Supplementary information

Cellular uptake of PLA nanoparticles studied by light and electron microscopy: Synthesis, characterization and biocompatibility studies using an iridium(III) complex as correlative label

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Experimental section

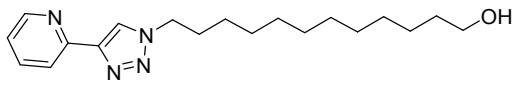
The solvents dichloromethane, methanol and toluene were dried with a PureSolv-EN™ Solvent Purification System (Innovative Technology). Unless otherwise noted, the starting materials were purchased from commercial sources and used as obtained. Reaction progress was monitored by thin layer chromatography on 0.2 mm Merck silica gel plates (60 F254). Column chromatographic purifications were performed on silica gel 60 (Merck).

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 250 MHz respectively AC 300 MHz spectrometer at room temperature. Chemical shifts are reported in parts per million (ppm, δ scale) relative to the residual proton signal of the deuterated solvent. Elemental analyses were carried out on a Vario ELIII–Elementar Euro and an EA–HekaTech. Size-exclusion chromatography was performed on an Agilent 1200 series system, equipped with a G1362A refractive index detector, and both a PSS Gram30 and a PSS Gram1000 column in series, applying *N,N'*-dimethylacetamide (DMAc) + 0.21% LiCl as eluent, flow rate 1 mL/min, temperature: 40 °C. MALDI-ToF MS spectra were measured on an Ultraflex III TOF/TOF (Bruker Daltonics GmbH) that was equipped with a Nd:YAG laser and a collision cell. The spectra were recorded in the positive reflector or linear mode using DCTB (*trans*-2-[3-(4-tert.-butylphenyl)-2-methyl-2-propenylidene]malononitrile) as matrix. ESI-Q-ToF MS measurements were executed on a micrOTOF (Bruker Daltonics GmbH) mass spectrometer, which was equipped with an automatic syringe pump for sample injection. The pump was supplied from KD Scientific. It was operated in the positive ion mode. The standard electrospray ion (ESI) source was used to generate ions. Mixtures of dichloromethane and acetonitrile were utilized as solvent. The ESI-Q-TOF-MS instrument was calibrated in the m/z range of 50 to 3,000 using an internal calibration standard (Tunemix solution) which was supplied from Agilent. UV/vis absorption spectra were measured on a Perkin-Elmer Lambda 750 UV/vis spectrophotometer. Emission spectra were recorded with a Perkin-Elmer LS 50 spectrometer and corrected according to the spectral sensitivity function of the detector.

Spectroscopic measurements were carried out in a 1 cm quartz cuvette using spectroscopy grade solvents.

Dynamic light scattering (DLS) experiments were carried out using a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany). For this purpose, 3×30 runs were recorded at 25 °C (wavelength of 633 nm). The counts were detected under an angle of 173°. Each measurement was performed three times. The mean particle size was approximated as the effective diameter (z-average diameter). The width of the distribution as the polydispersity index of the particles (PDI) was obtained by the cumulants method assuming a spherical shape of the particles. The zeta potential was determined by electrophoretic light scattering. The measurements were carried out on a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) by applying laser Doppler velocimetry. Therefore, 10 runs were carried out using the slow-field and fast-field reversal mode at 150 V (three times at 25 °C).

12-[4-(Pyridin-2-yl)-1*H*-1,2,3-triazol-1-yl]dodecanol (*1*)



12-[4-(Pyridin-2-yl)-1*H*-1,2,3-triazol-1-yl]dodecanol was synthesized according to a procedure described in literature.[1] Sodium azide (195 mg, 3.0 mmol), 2-ethinylpyridine (290 mg, 2.8 mmol), 12-bromododecanol (740 mg, 2.8 mmol), copper(II) sulfate pentahydrate (23 mg, approx. 5 mol-%) and sodium ascorbate (90 mg, approx. 25 mol-%) were suspended in 8 mL ethanol/water (7:3 v/v) in a microwave vial which was subsequently sealed. The suspension was heated to 125 °C under microwave irradiation for 20 min. After cooling to room temperature, the vial was opened and another portion of copper(II) sulfate pentahydrate (23 mg, approx. 5 mol-%) was added to the mixture. The mixture was then heated to 125 °C by microwave irradiation for further 25 min. The brown suspension was poured on 100 mL distilled water and the resulting brown precipitate was separated and then dried *in-vacuo*. The crude material was suspended in ethyl acetate and copper was removed by a short silica column (ethyl acetate as eluent). The resulting yellowish solid was dissolved in a small amount of dichloromethane and dropped to *n*-pentane, by what **1** precipitated as an off-white solid (678 mg, 2.1 mmol, 73 %). ^{1}H-NMR (CD_2Cl_2, 250 MHz): δ 8.57 (s, 1H), 8.19 (s, 2H), 7.81 (s, 1H), 7.25 (s, 1H) 4.43 (t, J = 7.2 Hz, 2H), 3.61 (t, J = 6.6 Hz, 2H), 1.97 (s, 1H), 1.84 (s, 1H), 1.55 (m, 2H), 1.33 (m, 16H) ppm. ^{13}C-NMR (CD_2Cl_2, 63 MHz): δ 150.5, 149.4, 148.1, 136.8, 122.7, 121.9, 119.8, 62.6, 50.4, 32.9, 30.6, 30.2, 29.5, 29.4, 29.4, 29.3, 28.9, 26.4, 25.7 ppm. ESI-MS: 331.0 (100) $[M+H]^+$, 353.3 (20) $[M+Na]^+$. Elemental Analysis: C: 69.05%, H: 9.15%, N: 16.95% (Calc.), C: 68.80%, H: 9.17%, N: 16.72% (Found).

