Supporting Information for the Manuscript Entitled Synthesis of Next Generation Dual-Responsive Cross-Linked Nanoparticles and their Application in Anticancer Drug Delivery†

Christina Schwarzenböck,[a] Peter J. Nelson,[b] Ralf Huss[c] and Bernhard Rieger*[a]

[a.] *WACKER-Lehrstuhl für Makromolekulare Chemie, Technische Universität München, Lichtenbergstraße 4, 85748 Garching bei München, Germany. E-Mail: rieger@tum.de*

[b.] *Medizinische Klinik und Poliklinik IV, Nephrologisches Zentrum und Arbeitsgruppe Klinische Biochemie, University of Munich, Munich, Germany.*

[c.] *Definiens AG, Bernhard-Wicki-Strasse 5, 80636 Munich, Germany.*

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1. Material and methods

General Information

All reactions were carried out under argon atmosphere using standard Schlenk or glovebox techniques. All glassware was heat dried under vacuum prior to use. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich, ABCR, Acros Organics or TCI Europe and used as received. Toluene and dichloromethane (DCM) were dried using a MBraun SPS-800 solvent purification system. The precursor complexes $Y(CH_2Si(CH_3)_3)_3(THF)_2$, $LiCH_2TMS$ and the catalyst $Cp_2Y(CH_2TMS)(THF)$ were prepared according to literature procedures.[1-4] Diethyl vinylphosphonate (DEVP) as well as 2-bromoethylphosphonic dichloride were synthesised according to literature procedures.[5-7] All monomers were dried over calcium hydride and distilled prior to use.

Nuclear Magnetic Resonance Spectroscopy

NMR spectra were recorded on a Bruker AVIII-300, AV-500HD and AVIII-500 Cryo spectrometer. ^{1}H-NMR spectroscopic chemical shifts δ are reported in ppm relative to the residual proton signal of the solvent. δ (^{1}H) is calibrated to the residual proton signal of the solvent. Deuterated solvents were obtained from Sigma-Aldrich and dried over 3 Å molecular sieves.

Elemental Analysis (EA)

Elemental analyses were measured on a Vario EL (Elementar) at the Laboratory for Microanalysis at the Institute of Inorganic Chemistry at the Technische Universität München.

Gel Permeation Chromatography

Gel Permeation Chromatography was performed on a *Varian* LC-920 equipped with two PL Polargel M columns with samples of 5 mg/mL. A mixture of 50% THF, 50% water, 9 g/L tetrabutylammonium bromide (TBAB) and 340 mg/L 3,5-Di-*tert*-butyl-4-hydroxytoluene (BHT) as stabilising agent was used as eluent. Absolute molecular weights have been determined by multiangle light scattering (MALS) analysis using a Wyatt Dawn Heleos II in combination with a Wyatt Optilab rEX as concentration source.

Turbidity Measurements

Turbidity measurements were performed on a Cary 50 UV/Vis spectrophotometer (Varian) in 40 mm × 10 mm × 2 mm quartz glass cuvettes with a magnetic stirring bar. The cloud point of the aqueous polymer solutions was determined by spectrophotometric detection of the changes in transmittance at λ = 500 nm, respectively 650 nm in case of the DMEM/PBS samples. The samples were heated/cooled at a rate of 1.0 K/min in steps of 1 K followed by a 5 min long period of constant temperature to ensure equilibration. The cloud point was defined as the temperature corresponding to a 10% decrease in optical transmittance.

Dialysis

Purification *via* dialysis was performed with a Spectra/Por 1 dialysis tubing (regenerated cellulose) with a molecular weight cut-off (MWCO) of 6-8 kDa (Spectrumlabs). Before use the membranes were treated with deionised water over night and then rinsed with deionised water. A 100:1 ratio of dialysis fluid to sample volume was applied. Specific solvents used as dialysis fluid are given for the corresponding procedures.

DLS Measurements

The samples were dissolved in water at a concentration of 2.5 mg/mL. The measurement was performed at a Zetasizer Nano ZS (Malvern). The diameter was averaged over three independent values consisting each of 10 measurements.

Transmission Electron Microscopy

The nanoparticle solutions were added onto a copper grid with a continuous carbon film for one minute and stained with a 2% uranyl acetate solution. Microscopy was done with a JEM 1400 plus microscope (JEOL, Tokyo) with a LaB6 filament operated at 120 kV. Images were collected with a Ruby (JEOL) CCD camera with a pixel size of 0.332 nm.

Surface Tension Measurements

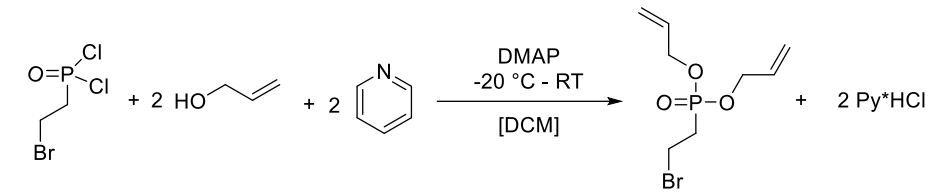
For the surface tension analysis, the samples were dissolved in deionised water and then a dilution series was prepared (0.025 mg/mL to 1.000 mg/mL). The measurements were performed on a Drop Shape Analyzer DSA100 (Krüss). The surface tension of each concentration was averaged over the values of three independent droplets.

Photoluminescence Measurements

Photoluminescence spectra were recorded on a AVA-Spec 2048 spectrometer (AVANTES) with a current controller as 365 nm light source (Prizmatix). A 90° cuvette holder and a 40 mm × 10 mm × 2 mm polystyrene cuvette (VWR) were used for the measurements. Intensity calibration of the spectrometer was performed by an AVANTES DH-Cal calibration light source using the halogen lamp.

2. Syntheses

2.1 Monomer synthesis[8]

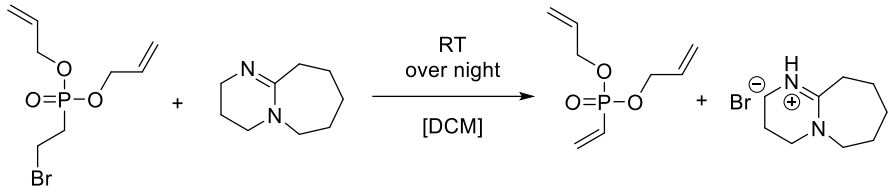


$C_2H_4BrCl_2OP$ 225.83 g/mol | C_3H_6O 58.08 g/mol | C_5H_5N 79.10 g/mol | $C_8H_{14}BrO_3P$ 269.07 g/mol

Allylic alcohol (2.1 eq., 93.0 mmol, 6.3 mL), pyridine (2.1 eq., 93.0 mmol, 7.5 mL) and a catalytic amount of DMAP were dissolved in anhydrous DCM. This solution was cooled to -10 °C, kept at this temperature for 30 min and cooled further to -20 °C. 2-Bromoethylphosphonic dichloride (1.0 eq., 44.3 mmol, 5.9 mL) was dissolved in DCM and added dropwise. After full addition the solution was kept 1 h at -20 °C and was slowly warmed to RT overnight. The resulting white solid is filtered off and the solution is washed three times with water. After drying with $MgSO_4$ the evaporation of the solvent under reduced pressure followed. The product was obtained as a clear slightly yellow liquid (95.6%, 42.3 mmol, 11.4 g).

^{1}H-NMR (300 MHz, $CDCl_3$, 300 K) δ [ppm] 5.99-5.87 (m, 2 H, 2 x $POCH_2CH$=CH_2), 5.39-5.25 (m, 4 H, 2 x $POCH_2CH$=CH_2), 4.57-4.53 (m, 4 H, 2 x $POCH_2CH$=CH_2), 3.58-3.49 (m, 2H, PCH_2CH_2-Br), 2.49-2.37 (m, 2H, PCH_2CH_2-Br).

^{31}P-NMR (121 MHz, $CDCl_3$, 300 K) δ [ppm] 26.51.



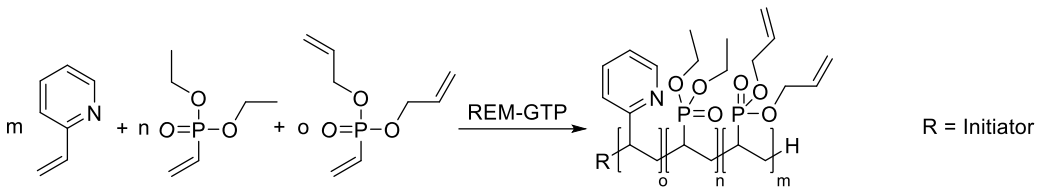
$C_8H_{14}BrO_3P$ 269.07 g/mol | $C_9H_{16}N_2$ 152.24 g/mol | $C_8H_{13}O_3P$ 188.16 g/mol

1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU, 2.8 eq.) was added dropwise to a stirred solution of diallyl-2-bromoethyl phosphonate in DCM. The resulting dark solution was washed three times with 2 M HCl and dried over $MgSO_4$. The solvent was evaporated under reduced pressure which resulted a viscous brown raw product. After testing varying purification methods the most successful one was vacuum distillation with addition of 1000 ppm phenothiazin to inhibit radical side reactions. The product was obtained as a clear liquid.

^{1}H-NMR (300 MHz, $CDCl_3$, 300 K) δ [ppm] 6.26-5.74 (m, 5 H, PCH=CH_2, PCH=CH_2, 2 x $POCH_2CH$=CH_2), 5.25-5.08 (q, 4 H, 2 x $POCH_2CH$=CH_2), 4.56-4.50 (t, 4 H, 2 x $POCH_2CH$=CH_2).

^{31}P-NMR (121 MHz, $CDCl_3$, 300 K) δ [ppm] 17.90.

2.2 Copolymerisation procedure and analysis



2,4,6-Trimethylpyridine (1.00 eq.) was added to a solution of $Cp_2Y(CH_2TMS)(THF)$ (1.00 eq.) in toluene (6 mg catalyst pro mL). After quantitative conversion was shown by ^{1}H-NMR spectroscopy, the respective equivalents of 2-vinylpyridine were added in one portion. The reaction mixture was stirred overnight. One aliquot (0.1 mL) was taken and quenched by the addition of 0.4 mL CD_3OD (calculation of conversion of 2-vinylpyridine *via* ^{1}H-NMR) in an NMR-tube while the calculated amount of DEVP was added to the reaction solution. The conversion of DEVP was determined by ^{31}P-NMR spectroscopy after 90 min. The DEVP procedure was repeated with DAlVP (5 eq.). The reaction was quenched by addition of methanol (0.50 mL) and the polymer was precipitated by pouring the reaction mixture into pentane (150 mL). The clear solution was decanted of, residual solvent was removed by drying at ambient temperature and the polymer was dissolved in water and lyophilised.

