Electronic Supplementary Material (ESI) for Chemical Science.

Ruthenium-Initiated Polymerization of Lactide: A Route to Remarkable Cellular Uptake for Photodynamic Therapy of Cancer

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Experimental

Synthesis and characterization of Ru-PLA nanoconjugates

Materials. All polymerizations were carried out under a purified argon atmosphere using Schlenk techniques or a glovebox (< 1 ppm O_2, < 2 ppm H_2O). Deuterated acetonitrile (CD_3CN) from Eurisotop and acetonitrile from Carlo Erba was freshly distilled from CaH_2 prior to use. D,L-lactide and L-lactide from Alfa Aesar and D-lactide from Fluorochem were recrystallized from isopropanol then toluene and sublimated before being stored in the glovebox. $Zn(N(SiMe_3)_2)_2$ was prepared according to the literature[1] and stored in the glove box freezer. **RuOH** was synthesised as previously reported by our group and dried over CaH_2 overnight.[2] PVA (M_n = 133 000 g/mol, >99% hydrolysed) was purchased from Polysciences, Inc. and used as received. *Trans*-2-[3-(4-*ter*-butylphenyl)-2-propenylidene]malonitrile (DCTB) was purchased from Sigma-Aldrich and used without further purification. Tetrahydrofuran (THF), dichloromethane (DCM), pentane and diethyl ether were purchased from VWR. Phosphate-buffered saline (PBS) was purchased from Gibco.

Instrumentation and Methods. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer at room temperature. NMR spectra were calibrated using residual ^1H resonances of deuterated solvents (δ = 1.94 ppm for CD_3CN, δ = 7.26 ppm for $CDCl_3$) and ^{13}C resonances of deuterated solvents (δ = 1.32 ppm, 118.26 for CD_3CN). MALDI-TOF MS analyses of polymers were performed at Institut de Chimie des Substances Naturelles, UPR CNRS 2301, Université Paris-Saclay, using an UltrafleXtreme mass spectrometer (Bruker Daltonics). Acquisitions were performed in reflector ion mode. The laser intensity was set just above the ion generation threshold to obtain peaks with the highest possible signal-to-noise (S/N) ratio without significant peak broadening. The mass spectrometer was externally calibrated using PEG3400. All data were processed using the program Flex-Analysis (Bruker Daltonics, Bremen). DCTB was used as the matrix for MALDI-TOF MS. Polymer sample for MALDI analysis was prepared at a concentration of 60 mM in THF. The matrix solution was prepared at a concentration of 6 mM in THF. The sample was prepared by mixing the polymer solution with matrix solution at a volume ratio of 1:9. The nanoparticle intensity-average diameters D_z and the polydispersity index (PdI) were determined by dynamic light scattering (DLS) using a Malvern ZetaSizer Nano ZS (scattering angle = 173°) at a temperature of 25 °C with an equilibrium time of 120 s. Differential scanning calorimetry (DSC) was carried out with Setaram DSC 131 EVO using high pressure crucibles M30. Polymer samples were analysed under nitrogen flow with a heating/cooling rate of 5 °C/min in the range of 40 to 230 °C. Two heating/cooling cycles were performed and the melting temperature (T_m) was determined using the second heating run. DSC data were processed with Calisto software. RP-HPLC equipped with an Agilent Pursuit XRs 5C18 (Analytic: 100 Å, C18 5 µm 250 × 4.6 mm, Preparative: 100 Å, C18 5 µm 250 × 300 mm) Column was used to assess the hydrolytic release of **RuOH** from

Ru-PLA nanoconjugates. The C18 reverse phase column was used with a flow rate of 1 $mL.min^{-1}$ and UV absorption was measured at 300 nm. The runs were performed with a linear gradient of A (CH_3CN containing 0.01 % TFA) and B (distilled water containing 0.01 % TFA): t=0–3 min, 20% A; t=7 min, 50 % A; t=20 min, 90 % A.

Ring opening polymerization of lactide. In a typical polymerization (**P5**), $Zn(N(SiMe_3)_2)_2$ (7.6 mg, 0.020 mmol, 1 equiv.) and **RuOH** (40 mg, 0.039 mmol, 2 equiv.) was dissolved in 0.47 mL of dry acetonitrile. The red solution was stirred at room temperature for a couple of minutes. The solvent was then removed under vacuum to give a red solid which was washed three times with pentane to get rid of the released $HN(SiMe_3)_2$. The resulting product was dissolved in 0.47 mL of dry acetonitrile and D,L-lactide (200.5 mg, 1.4 mmol, 70 equiv.) was added. The reaction mixture was heated at 60 °C for 1 hour. The polymerization was quenched by contact to air and the reaction mixture was, then, precipitated in cold diethyl ether to remove unreacted monomer, yielding an orange powder.