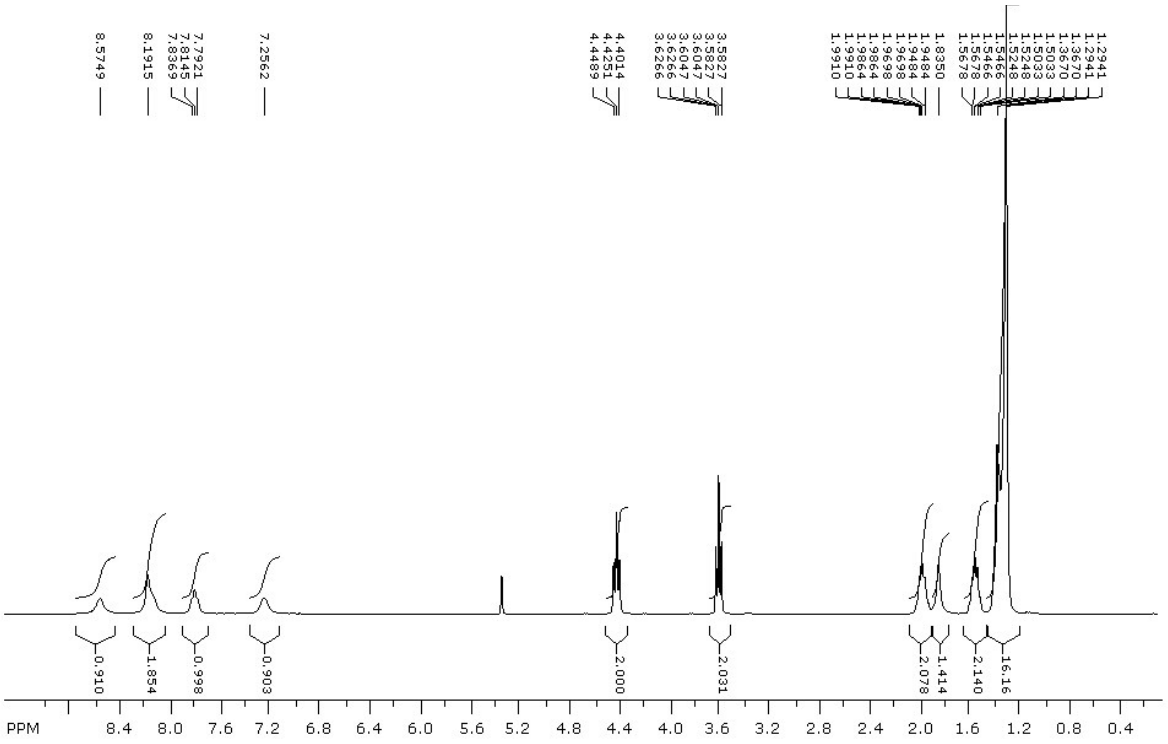


Figure S1: ^{1}H-NMR spectrum of compound ***1*** (CD_2Cl_2, 250 MHz).

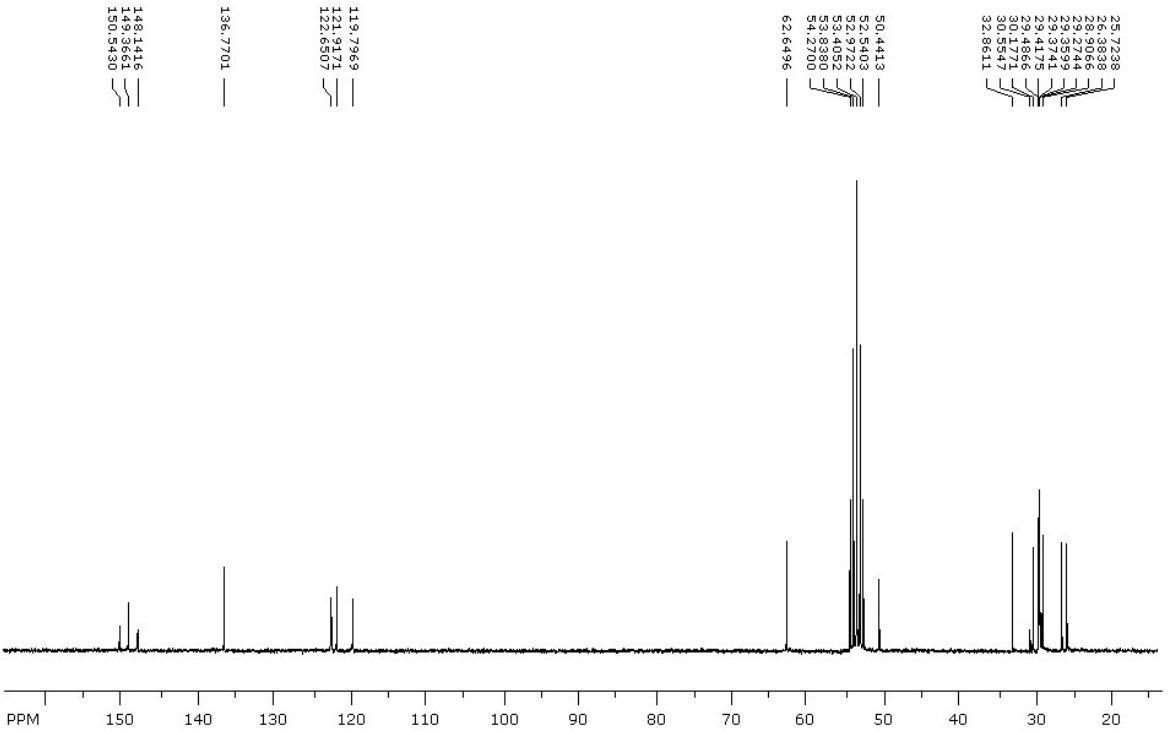
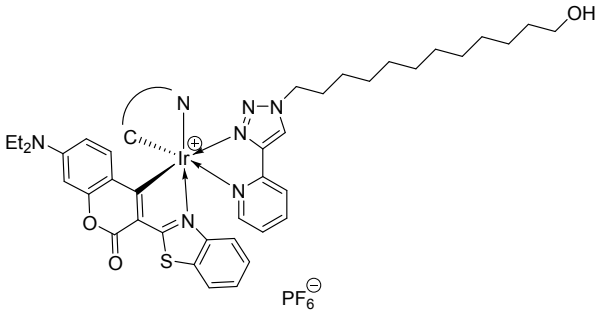


Figure S2: ^{13}C-NMR spectrum of compound ***1*** (CD_2Cl_2, 63 MHz).

Tetrakis[3-(2-benzothiazolyl)-7-(diethylamino)-2H-1-benzopyran-2-onato-N',C^{4}]-di-μ-chlorodi-iridium(III) (*2*)

The precursor complex was synthesized according to a standard method described in literature.[2]

Iridium(III) bis[3-(2-benzothiazolyl)-7-(diethylamino)-2H-1-benzopyran-2-onato-N',C^4]- 12-[4-(pyridin-2-yl)-1*H*-1,2,3-triazol-1-yl]dodecanol hexafluorophosphate (*3*)