Molecular weights and molecular weight distributions of the first polymerisation sequence were measured *via* GPC-MALS analysis of the first aliquot. Composition A/BB' [2VP/DEVP DAlVP] is determined *via* ^{1}H-NMR-spectroscopy of the dried block copolymer. The molecular weight of the block copolymer is determined with the help of the composition A/BB' and the molecular weight of the first block. Molecular weight distributions are determined *via* GPC-MALS analysis.

Table S1 Composition, molecular weight (M_n) and $Đ$ of blockcopolymer substrates

	Feed A_{eq}/B_{eq}	**Composition A/B [2VP/DEVP]**	**$Eq_{,NMR}$ (B')/Chain**	**M_n (A) [* 10^4 g/mol]**	**$M_{n,NMR}$ (AB) [* 10^4 g/mol]**	**$Đ$**
AB1	$2VP_{100}/DEVP_{100}$	1/1.3	6	1.3	4.1	1.17
AB2	$2VP_{100}/DEVP_{200}$	1/2.2	5	1.2	5.4	1.12
AB3	$2VP_{200}/DEVP_{200}$	1/1.4	5	2.5	7.9	1.10

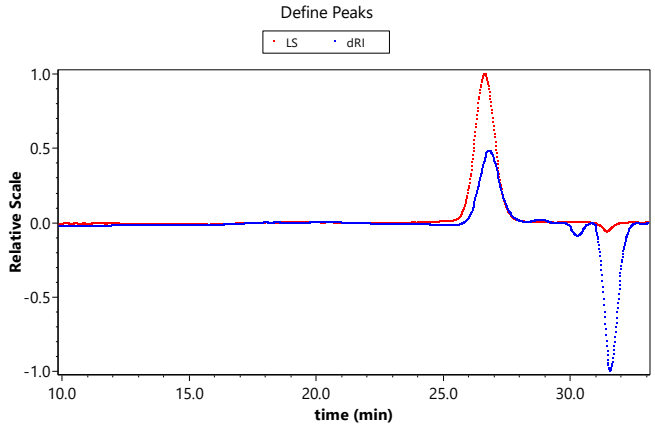


Fig. S1 GPC-traces of P2VP block of AB1.

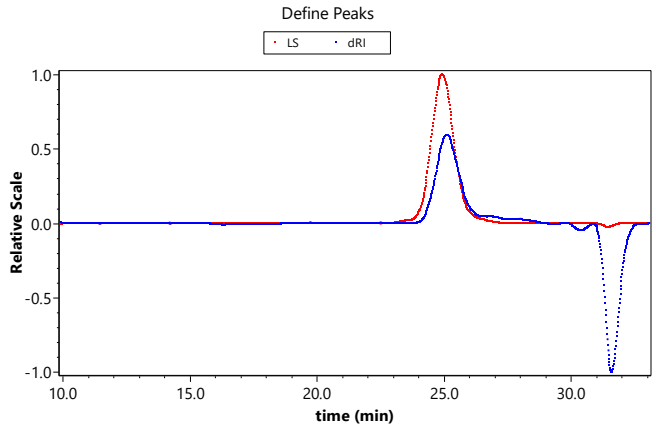


Fig. S2 GPC-traces of 2VP/DEVP blocks of AB1.

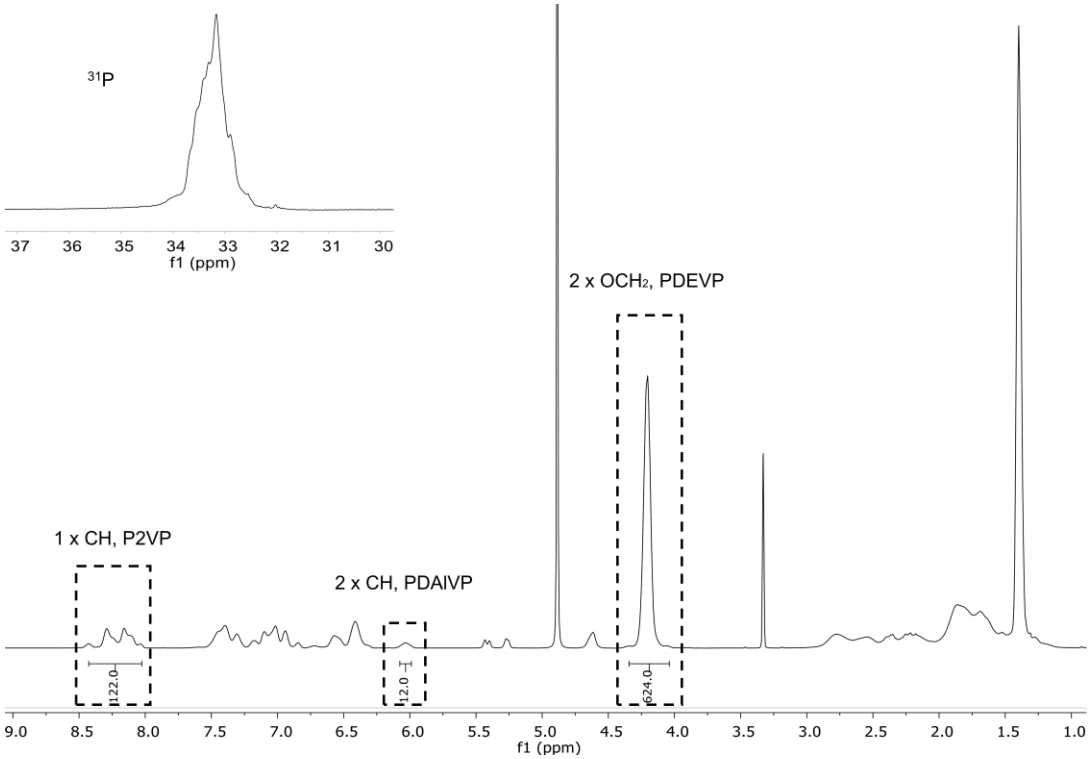


Fig. S3 ^{1}H- and ^{31}P-NMR spectrum of AB1 in MeOD.

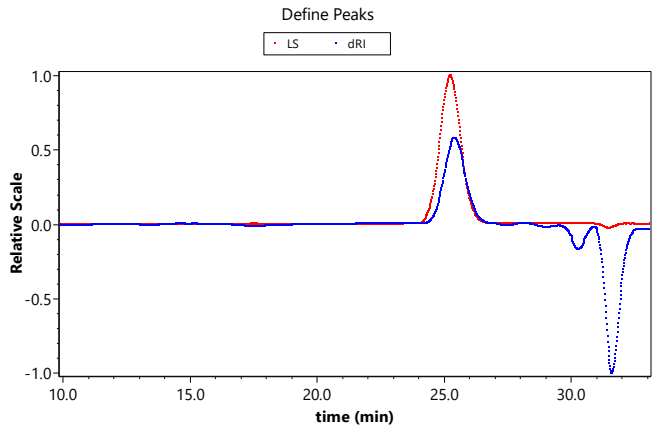


Fig. S4 GPC-traces of P2VP block of AB2.

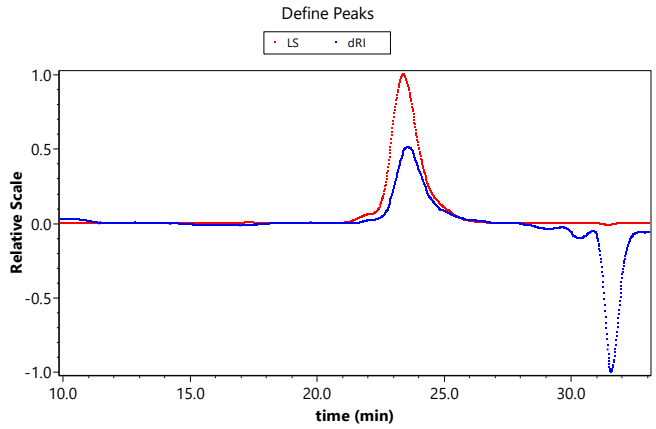


Fig. S5 GPC-traces of 2VP/DEVP blocks of AB2.

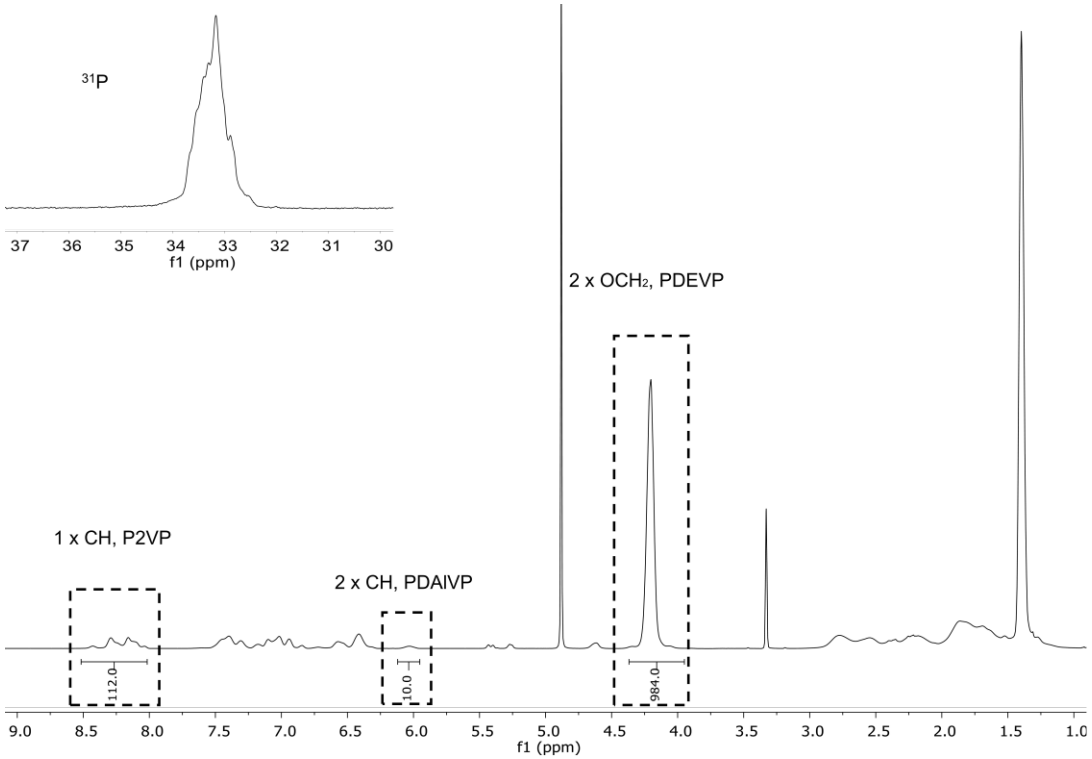


Fig. S6 ^{1}H- and ^{31}P-NMR spectrum of AB2 in MeOD.

