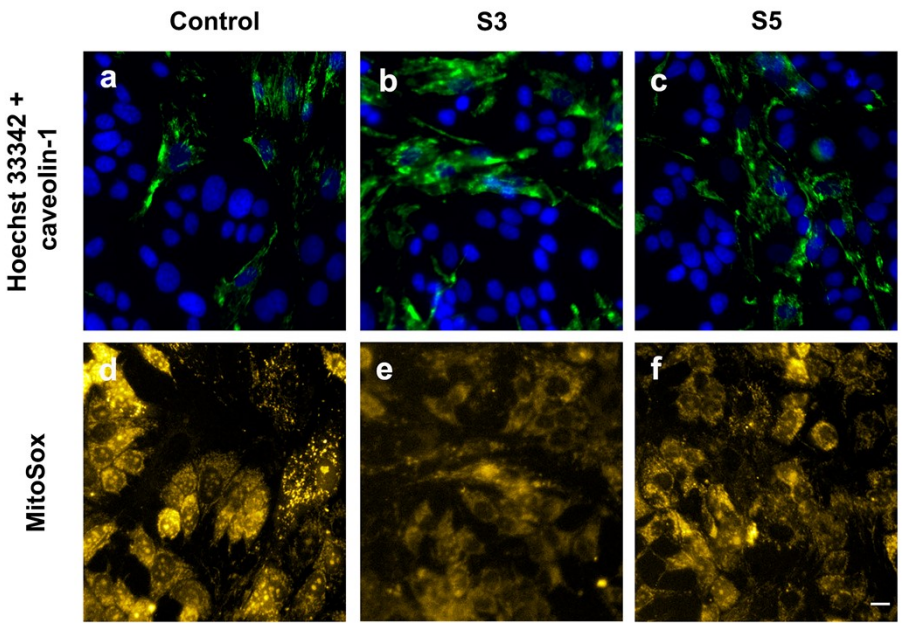
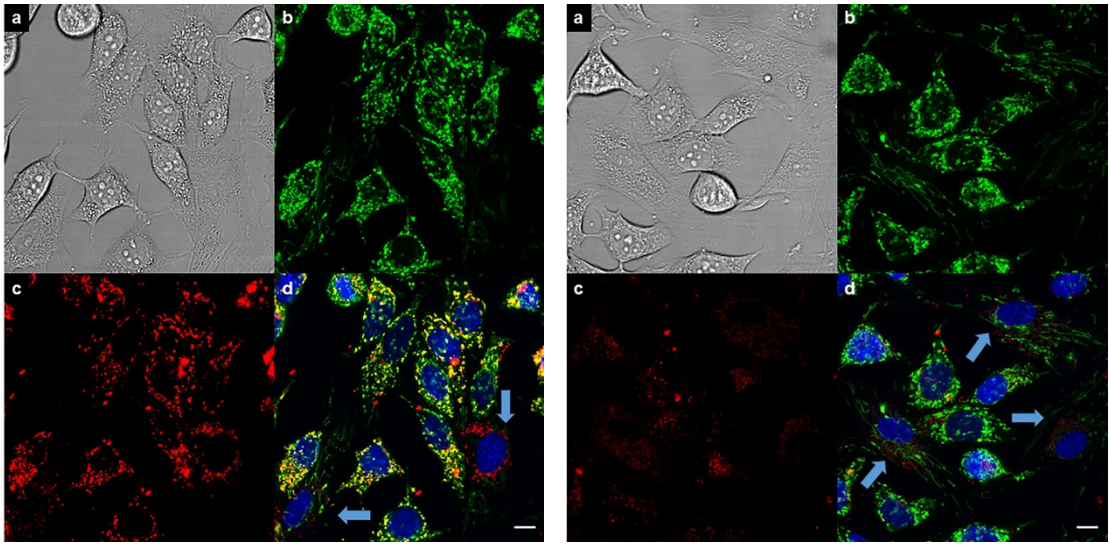
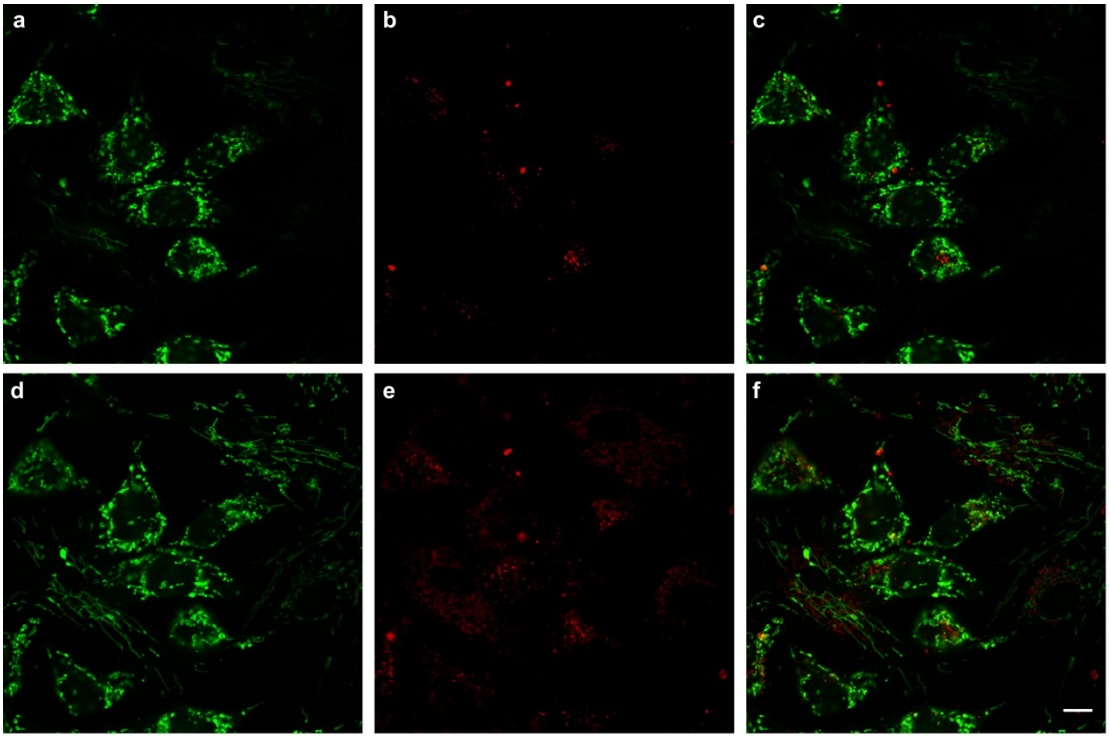