Tetrakis[3-(2-benzothiazolyl)-7-(diethylamino)-2H-1-benzopyran-2-onato-N',C^4]-di-μ-chlorodi-iridium(III) (50 mg, 0.027 mmol) and 12-[4-(pyridin-2-yl)-1*H*-1,2,3-triazol-1-yl]dodecanol (18 mg, 0.054 mmol) were suspended in 15 mL of a degassed mixture of dichloromethane/methanol (5:4 v/v) and heated under reflux for 24 h, whereupon all insoluble compounds dissolved. The solution was then cooled to room temperature and stirred for three hours in the presence of NH_4PF_6 (58 mg, 0.355 mmol, 6.5 eq). The reaction mixture was evaporated to dryness. The residue was resuspended in 100 mL dichloromethane and the organic phase was washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude product was purified by silica gel chromatography with dichloromethane/methanol 15:1 (v/v) as eluent to give **3** (65 mg, 88%) as an orange powder. ^{1}H-NMR (CD_2Cl_2, 250 MHz): δ 8.61 (m, 2H), 8.01 (m, 2H), 7.83 (dd, J = 7.8, 2.5 Hz, 2H), 7.55 (t, J = 6.4 Hz, 1H), 7.24 (q, J = 7.0 Hz, 2H), 7.03 (t, J = 7.8 Hz, 1H), 6.90 (t, J = 7.8 Hz, 1H), 6.38 (dd, J = 9.1, 2.5 Hz, 2H), 6.27 (d, J = 8.2 Hz, 2H), 6.19 (d, J = 9.5 Hz, 1H), 5.59 (m, 3H), 4.49 (t, J = 6.6 Hz, 2H), 3.59 (t, J = 6.4 Hz, 2H), 3.27 (m, 8H), 2.55 (s, 1H), 1.90 (m, 2H), 1.52 (m, 2H), 1.92 (m, 16 H), 1.06 (t, J = 7.5 Hz, 12H) ppm. ^{13}C-NMR (CD_2Cl_2, 63 MHz): δ 181.3, 178.0, 177.1, 176.5, 157.7, 157.4, 155.2, 154.8, 152.7, 152.4, 149.6, 149.5, 148.3, 148.1, 147.7, 141.3, 132.5, 131.9, 131.3, 131.2, 127.6, 127.3, 127.2, 126.1, 124.7, 124.5, 123.3, 123.2, 123.0, 121.9, 118.9, 118.8, 116.2, 115.6, 109.8, 109.5, 96.5, 96.4, 62.7, 44.8, 44.7, 41.0, 32.9, 30.9, 30.1, 29.52, 29.45, 29.4, 29.3, 28.8, 26.0, 25.7, 12.2 ppm. HRMS (ESI, m/z): 1221.4066 (calc. for $C_{59}H_{64}IrN_8O_5S_2$ $[M\text{-}PF_6]^+$), 1221.4033 (found).

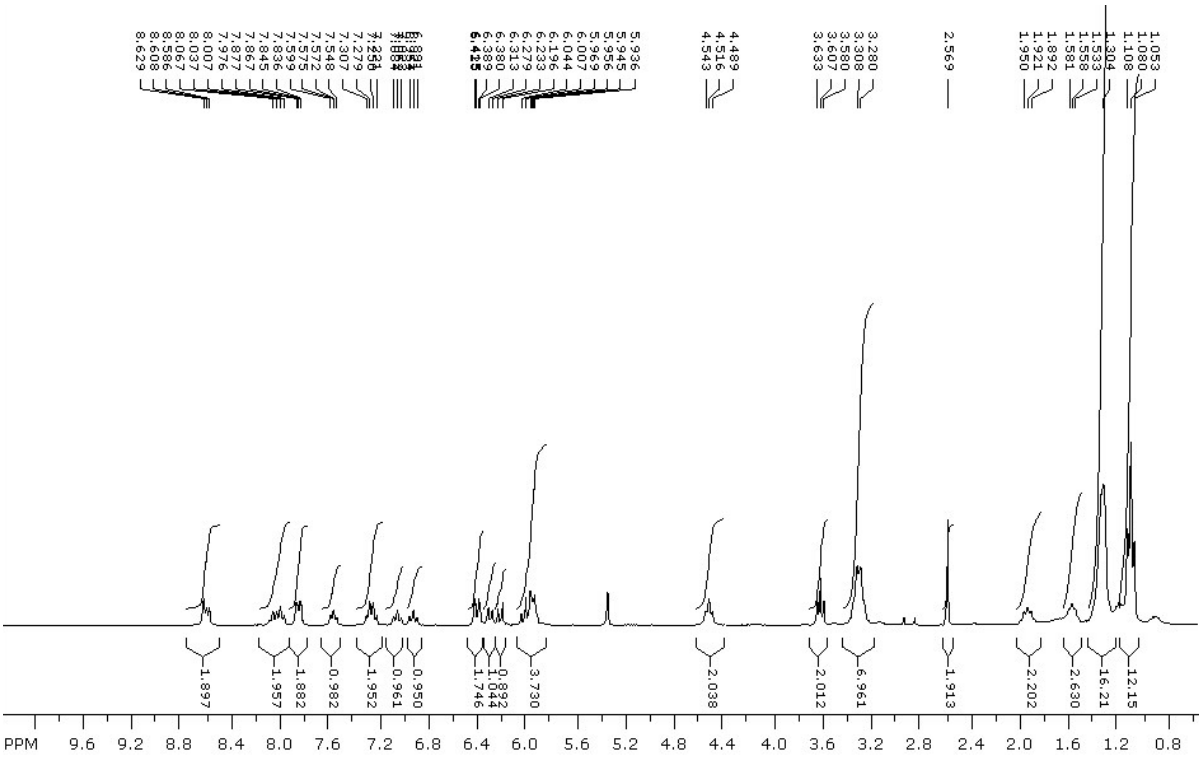


Figure S3: ^{1}H-NMR spectrum of compound ***3*** (CD_2Cl_2, 250 MHz).

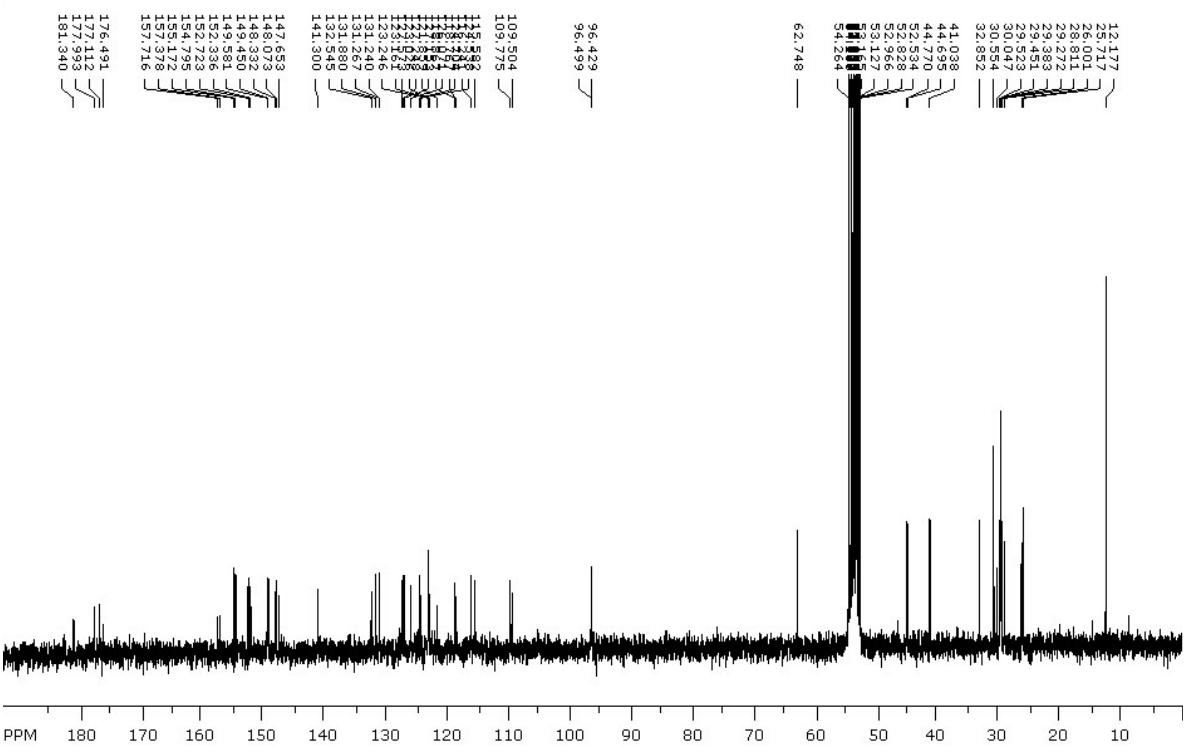
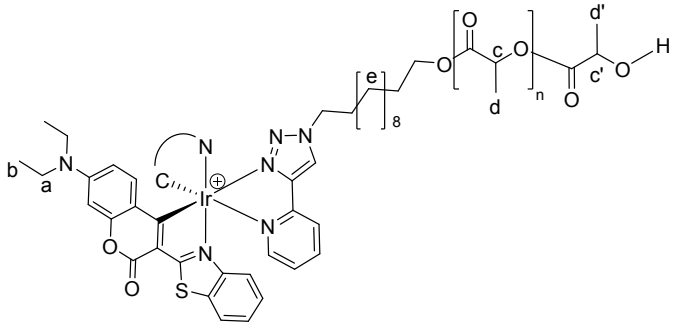