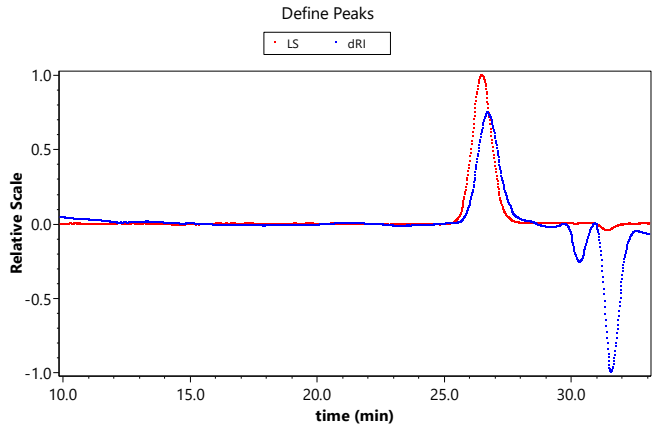


Fig. S7 GPC-traces of P2VP blocks of AB3.

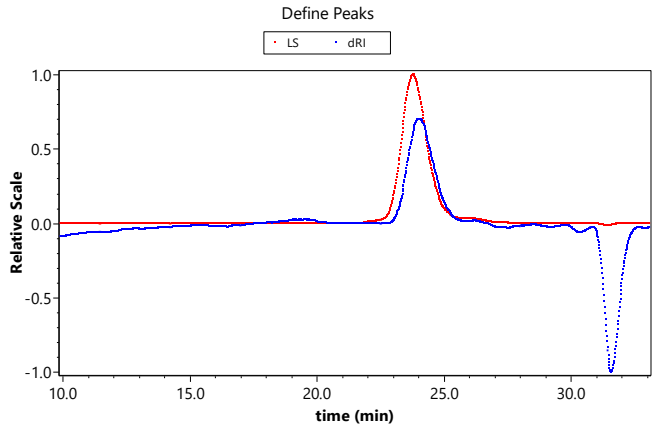


Fig. S8 GPC-traces of 2VP/DEVP block of AB3.

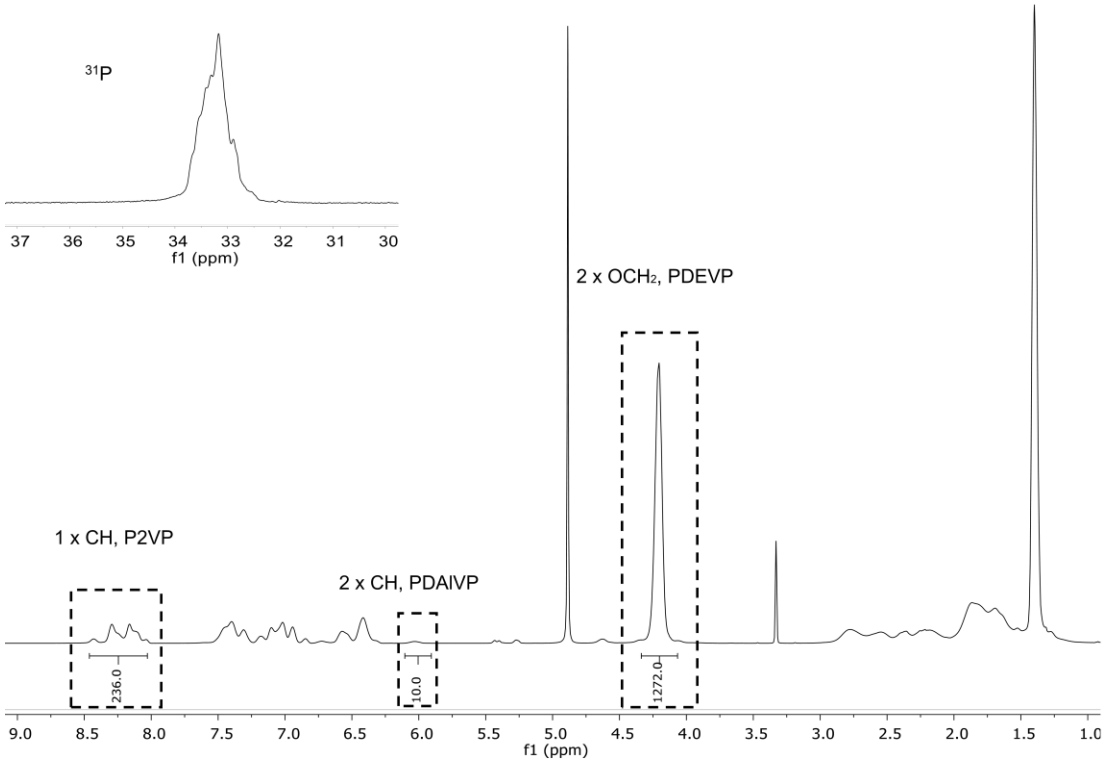
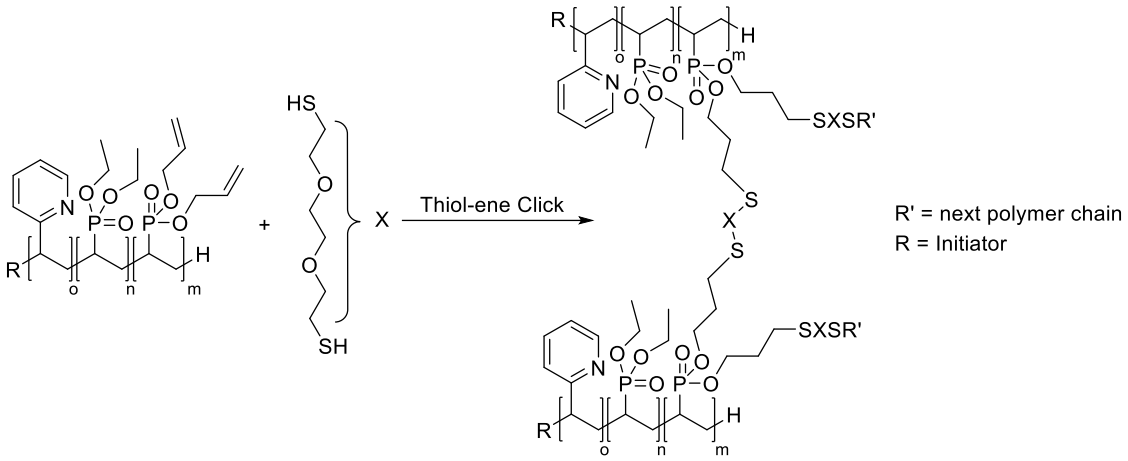


Fig. S9 ^{1}H- and ^{31}P-NMR spectrum of AB3 in MeOD.

2.3 Nanoparticle synthesis *via* thiol-ene click reactions

General procedure: Thiol-ene coupling of polymer micelles towards stable nanoparticles



4.5 eq of 3,6-dioxa-1,8-octanedithiol corresponding to each double bond in the polymer are dissolved in DCM and added to the chosen polymer dissolved in DCM to reach an overall concentration of polymer in DCM of 4.6 mg/mL. After the addition of a catalytic amount of azobisisobutyronitrile (AIBN) the reaction mixture was degassed and heated to reflux for 16 h. The solvent was fully evaporated under reduced pressure and the resulting nanoparticles were dissolved in water, dialysed overnight and lyophilised to obtain a white powder.

Following NMR and elemental analysis were used to proof the full conversion of all double bonds and the presence of the according amounts of sulphur in the nanoparticles.

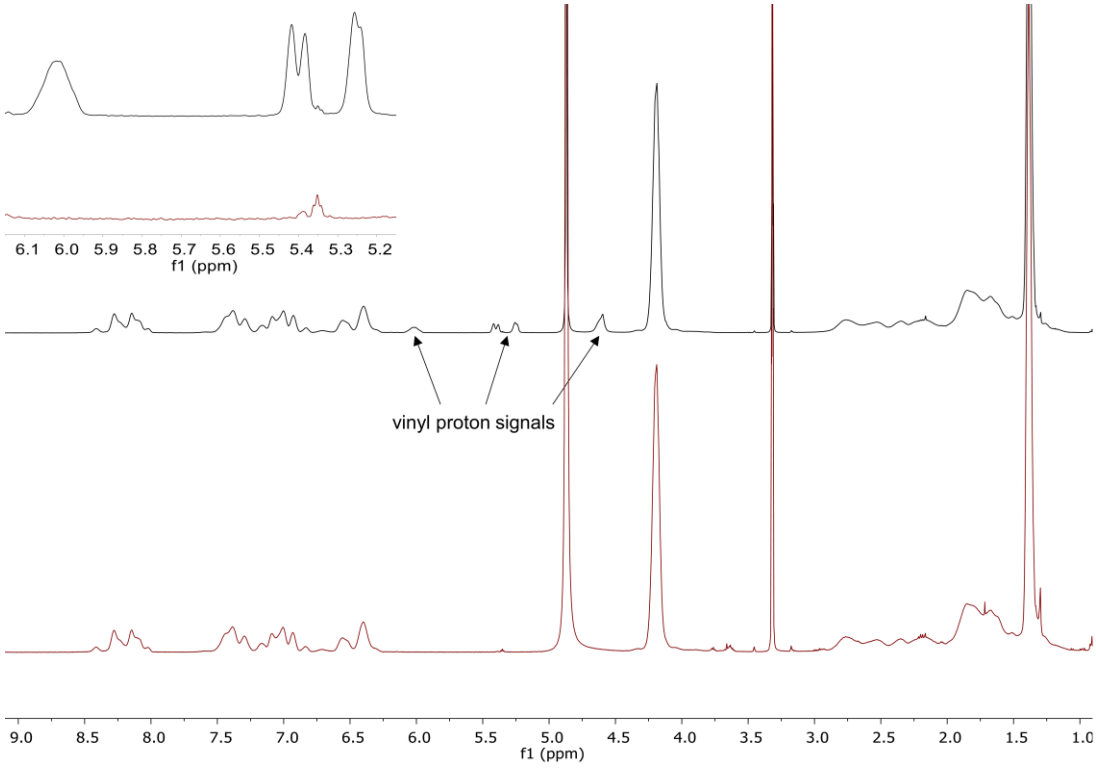


Fig. S10 ^{1}H-NMR spectra of AB1 and NP1 in MeOD.