Figure S12. Merged channels of Hoechst 33342 (blue) and caveolin-1 (green) (a, b, c) and the corresponding MitoSOX channels (d, e, f) of BJ-5ta - MCF-7 co-cultured cells when treated with TEMPO-star **S3** (b, e) or its ascorbic acid-reduced analogue **S5** (c, f) or left untreated for 24 hours (a, d). Polymers were employed at 0.125 mg/mL. MitoSOX Red was employed for staining mitochondrial superoxide anion before the images (d, e, f) were captured. Subsequently, cells were fixed and stained for visualisation of caveolin-1 (a fibroblast marker[3]) and nuclei and the images were captured at the same position (a, b, c). Images were obtained from an Operetta High content imaging system with a 20X magnification objective. Scale bar 20 µm.



A B



C

Figure S13. Brightfield images (a), mitochondrial channels (b), Cy5 channels (c) and the merged channels of mitochondria, Cy5 and nuclei (blue) (d) of co-cultured BJ-5ta and MCF-7 breast cancer cells after 24-hour treatment with reduced-TEMPO-Cy5 star **S6** (A) and cyclohexyl-Cy5 star **S7** (B). Fluorescent images with the maximum intensity of a z-stack projection are shown. In panel C, mitochondria channels (a, d), Cy5 channels (b, e) and their merged channels (c, f) at two different z-stacks were illustrated instead of maximum

projections in panel B. Cells were loaded with TMR and Hoechst 33342 for 30 minutes for visualisation of mitochondria and nuclei, respectively. Fibroblasts are denoted by blue arrows. Scale bar: 10 µm. Data were taken from the Leica TCS-SP8 confocal lightning system using a 40× magnification objective.

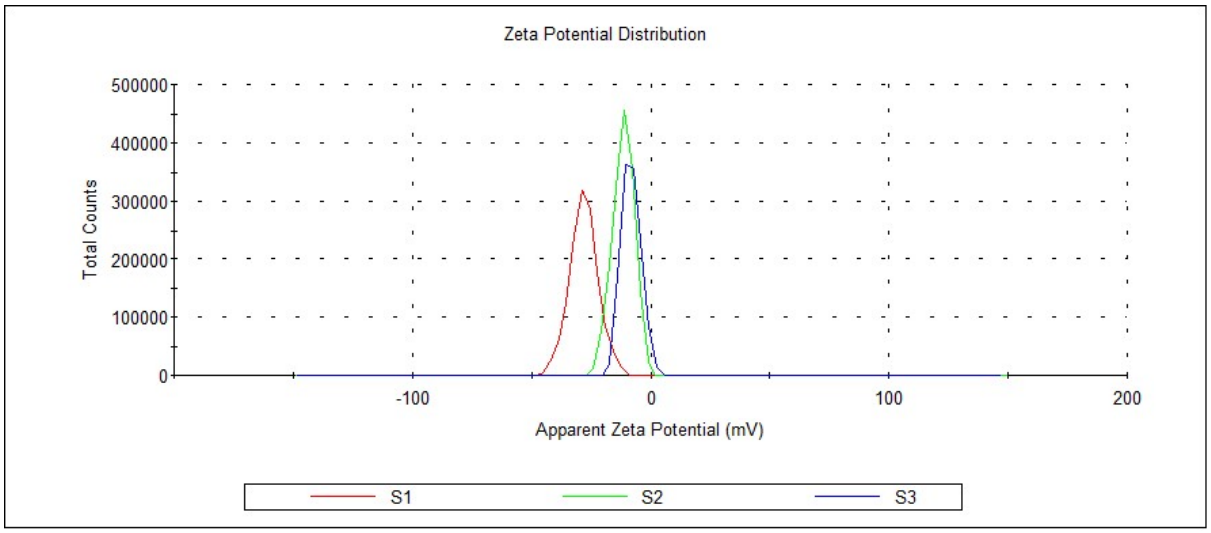


Figure S14. Representative zeta potential distributions of non-labelled TEMPO-conjugated stars (**S1**, **S2**, **S3**) suspended in water. Average zeta potentials of particles are: -28.4 mV (**S1**), -11.6 mV (**S2**) and -7.9 mV (**S3**).

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