Figure S4: ^{13}C-NMR spectrum of compound ***3*** (CD_2Cl_2, 63 MHz).

End-functionalized polylactide (P1, P2)



General procedure

All polymerization reactions were carried out in oven-dried glassware under an argon atmosphere using standard Schlenk technique. All solvents were dried using standard techniques and stored over molecular sieves. (3*S*)-*cis*-3,6-Dimethyl-1,4-dioxane-2,5-dione was recrystallized from dried toluene and tin(II) 2-ethylhexanoate was distilled *in-vacuo* prior to usage and stored under an argon atmosphere.

(3*S*)-*cis*-3,6-Dimethyl-1,4-dioxane-2,5-dione, tin(II) 2-ethylhexanoate (catalyst C) and complex **3** (initiator I, molar conc. ratio [I]:[C] = 1:1) were dissolved in degassed toluene and heated to reflux for 21 min. The orange solution was cooled to room temperature and exposed to air moisture for further 20 min. The reaction solution was precipitated in ice-cold *n*-pentane, redissolved in dichloromethane and precipitated two more times in *n*-pentane. The polymers were obtained as orange amorphous substances.

P1

(3*S*)-*cis*-3,6-Dimethyl-1,4-dioxane-2,5-dione (212 mg, 1.47 mmol, 0.5 mmol·mL^{-1}), complex **3** (20 mg, 0.016 mmol), tin(II) 2-ethylhexanoate (6 mg, 0.016 mmol). Conversion: 80% (according to ^{1}H-NMR). ^{1}H-NMR ($CDCl_3$, 300 MHz): 8.52, 8.12, 8.07, 7.71, 7.17, 6.31, 6.14, 5.91, 5.81 (complex aromatic region), 5.00-5.21 (c), 4.43-4.32 (c'), 4.05 (a), 3.20, 1.86, 1.34-1.64 (d, d') 1.33-1.12 (e), 1.11-0.92 (b) ppm. SEC (eluent: DMAc-LiCl, PMMA calibration, RI detection): M_n = 6,800 g mol^{-1}, M_W = 7,800 g mol^{-1}, Đ = 1.18. MALDI-ToF (DCTB, NaCl): M_n = 5,000 g mol^{-1}, M_W = 5,400 g mol^{-1}, Đ = 1.04.

P2

(3*S*)-*cis*-3,6-Dimethyl-1,4-dioxane-2,5-dione (21 mg, 0.15 mmol, 0.3 mmol·mL^{-1}), complex **3** (12 mg, 0.01 mmol), tin(II) 2-ethylhexanoate (4 mg, 0.01 mmol). Conversion: 82% (according to ^{1}H-NMR). ^{1}H-NMR: 8.52, 8.41, 8.12, 7.94, 8.07, 7.42, 7.71, 7.17, 6.94, 6.80, 6.31, 6.14, 5.91, 5.81 (complex aromatic region), 5.00-5.21 (c), 4.43-4.32 (c'), 4.05 (a), 3.20, 1.86, 1.34-1.64 (d, d') 1.33-1.12 (e), 1.11-0.92 (b). SEC (eluent: DMAc-LiCl, PMMA-standard, RI detection): M_n = 2,200 g mol^{-1}, M_W = 2,700 g mol^{-1}, Đ = 1.22. MALDI-ToF (DCTB, NaCl): M_n = 2,460 g mol^{-1}, M_W = 2,600 g mol^{-1}, Đ = 1.06.

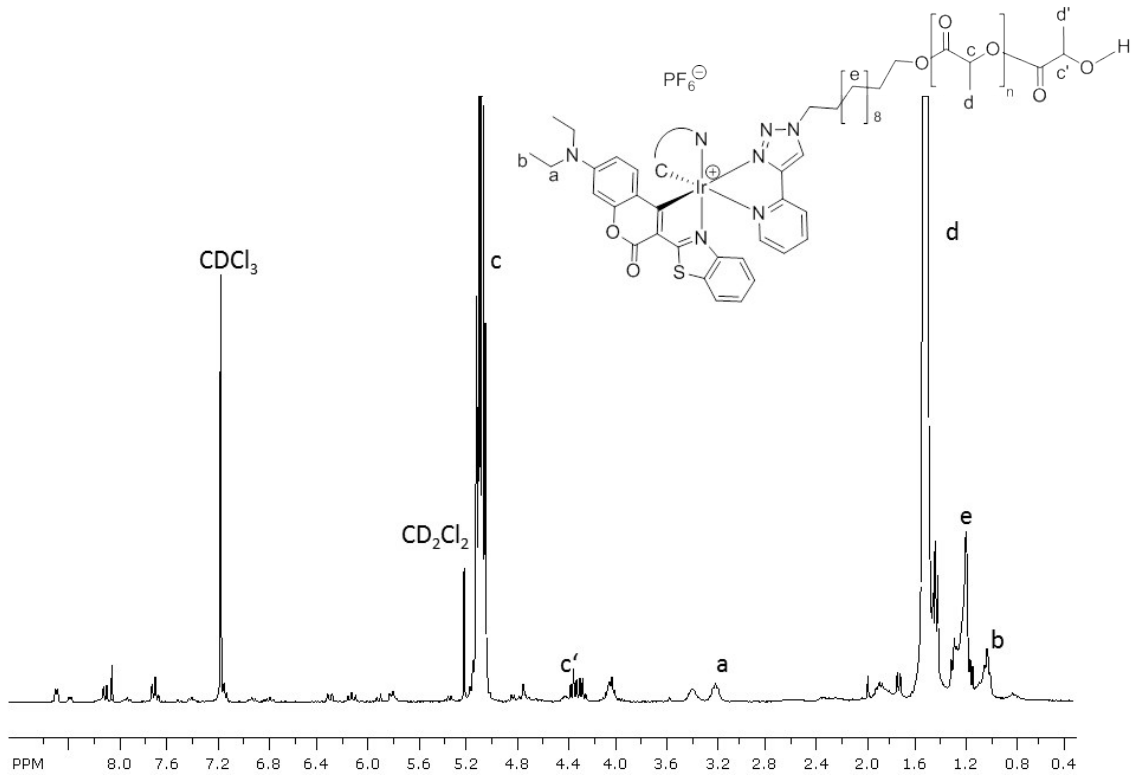


Figure S5: ^{1}H-NMR spectrum of compound ***P1*** ($CDCl_3$, 300 MHz).