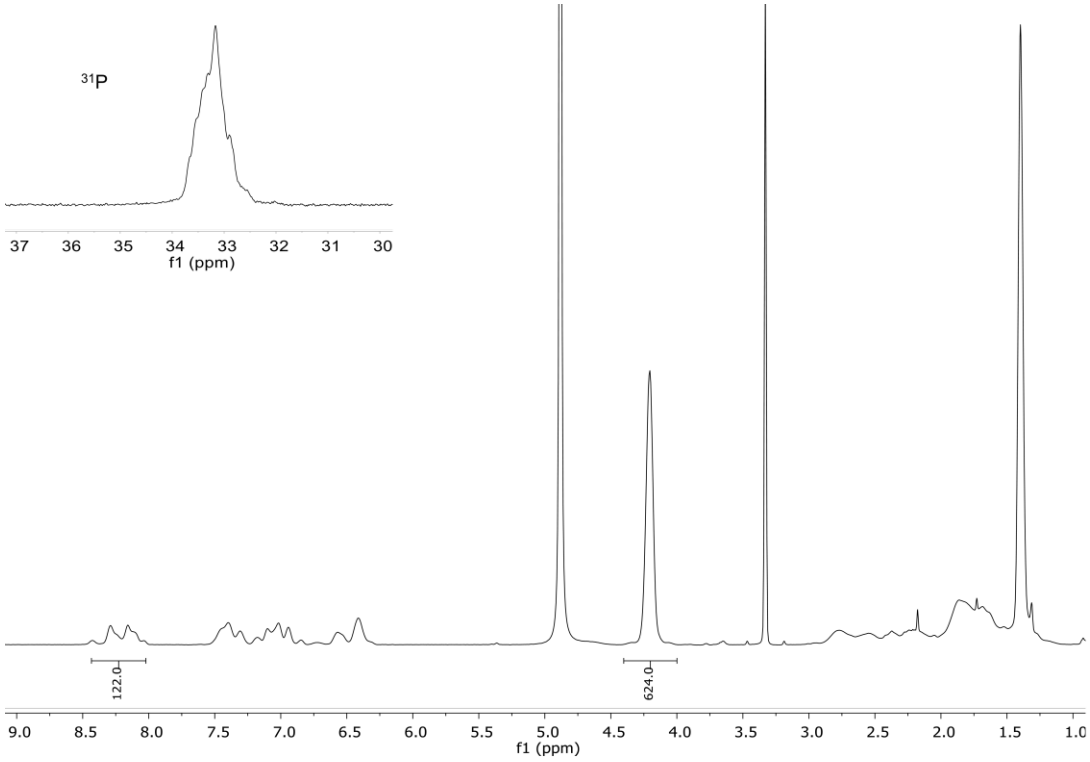


Fig. S11 ^{1}H- and ^{31}P-NMR spectrum of NP1 in MeOD.

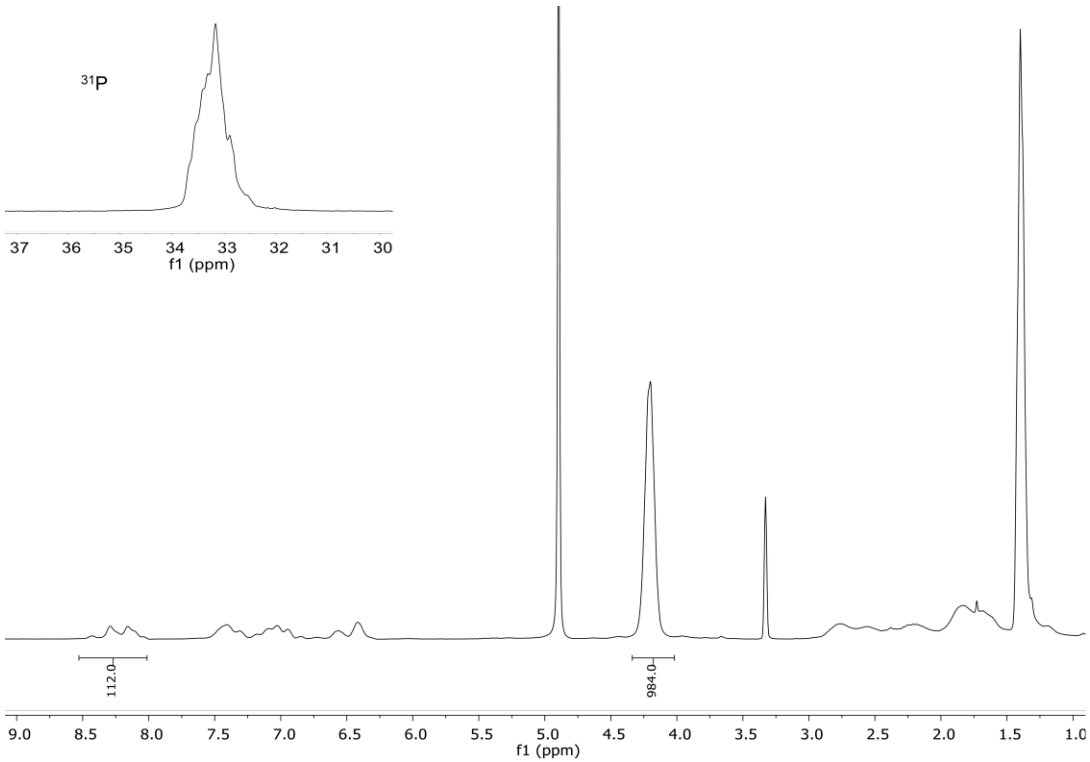


Fig. S12 ^{1}H- and ^{31}P-NMR spectrum of NP2 in MeOD.

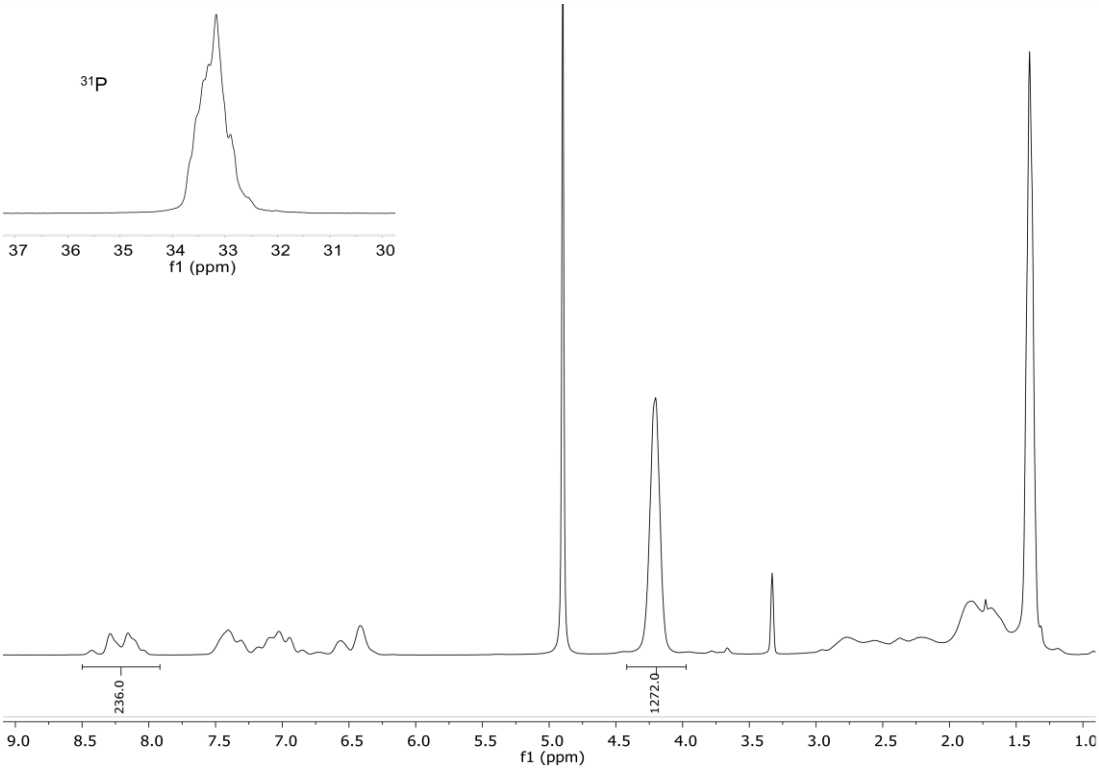


Fig. S13 ^{1}H- and ^{31}P-NMR spectrum of NP3 in MeOD.

Table S2 Elemental analysis of nanoparticle samples

		C	H	N	S
NP1	**calculated**	55.43	7.57	4.23	0.94
NP1	**found**	54.95	7.63	4.11	1.02
NP2	**calculated**	51.83	7.71	2.93	0.59
NP2	**found**	51.50	7.77	2.91	0.42
NP3	**calculated**	55.31	7.58	4.20	0.41
NP3	**found**	54.85	7.72	4.14	0.43

3. Characterisation of polymer micelles and nanoparticles

3.1 Lower critical solution temperature

Table S3 Cloud points (T_c) of blockcopolymer substrates and nanoparticles

	Substrate	T_C [°C] (H_2O)	T_C [°C] (DMEM)
AB1	-	40.0	36.0
AB2	-	40.0	36.0
AB3	-	37.0	32.0
NP1	**AB1**	46.0	37.5
NP2	**AB2**	45.0	37.0
NP3	**AB3**	39.5	33.0

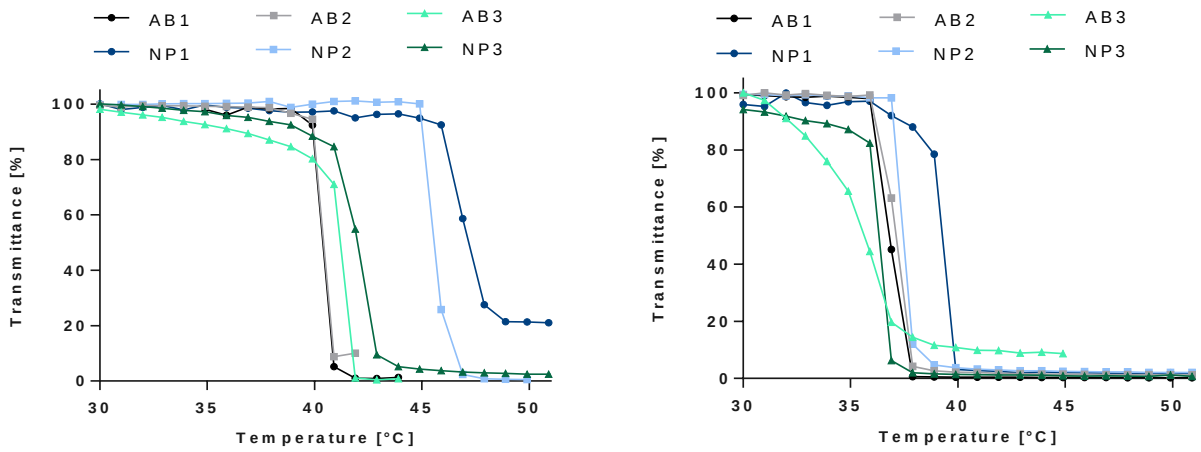


Fig. S14 Determination of the LCST of the samples AB1, AB2, AB3, NP1, NP2 and NP3 in water (left) and DMEM/PBS (2/1) (right) (2.50 mg/mL). The cloud point was determined at 10% decrease of transmittance.

3.2 Critical micelle concentration studies

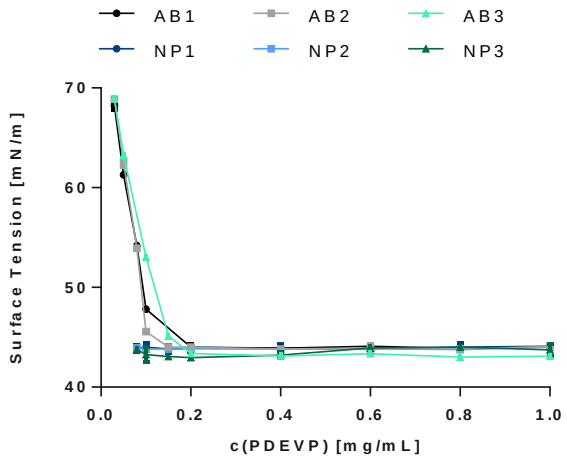


Fig. S15 Surface tension plot of the polymers AB1, AB2, AB3 and the particles NP1, NP2 and NP3

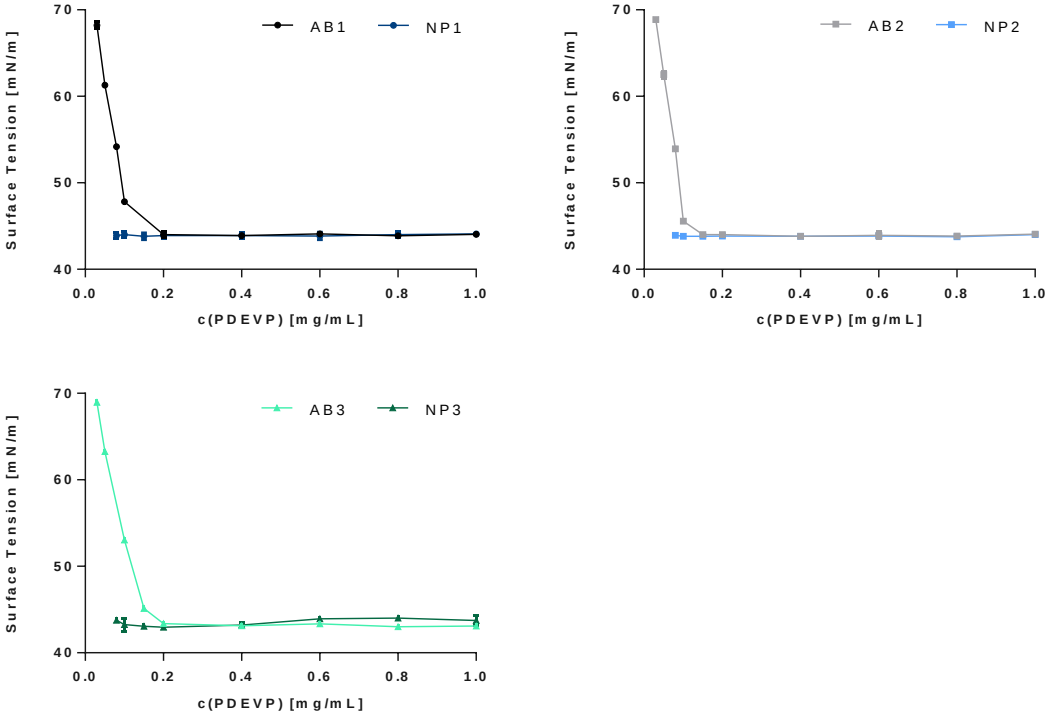


Fig. S16 Surface tension plots of AB1, NP1 (top left), AB2, NP2 (top right) and AB3, NP3 (bottom left).

3.3 Dynamic light scattering

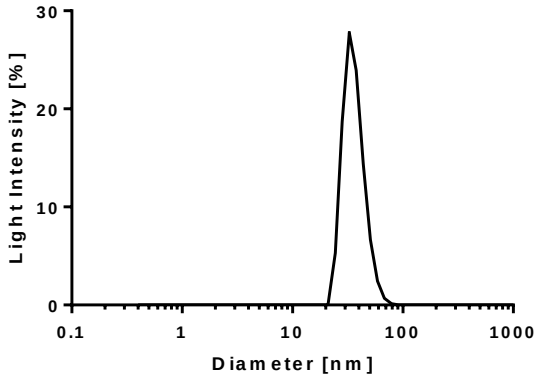


Fig. S17 Size distribution of NP1 determined *via* DLS measurement at a concentration of 2.5 mg/mL in water.

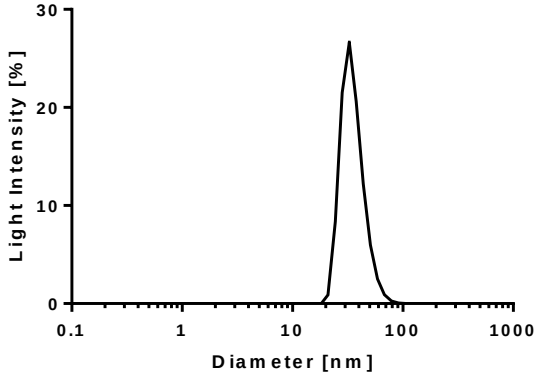


Fig. S18 Size distribution of NP2 determined *via* DLS measurement at a concentration of 2.5 mg/mL in water.

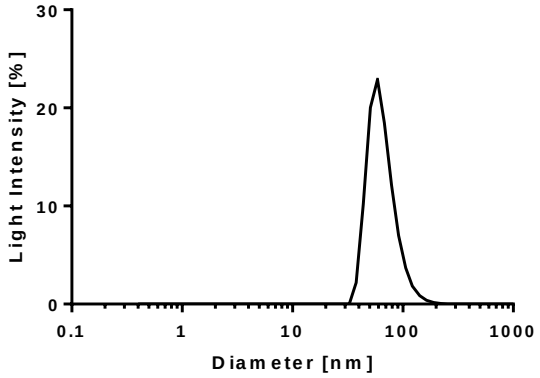


Fig. S19 Size distribution of NP3 determined *via* DLS measurement at a concentration of 2.5 mg/mL in water.

3.4 Transmission electron microscopy

The following figures S20-22 show the TEM images and their analysis. For each histogram plot with Gaussian regression fit fifty particles were measured. The mean sizes are depicted in the following table also in comparison to the DLS data.

Table 4 Diameter, polydispersity and zeta potential ζ of nanoparticles

	d [nm] (DLS)	PDI (DLS)	ζ [mV]	d [nm] (TEM)	PDI (TEM)
NP1	36.39 ± 1.09	0.030	-5.02 ± 0.10	32.54 ± 1.89	0.058
NP2	35.57 ± 2.31	0.065	-5.30 ± 0.35	28.70 ± 1.64	0.057
NP3	65.99 ± 7.65	0.116	-3.97 ± 0.21	53.19 ± 2.70	0.051

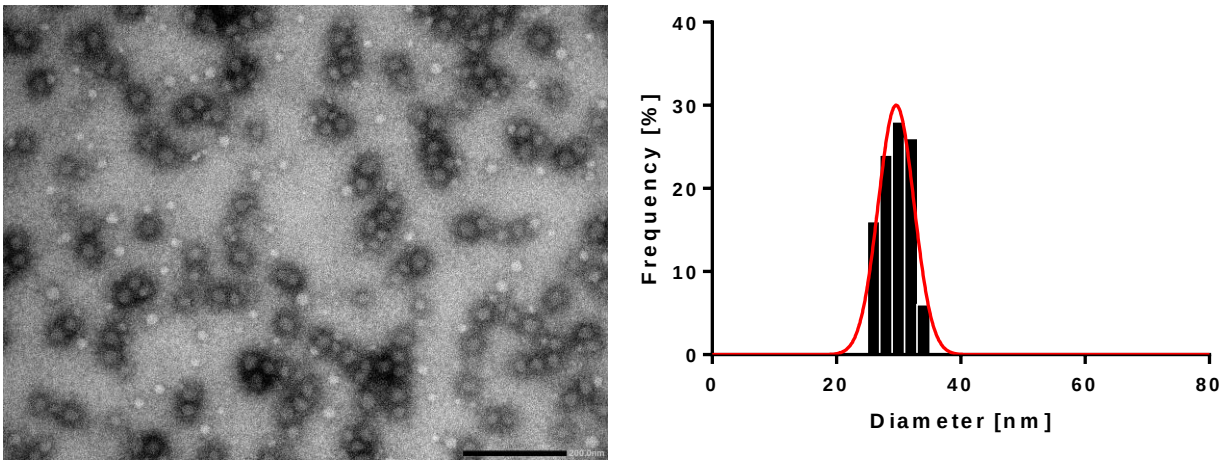


Fig. S20 TEM image of NP1 (left) and histogram plot with gaussian regression fit (right).

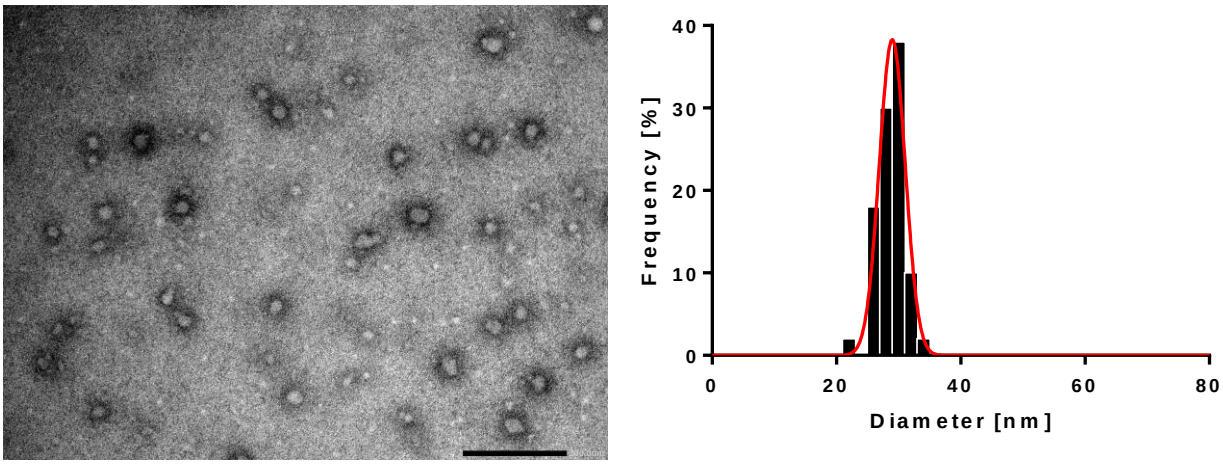


Fig. S21 TEM image of NP2 (left) and histogram plot with gaussian regression fit (right).