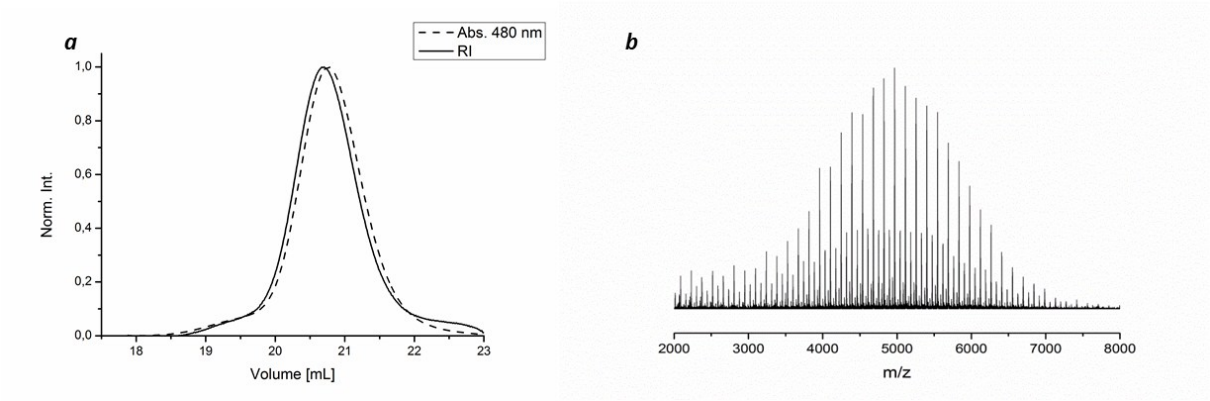


Figure S6: (a) SEC traces of polymer ***P1*** (DMAc-LiCl). Overlay of the RI signal and abs. UV/vis signal at 480 nm. (b) MALDI-ToF MS spectrum (DCTB, NaCl).

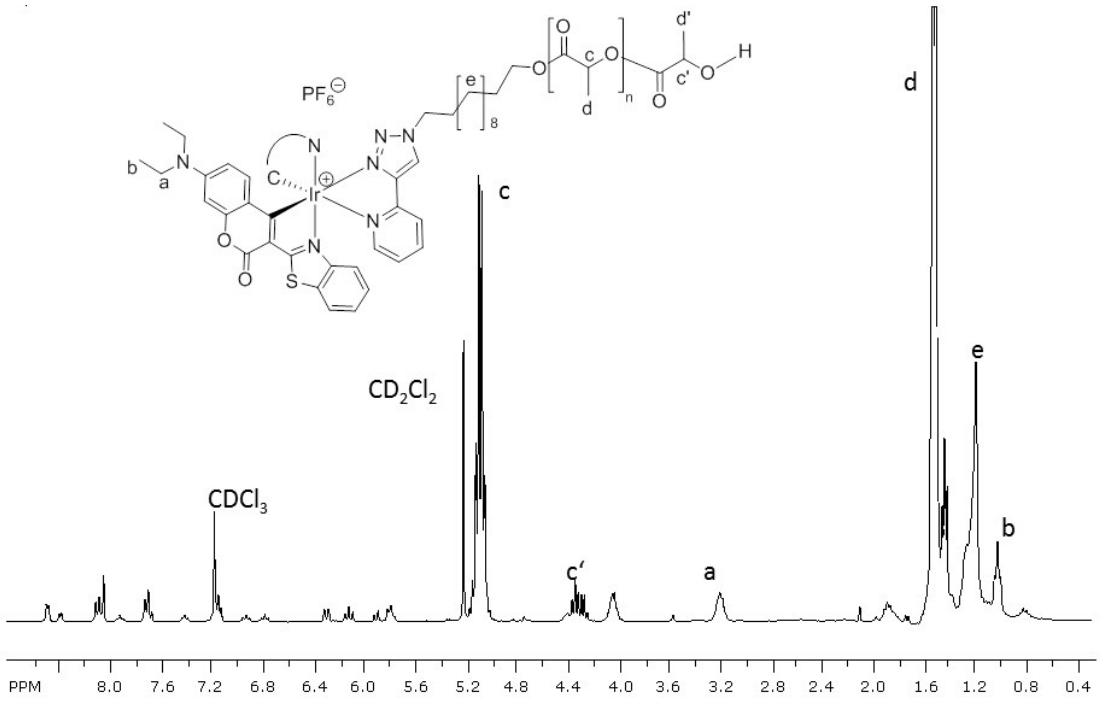


Figure S7: ^{1}H-NMR spectrum of compound ***P1*** ($CDCl_3$, 300 MHz).