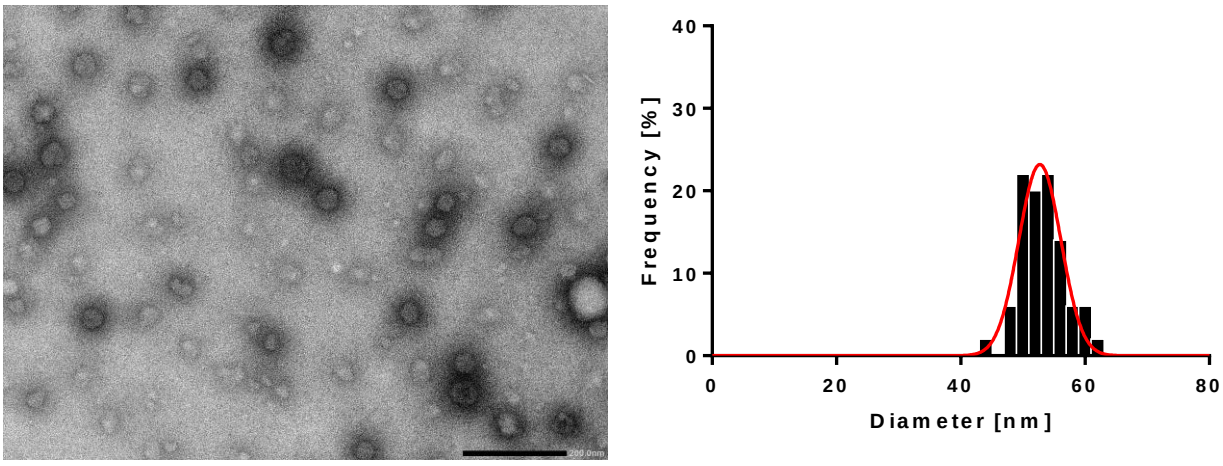


Fig. S22 TEM image of NP1 (left) and histogram plot with gaussian regression fit (right).

3.5 Loading and release properties

To obtain fluorescein loaded vehicles, firstly fluorescein was dissolved in DMSO (c = 0.3 mg/mL). Under continuous stirring the fluorescein solution was added dropwise to an ice cooled polymer solution (Polymer/Fluorescein = 12/1). The mixture was then stirred for 1 h on ice and 90 min at ambient temperature, followed by overnight dialysis against water (MWCO: 6000 – 8000).

The next day the dialysis tubes were placed in separate beakers and the release study was started. The first sample (reference) was put into 90 mL deionised water, the second, third and fourth samples into 90 mL deionised water and put into a preheated water bath (37 °C, 42 °C + 44 °C) (temperature release), and the fifth and sixth samples were put into 90 mL citrate buffer (pH = 4.3 + 6.0) (pH release). Samples of 2 mL were drawn regularly for 48 h and the beaker refilled with the appropriate amount of fresh water/buffer. The samples were then measured *via* fluorescence spectrometry and quantitatively analysed against a freshly prepared calibration curve consisting of 10 known concentrations.

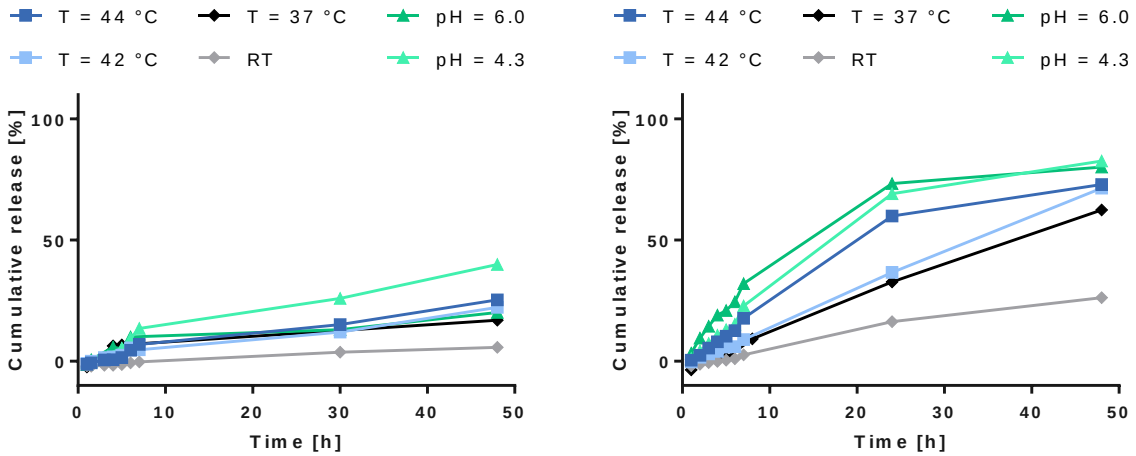


Fig. S23 Cumulative release of fluorescein from the AB1 (left) and NP1 (right) under varying conditions.

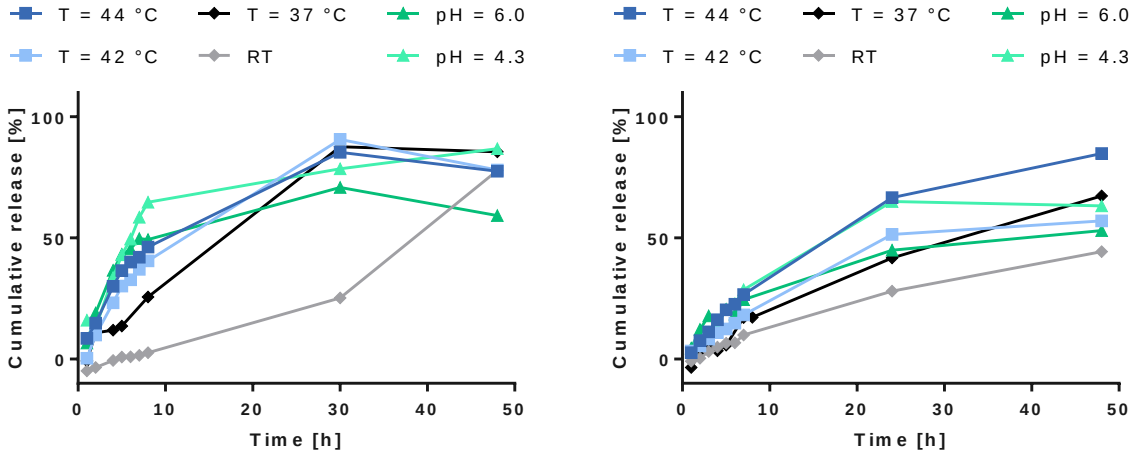


Fig. S24 Cumulative release of fluorescein from the AB2 (left) and NP2 (right) under varying conditions.

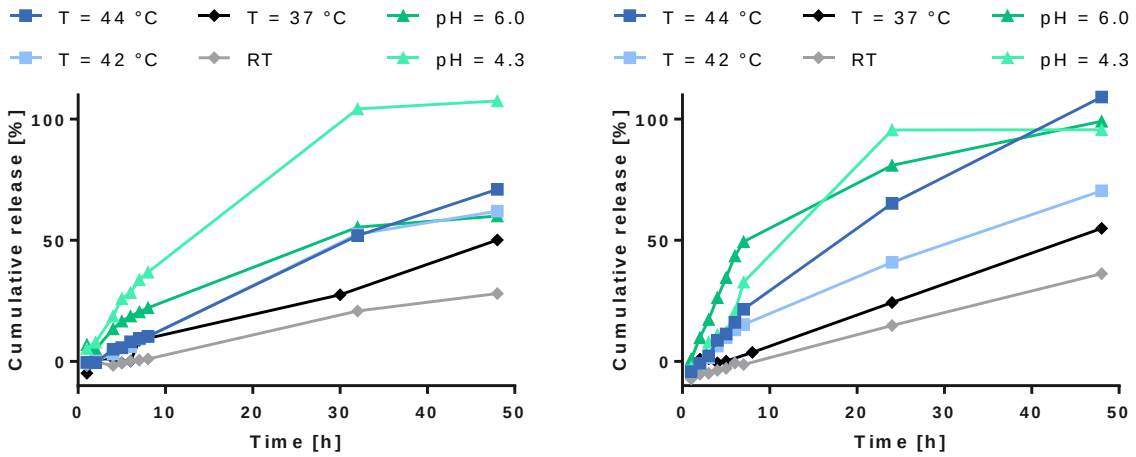


Fig. S25 Cumulative release of fluorescein from the AB3 (left) and NP3 (right) under varying conditions.

4. In vitro experiments

4.1 Cell culture

In vitro studies on nanoparticle samples were performed in HeLa and MCF-7 cells (ATCC). Cells were cultured in Dulbecco's Modified Eagle Medium (Life Technologies) equipped with 10% (v/v) Fetal Bovine Serum (Biochrom) and 1% Penicillin/Streptomycin 10000 U/mL /10000 µg/mL (Biochrom) at 37 °C in a humidified atmosphere containing 5% CO_2. For splitting and sub-culturing of cells Trypsin 0.05%/EDTA 0.02% in PBS (PAN Biotech) was used.

4.2 Cell viability studies

The growth inhibition of the nanoparticle samples on HeLa and MCF-7 cells, was determined by analysing their cell viability in presence of increasing polymer concentrations (0.08 to 5.00 mg/mL) or increasing doxorubicin (Dox) concentrations (0.09 to 6.00 µg/mL) incorporated into the particles. Prior to the addition of the samples, the cells were cultured for 24 h in 96 well flat bottom plates (TPP) with a density of 10000 cells/well. After 3 or 24 h of incubation at 37 °C in a humidified atmosphere with 5% CO_2, the cell viability of the treated cells was determined using the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide reagent (MTT) (Sigma Aldrich). Therefore, MTT was dissolved at a concentration of 5 mg/mL in RPMI-1640 without phenol red (Life Technologies). PBS treated cells were used as positive control (100% viability). DMSO treated cells were used as negative control (0% viability). After 3 h of incubation with 50 µL of MTT per well at 37 °C, the blue formazan crystals were dissolved for 15 min on a plate shaker at 550 min^{-1} with 100 µL 0.04 N HCl in isopropanol and exclusion of light. Following the absorbance of each well was measured at 570 nm with 690 nm as background wavelength at a Tecan Genios Plus plate reader. Shown are mean values of at least three independent biological replicates and the respective standard deviations are indicated.

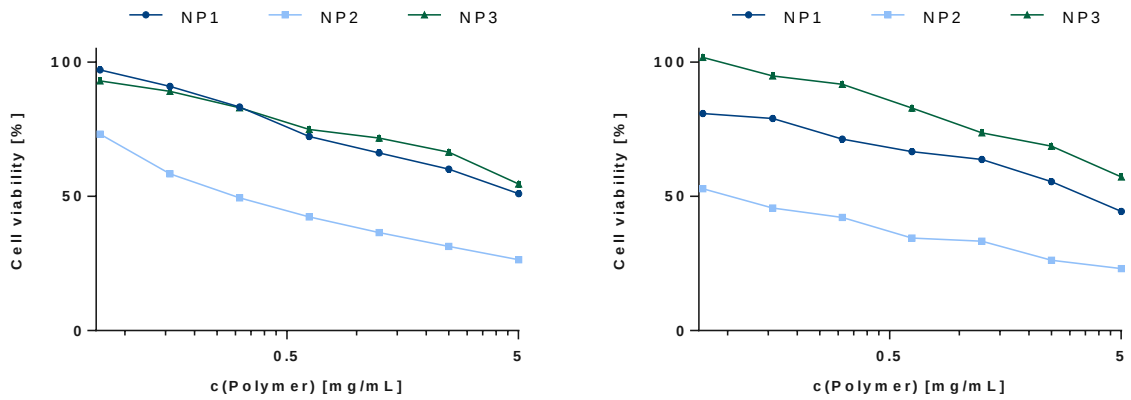


Fig. S26 Cell viability of HeLa (left) and MCF-7 cells (right) after 24 h of incubation with unloaded nanoparticle samples (measured in triplicate, standard error of the mean indicated).