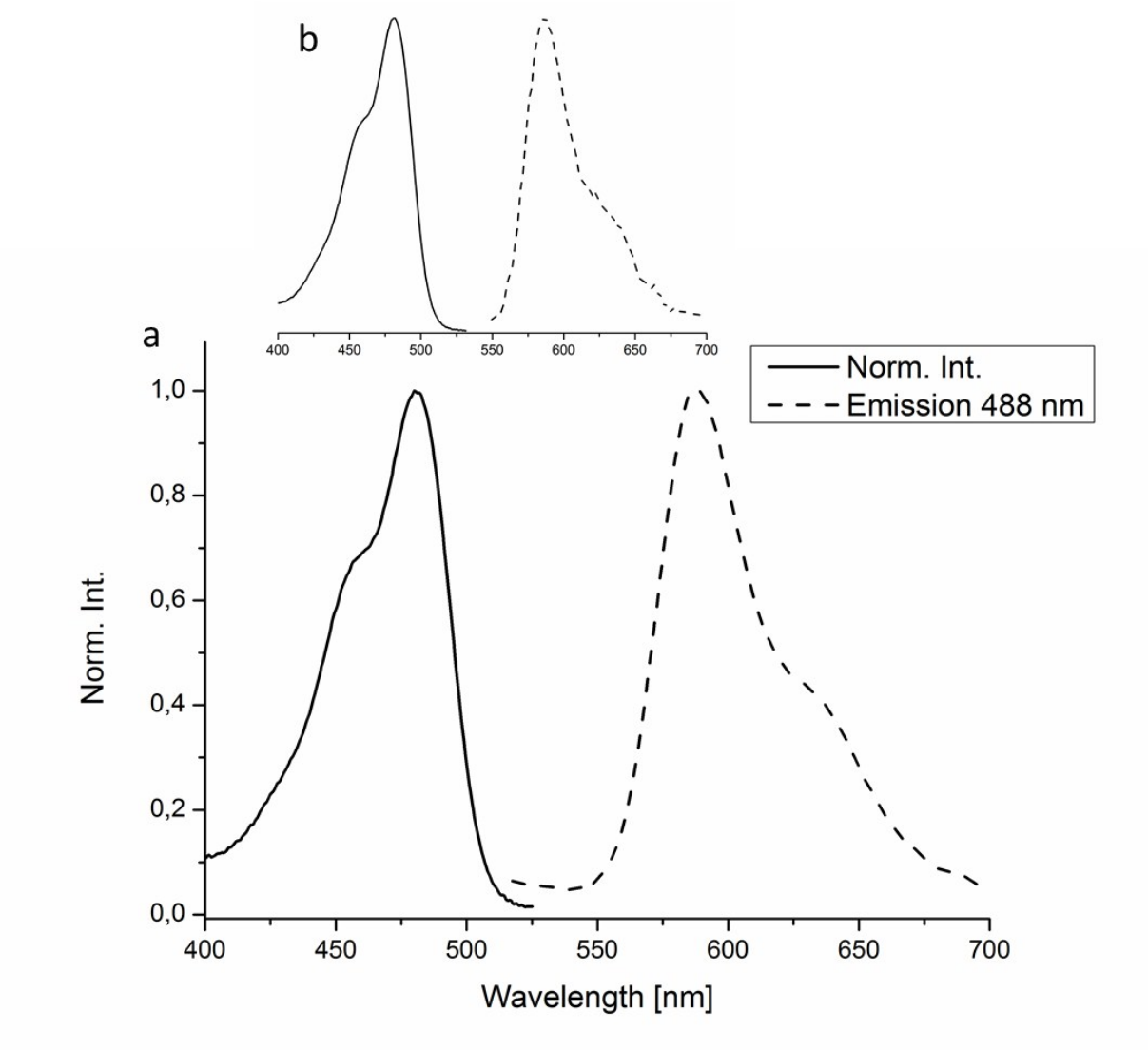


Figure S8: Absorption and emission spectrum of (a) polymer ***P2*** and (b) complex ***3*** (10^{-6} mol·L^{-1}). Absorption and emission spectra of both compounds are in good accordance (excitation slit width 5 nm, emission slit width 10 nm).

Kinetic measurements

For kinetic measurements, (3*S*)-*cis*-3,6-dimethyl-1,4-dioxane-2,5-dione (212 mg, 1.47 mmol, 0.5 mmol·mL^{-1}), complex ***3*** (20 mg, 0.016 mmol) and tin(II) 2-ethylhexanoate (6 mg, 0.016 mmol, [M]:[I] = 90:1) were dissolved in 3 mL toluene. From the reaction mixture, aliquots of 200 µL were taken with a syringe purged with argon. The aliquots were analyzed by ^{1}H-NMR spectroscopy to estimate the conversion. Therefore, the ratio of the integrals of the signals at 5.04 ppm (methine proton of monomer) and 5.13–5.25 ppm (methine proton of polymer)[3] were calculated. SEC measurements were carried out using DMAc-LiCl in order to estimate the molar masses using a PMMA calibration.

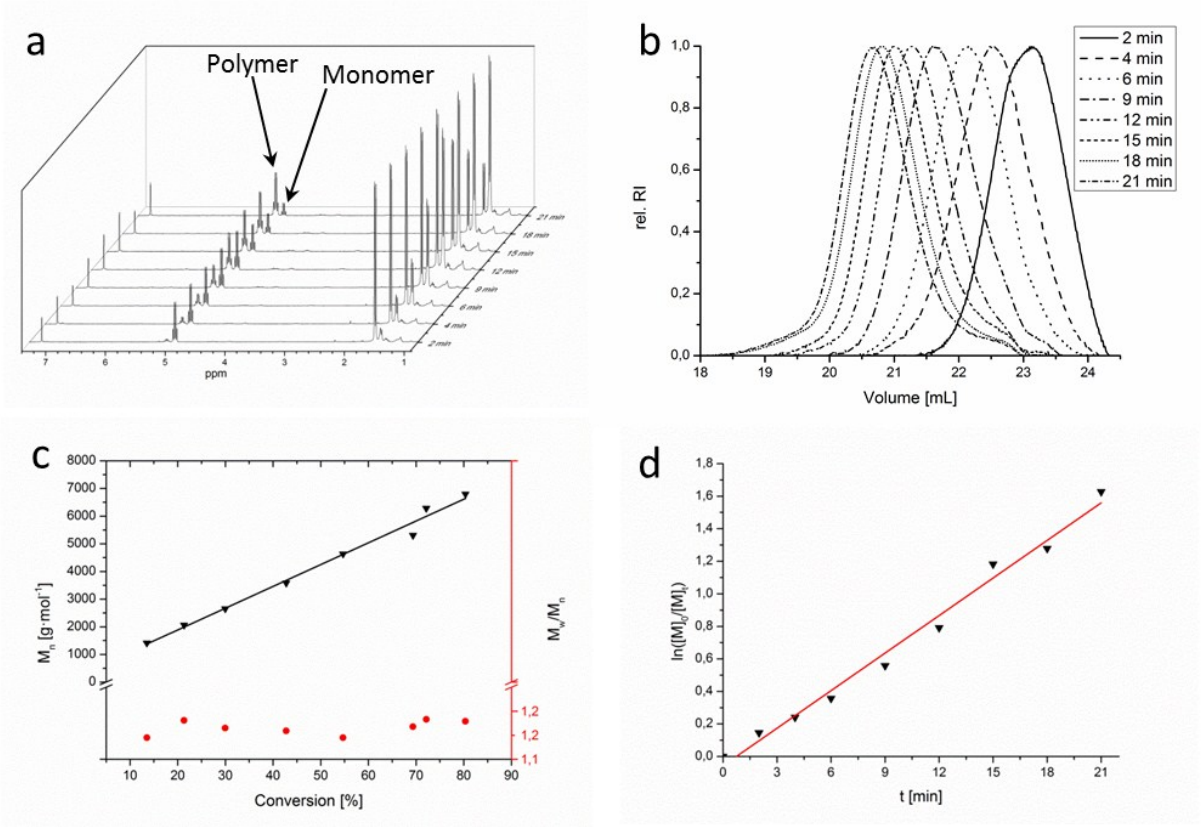


Figure S9: (a) ^{1}H-NMR spectra and (b) SEC traces of the samples taken during kinetic measurements (DMAc-LiCl, PMMA calibration and RI detection). (c) Molar mass versus conversion. Conversions were determined by ^{1}H-NMR measurements of the polymerization mixtures and molar masses as well as polydispersity indices by SEC measurements (DMAc-LiCl, PMMA calibration and RI detection). (d) Semilogarithmic plot. k_P = 0.15 L·mol^{-1}·s^{-1}.

pH-dependence

For the determination of the pH-dependence of the complex, a suspension of 10 µg·mL^{-1} complex in buffer solution was prepared. Therefore, ***2*** was predissolved in acetonitrile (1 mg·mL^{-1}) and 10 µL stock solution was given to 1 mL buffer solution (pH 4-6: acetate buffer, 0.1 mol·L^{-1}, pH 7-9: phosphate buffer, 0.1 mol·L^{-1}).