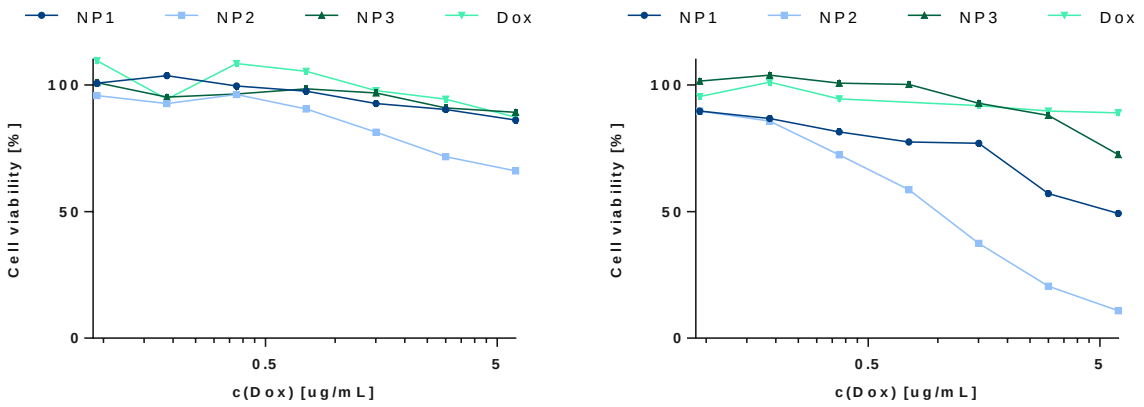


Fig. S27 Cell viability of HeLa (left) and MCF-7 cells (right) after 3 h of incubation with doxorubicin loaded nanoparticle samples (measured in triplicate, standard error of the mean indicated).

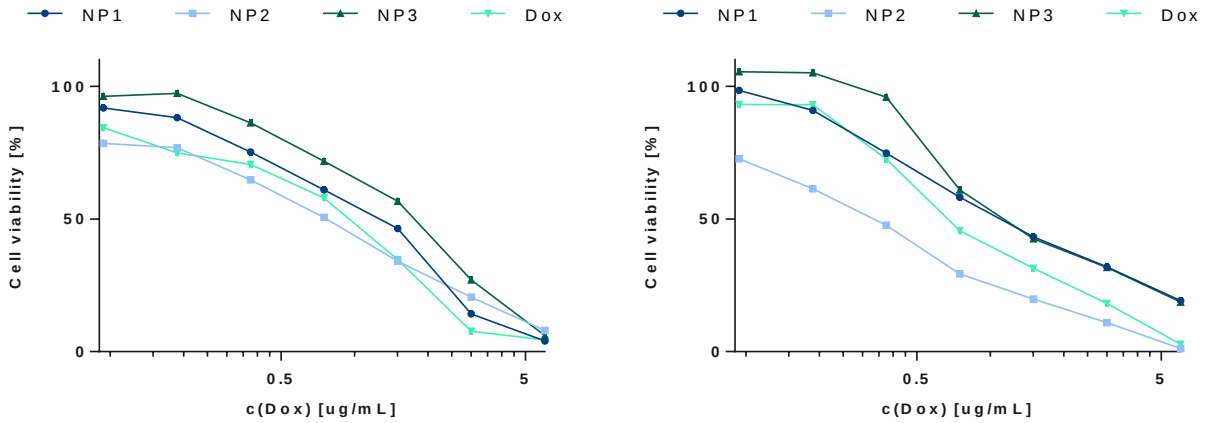


Fig. S28 Cell viability of HeLa (left) and MCF-7 cells (right) after 24 h of incubation with doxorubicin loaded nanoparticle samples (measured in triplicate, standard error of the mean indicated).

4.3 Fluorescence microscopy

For microscopic studies HeLa and MCF-7 cells were cultured for 24 h in 8-well glass chamber slides (BD Falcon). Following they were treated with a total concentration of 3.0 µg/mL of doxorubicin or particles loaded with the respective dox amount, dissolved in PBS, for 1 or 3 h at 37 °C in a humidified atmosphere with 5% CO_2. After this incubation time the samples were removed, the cells were washed with PBS and fixed with 4% Paraformaldehyd in PBS for 10 min. After three more washing steps, the chambers were removed, Vectashield (Vector Laboratories, Inc.) and the cover slip were put on top of the microscope slide. The measurements were performed on a Leica DM IL inverted fluorescence microscope with 20x magnification (scale bar = 50 µm).

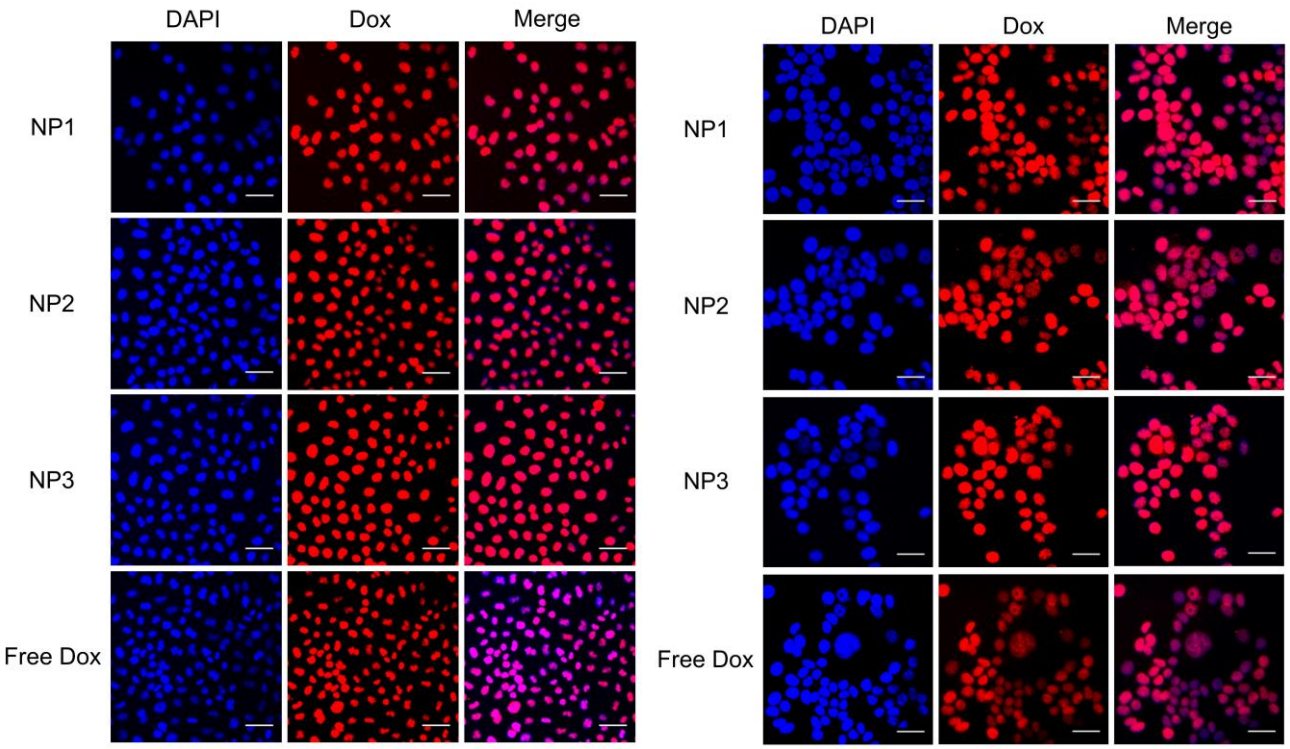


Fig. S29 Fluorescence microscopic pictures of HeLa (left) and MCF-7 (right) cells incubated with NP1, NP2, NP3 and doxorubicin for 1 h at 37 °C

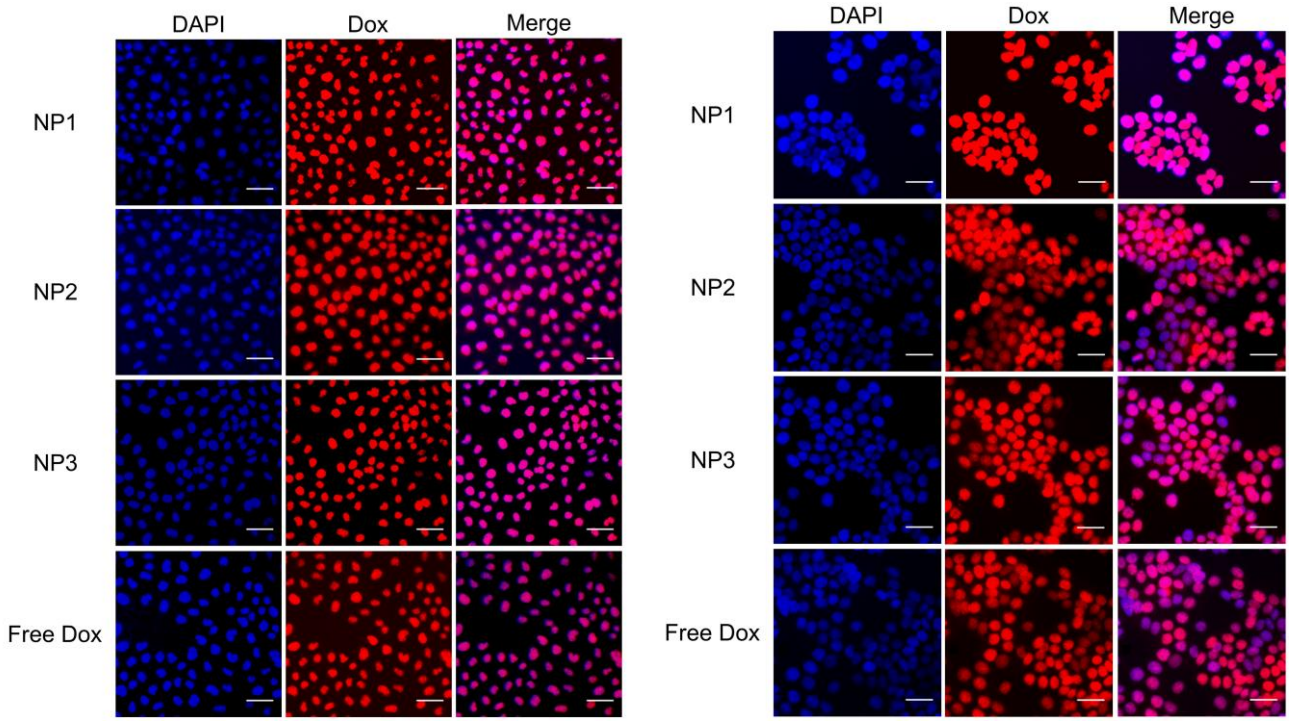


Fig. S30 Fluorescence microscopic pictures of HeLa (left) and MCF-7 (right) cells incubated with NP1, NP2, NP3 and doxorubicin for 3 h at 37 °C.