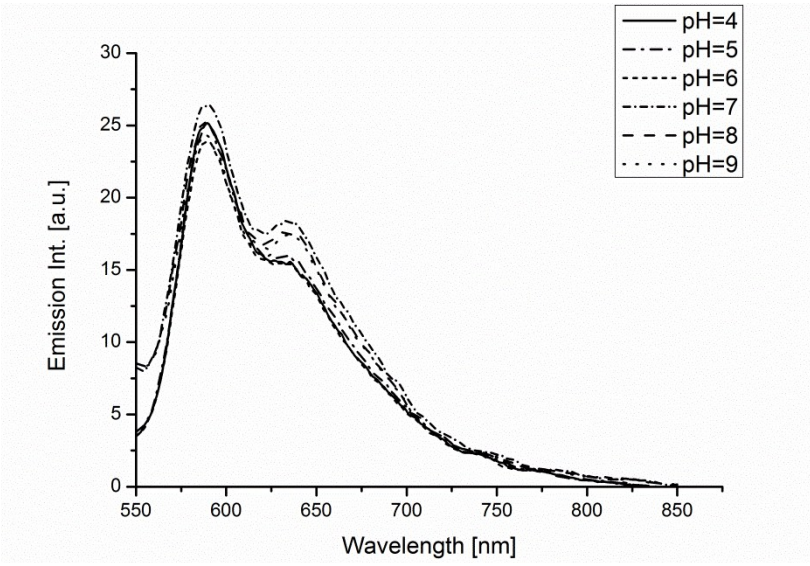


Figure S10: Emission from complex suspended in aqueous buffers. The emission intensity is barely dependent of the pH value of the surrounding medium.

Nanoparticle preparation

The nanoparticle suspensions were prepared using a single emulsion technique. Therefore, 25 mg of ***P1*** for ***NP1*** (1:1 mixture of ***P1*** and PLA, isopropanol initiated, M_n = 5,800 g mol^{-1}, $Đ$ = 1.18 acc. to SEC measurements, THF eluation and PLA calibration for ***NP2***) were dissolved in 0.5 mL dichloromethane and added to 1 mL poly(vinyl alcohol) (PVA) solution (3 wt%). The two-phase system was emulsified with a sonicator tip (10 s, 50 W), poured into 10 mL of 0.3 wt% PVA solution and stirred overnight at room temperature. The particles were washed twice by centrifugation and resuspension. A solution of Hepes buffered glucose (0.5 mL) was added as cryoprotectant to stabilize the particles during lyophilization. DLS measurements before and after lyophilization prove that the particles can be easily resuspended resulting in same particle size distributions.

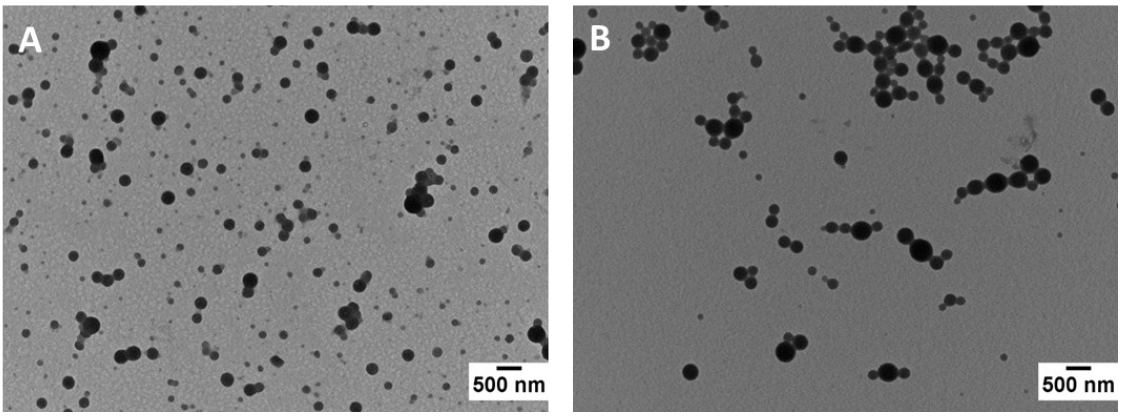


Figure S11: TEM images of (a) NP1 and (b) NP2.

The iridium content of the particles was determined *via* ICP-OES. For this purpose, the lyophilized particle samples were weighted out into a beaker and heated to reflux in suprapure nitric acid (approx. 35%, diluted with miliQ water) for 20 minutes and diluted with miliQ water prior to the measurement. The measurements were carried out using a Varian 725-ES spectrometer (ICP-725).

NP1: 0.262% Ir (w/w, referred mass of the lyophilized particles including stabilizers and buffer) and ***NP2:*** 0.182% Ir (w/w).

The luminescence behavior of the particles at different concentrations (miliQ water) was determined using a Tecan M200 Pro fluorescence microplate reader (Crailsheim, Germany), excitation wavelength 488 nm (exc. bandwith 9 nm), emission wavelength 580 nm (em. bandwith 20 nm).

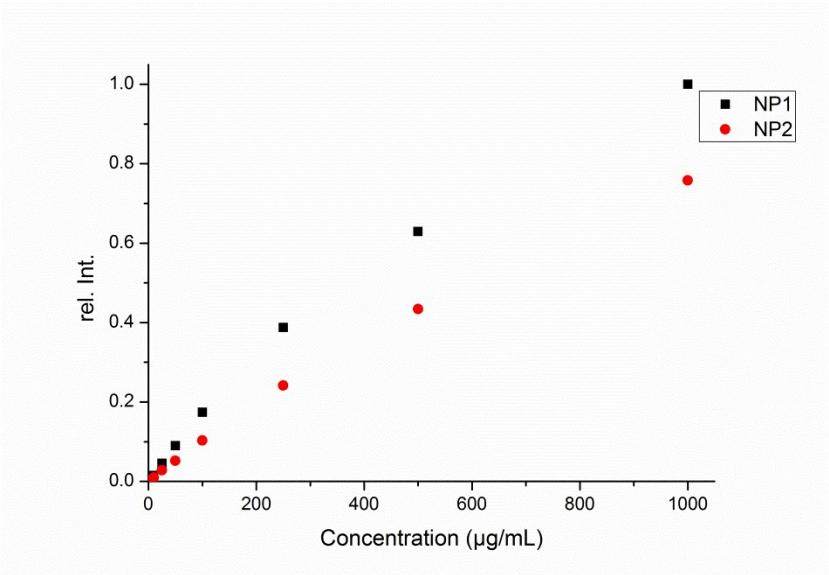


Figure S12: Luminescence intensities in dependence of the particle concentration. At concentrations up to 250 µg·mL^{-1}, a linear dependence was observed. For higher concentration, a saturation behavior was observed.

Cell Cultivation, Toxicity and Flow Cytometry Tests

Cell cultivation

The human embryonic kidney cell line HEK-293 used in the biological experiments was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 2 mmol L^{-1} L-glutamine, 1% non-essential amino acids, 1.0 mmol sodium pyruvate, 10% fetal bovine, 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (all components from Biochrom, Berlin, Germany) at 37 °C in a humidified atmosphere with 5% (v/v) CO_2.

Toxicity tests

The *in-vitro* cytotoxicity experiments were performed via an XTT assay according to the German standard institution guideline DIN ISO 10993-5 as a reference for biomaterial testing. HEK-293 cells were seeded in 96-well plates at a density of $1 \cdot 10^4$ cells/well and were grown as monolayer cultures for 24 h. The cells were then incubated separately with different concentrations of the NP (1.0, 0.5, 0.25, 0.1, 0.05 mg·mL^{-1}) and the complex ***3*** (0.25, 0.125, 0.0625, 0.025 and 0.0125 mg·mL^{-1}) for 24 h. Control cells were incubated with fresh culture medium. After incubation, cells were washed once and 150 µL of medium containing 50 µL XTT solution prepared according to the manufacturer's instructions were added to each well. After 4 h at 37 °C 100 µL of each solution was transferred to a new microtiter plate and the optical density (OD) at 450 nm was measured photometrically. The negative control was standardized as 0% of metabolism inhibition and referred as 100% viability.

Cellular uptake studied by flow cytometry

The concentration dependent uptake of the complex containing NP1 and NP2 was quantified by flow cytometry measurements. For this purpose, HEK cells were incubated with different concentrations (1, 0.5, 0.25, 0.1 and 0.05 mg·mL^{-1}) of the NP for 24 h at 37 °C. Following the incubation, the excess of NP was removed by washing with PBS. Subsequently, the adherent cells were detached by trypsin treatment, the cell suspension was washed twice with PBS supplemented with 10% fetal calf serum. For the identification of dead cells, a propidium iodide (PI) staining was performed for 15 minutes at room temperature using 100 µg PI/mL PBS. A total of $2 \cdot 10^4$ cells were resuspended and directly subjected to flow cytometry on a FACS Canto II (BD, Heidelberg, Germany) using gates of forward and side scatters to exclude debris and cell aggregates.

Microscopy

Structured illumination microscopy

HEK-293 cells were grown on coverslips (Zeiss, thickness no 1 ½, high-performance 18 mm × 18 mm, thickness 170±5 µm) and incubated for 24 h with the respective nanoparticle samples (250 µg·mL^{-1}). After rinsing with PBS to remove the excess of NP, the cells were stained for fluorescence microscopy according to manufactures instructions. The Hoechst dye was applied to viable cells at a concentration of 1 µg·mL^{-1}. Cells were then fixed for 10 minutes at room temperature using 4% paraformaldehyde dissolved in PBS, permeabilized with 0.1% TritonX100 solution and subsequently stained with AlexaFluor488 Phalloidin (Thermo Fisher Scientific, Schwerte, Germany) at a concentration of 165 nmol·mL^{-1} for 20 min. After rinsing with PBS, the coverslips were mounted on glass slides using 25 µL Mowiol 4-88 solution containing 625 µg 1,4-diazabicyclo-(2,2,2)octane (Sigma-Aldrich, Buchs, Switzerland). Imaging was performed on an Elyra S1 system (Zeiss, Oberkochen, Germany, excitation wavelengths of 405 nm, 488 nm, 561 nm and 642 nm) applying an 63× 1.4 NA plan apochromat oil objective. For structured illumination, excitation gratings with 23

resp. 28 µm were applied. Three channels were acquired: blue displaying nucleus (Hoechst 33342 staining, excitation wavelength 405 nm, BP 420-480 + LP 750), green displaying cytoskeleton (Alexa Fluor 488™ Phalloidin staining, excitation wavelength 488 nm, BP 495-550 + LP 750), red displaying nanoparticles (complex ***3***, excitation wavelength 488 nm, BP 570-620 + LP 750). The grating position and axial position of the sample table were controlled by piezo controllers (Physik Instrumente, Germany). An axial stack of images was acquired (Δz = 120 nm). Images were recorded with a CCD camera (Andor, USA), cooled to −63 °C. Reconstructions were performed with the commercial ZEN software installed on the system.

Electron microscopy

For electron microscopy, HEK-293 cells were grown in a 6-well plate and incubated for 24 h with the respective nanoparticle samples (250 µg·mL^{-1}). Subsequent to incubation, cells were suspended by treatment with trypsin. The cell suspension was fixed for 2 h with glutaraldehyde (2% in PBS 1x, prepared from 8% EM grade stock solution) on ice and subsequently fixed with osmium tetroxide (1% in PBS, prepared from 4% EM grade stock solution, both purchased from EMS, Hatfield). After washing with pure water, the samples were dehydrated by an ethanol/water series (50%, 70%, 90%, 2 × 100% dry EtOH, purified with a Solvent Purification System, stored over molecular sieves). The dehydrated samples, which were transferred into BEEM capsules (Plano, Wetzlar), were immersed in mixtures of Embed 812 (EMS, Hatfield) and ethanol (Embed/EtOH = 1:1 *v/v* for 1 hour, 2:1 *v/v* for 12 h) and subsequently in pure Embed 812 for 4 h. After a further change of the embedding medium, the samples were stored in a drying oven at 70 °C for 24 h, until the resin hardened. Ultrathin sections of 80 nm were cut with an ultramicrotome (PT-XL PowerTome, RMC, Tucson) using a diamond knife (RMC, Tucson). The sections were put on a carbon supported copper grid (400 mesh, Quantifoil, Jena) imaged with a Technai G^2 system (FEI), with 200 kV acceleration voltage in STEM mode (HAADF detection).

Comparison of contrast generated by gold nanoparticles and polymer nanoparticles

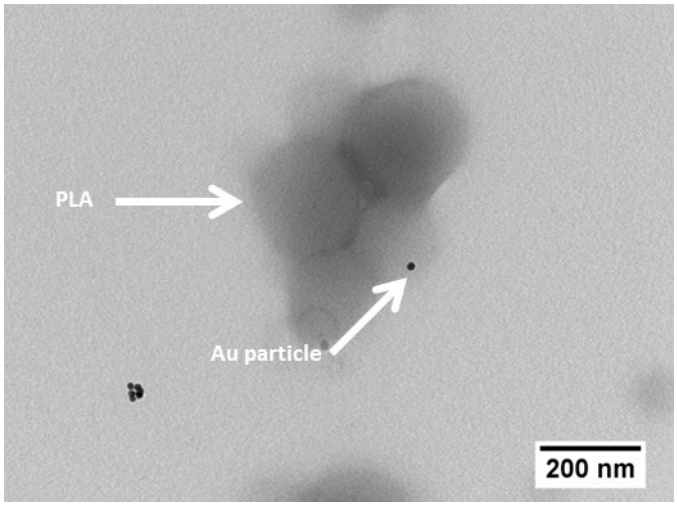


Fig. S13: Comparison of the contrast generated by gold nanoparticles and polymer nanoparticles PLA particles. PLA provides poor contrast in comparison to the gold particles. The Au particles were prepared according to a standard procedure[4] (diameter 23.9 nm, PDI 0.204 according to DLS).

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