4.4 Fluorescence activated cell sorting

For flow cytometry HeLa and MCF-7 cells were cultured for 24 h in 6-well flat bottom plates (TPP) with a density of 500000 cells/well at 37 °C in a humidified atmosphere with 5% CO_2. Following they were treated with a total concentration of 3.0 µg/mL of doxorubicin or particles loaded with the respective dox amount, dissolved in PBS, for 10 min or 3 h. After this incubation time the samples were removed, the cells were washed with PBS and scraped from the plates' surface. Suspended in 0.5 mL PBS the cells were transferred to an Eppendorf tube, centrifuged, the supernatant was removed and the pellet was resuspended in 0.2 mL ice cold PBS. The measurements were performed with a FACSCanto II (BD Biosciences) and a FACSCalibur (BD Biosciences) and the data were analysed with the help of the software FlowJo.

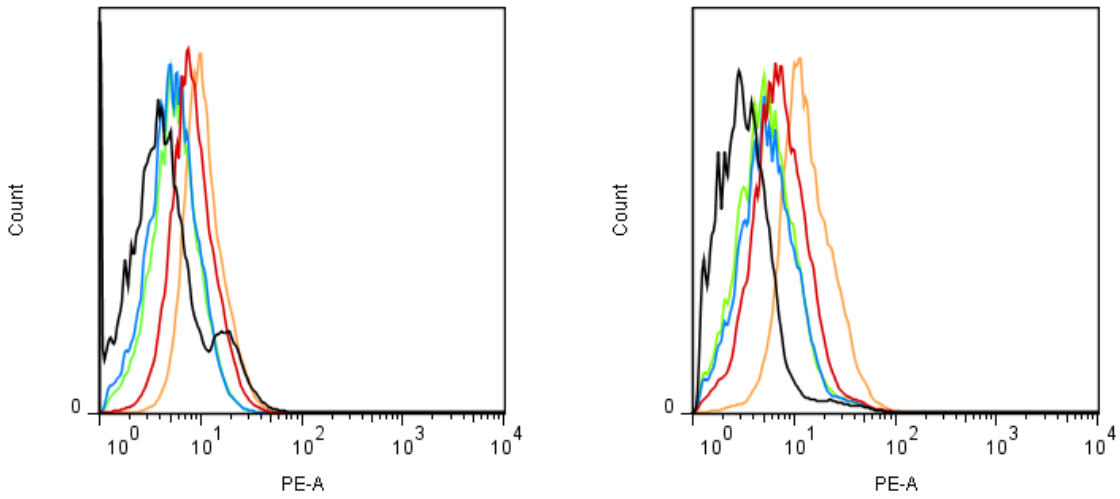


Fig. S31 Mean fluorescence intensity of HeLa (left) and MCF-7 cells (right) incubated with PBS (black) NP1 (blue), NP2 (green), NP3 (orange) and doxorubicin (red) for 10 min at 37 °C.

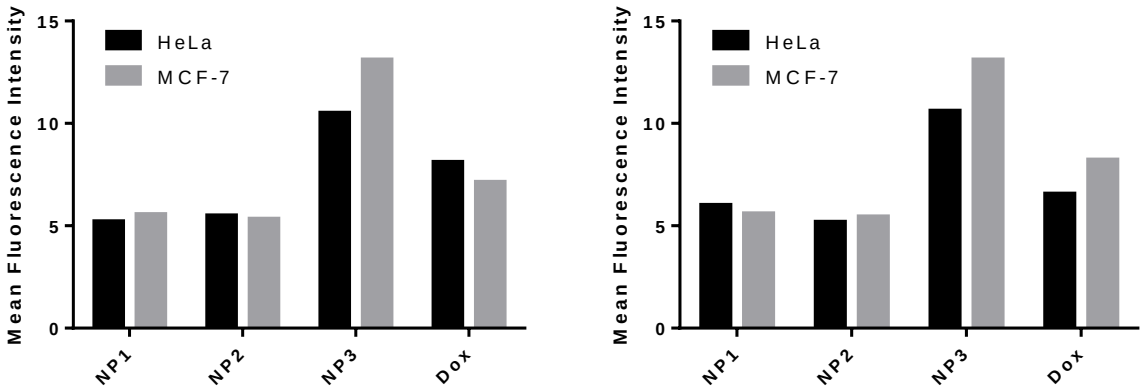


Fig. S32 Mean fluorescence intensity comparison of HeLa (black) and MCF-7 cells (grey) incubated with NP1, NP2, NP3 and doxorubicin for 10 min at 37 °C (left) and 42 °C (right).

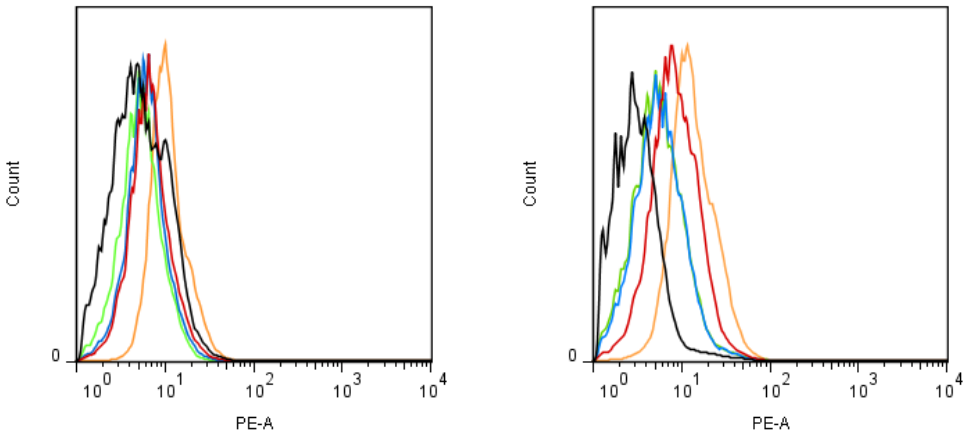


Fig. S33 Mean fluorescence intensity of HeLa (left) and MCF-7 cells (right) incubated with PBS (black) NP1 (blue), NP2 (green), NP3 (orange) and doxorubicin (red) for 10 min at 42 °C.

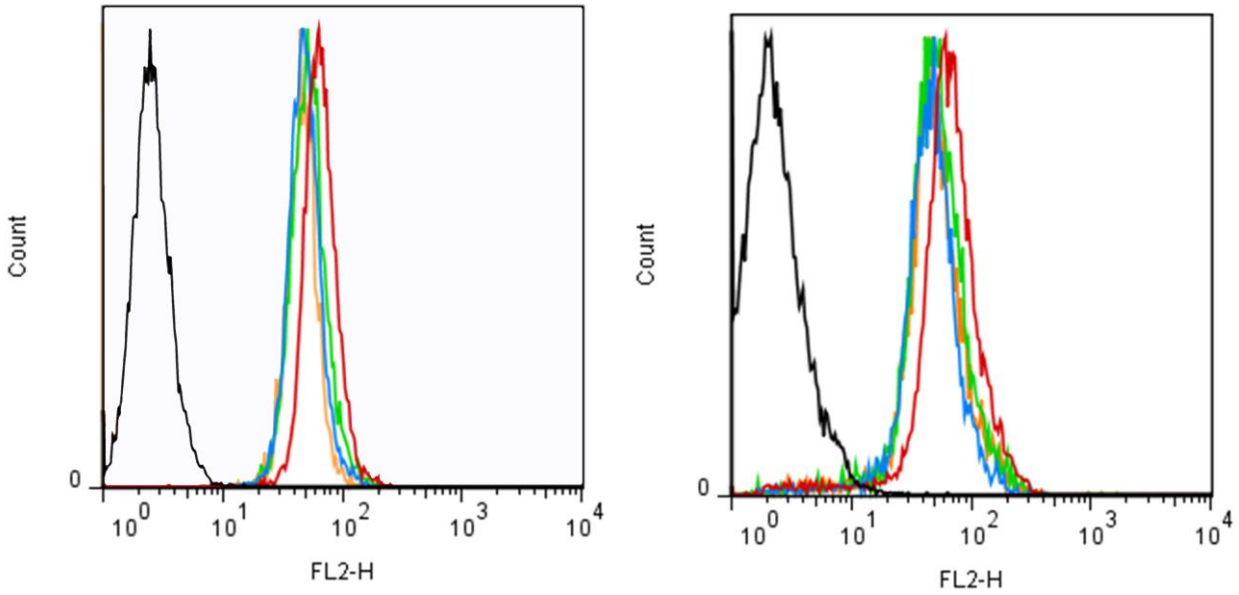


Fig. S34 Mean fluorescence intensity of HeLa (left) and MCF-7 cells (right) incubated with PBS (black) NP1 (blue), NP2 (green), NP3 (orange) and doxorubicin (red) for 3 h at 37 °C.

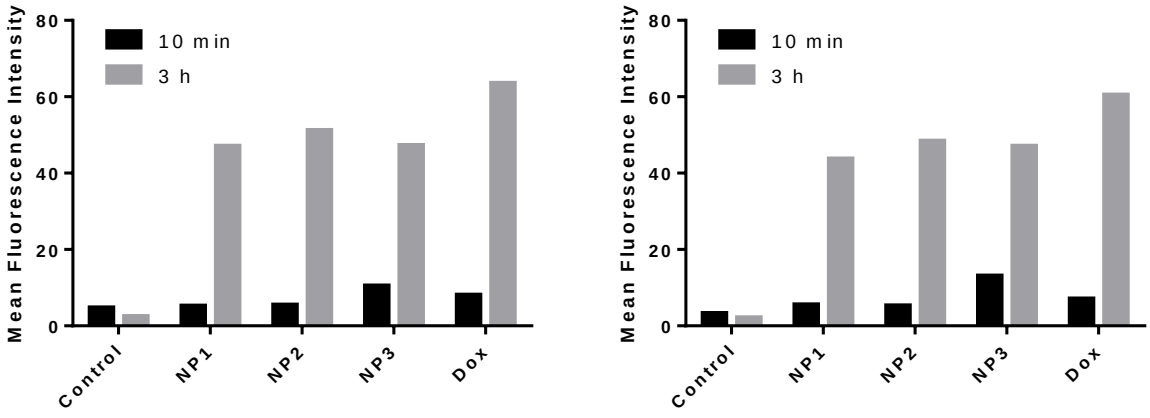


Fig. S35 Mean fluorescence intensity comparison of HeLa (left) and MCF-7 cells (right) incubated with NP1, NP2, NP3 and doxorubicin for 10 min (black) and 3 h (grey) at 37 °C.

5. Literature

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