^1H NMR (400 MHz, CD_3CN): δ/ppm = 9.67 – 9.65 (d, 2H), 8.56 – 8.51 (dd, 4H), 8.49 – 8.47 (d, 1H), 8.43 (s, 1H), 8.18 – 8.08 (m, 5H), 8.04 – 8.00 (t, 2H), 7.91 – 7.88 (t, 2H), 7.86 – 7.85 (d, 2H), 7.73 – 7.72 (d, 2H), 7.48 – 7.45 (t, 2H), 7.28 – 7.24 (t, 2H), 5.56 (s, 2H), 5.19 – 5.11 (m, PLA, $-CH_2C(O)-$),1.52 – 1.49 (m, PLA, CH_3)

^{13}C NMR (100 MHz, CD_3CN): δ/ppm = 175.25, 170.49 – 170.31 (PLA), 158.14, 157.94, 154.72, 153.04, 152.91, 151.43, 143.42, 141.62, 141.34, 141.21, 138.96, 138.96, 138.88, 134.46, 132.80, 131.73, 130.90, 128.59, 128.43, 125.29, 125.23, 70.48 - 69.94 (PLA), 67.72, 66.89, 20.72, 17.09 (PLA)

NMR determination of the M_n of Ru-PLA conjugates. $M_{n,NMR}$ was calculated by integrating the singlet at 5.54 ppm corresponding to two protons from the **RuOH** end-chain and the multiplet (in the case of atactic polymers) or quadruplet (in the case of isotactic polymers) at 5.16 ppm assigned to the protons of the PLA methine group, that allowed determination of LA unit content *DP*. Given the LA unit content, $M_{n,NMR}$ can be calculated according to: $M_{n,NMR}$ = *DP**MW(LA)+ MW(**RuOH**) with MW(LA) = 144.13 g mol^{-1} and MW(RuOH) = 1015.7 g mol^{-1}.

Nanoparticle preparation. Nanoparticles were prepared by nanoprecipitation. Briefly, 2 mg of polymer was dissolved in 0.5 mL of THF and added dropwise to 1 mL of a 0.3% w/v aqueous solution (Milli-Q water) of PVA under moderate stirring. THF was removed under reduced pressure using a rotary evaporator to give an orange nanoparticle suspension. In the case of **P1**, only 0.5 mg of polymer was dissolved in 0.5 mL of THF. For **NPs-3**, 1 mg of **P3** and 1 mg of **P4** were dissolved in 0.25 mL of THF, respectively. The two solutions were, then, mixed and added dropwise to a 0.3% w/v aqueous solution (Milli-Q water) of PVA under moderate stirring.

Stereocomplex formation for DSC measurements. 10 mg of **P3** and 10 mg of **P4** were dissolved in 1 mL of CH_2Cl_2, respectively and mixed together. The resulting solution was added

to an excess of pentane to give an orange powder which was dried under vacuum, prior to analysis.

Release kinetics of RuOH from nanoparticles. Briefly, 0.5 mL of nanoconjugates were added to 4.5 mL of PBS (1 X, pH = 7.0 - 7.2). The resulting PBS solution was divided into equal portions, added to five separate 1 mL Eppendorf tubes and incubated at 37 °C. At different time points, the corresponding Eppendorf tubes were taken out of the incubator and centrifuged at 10 000 g for 20 min. The supernatant was directly injected into RP-HPLC to quantify the released **RuOH**, based on the calibration curve of **RuOH** (figure S12).

Photophysical studies: emission, luminescence quantum yield, and lifetimes

Spectroscopic measurements. The absorbance was measured using a Lambda 30 UV/Vis spectrophotometer from Perkin Elmer. The emission was measured by irradiation of the sample in fluorescence quartz cuvettes (width 1 cm) using a NT342B Nd-YAG pumped optical parametric oscillator (Ekspla) at 450 nm. Luminescence was focused and collected at right angle to the excitation pathway and directed to a Princeton Instruments Acton SP-2300i monochromator. As a detector a XPI-Max 4 CCD camera (Princeton Instruments) has been used.

Luminescence quantum yield measurements. For the determination of the luminescence quantum yield, the samples were prepared in a degassed H_2O solution with an absorbance of 0.2 at 450 nm. This solution was irradiated in fluorescence quartz cuvettes (width 1 cm) using a NT342B Nd-YAG pumped optical parametric oscillator (Ekspla) at 450 nm. The emission signal was focused and collected at right angle to the excitation pathway and directed to a Princeton Instruments Acton SP-2300i monochromator. As a detector a XPI-Max 4 CCD camera (Princeton Instruments) has been used. The luminescence quantum yields were determined by comparison with the reference $[Ru(bipy)_3]Cl_2$ in H_2O (Φ_{em}=5.5%[3]) applying the following formula:

$$\Phi_{em,\,sample} = \Phi_{em,\,reference} * (F_{reference} / F_{sample}) * (I_{sample} / I_{reference}) * (n_{sample} / n_{reference})^2$$

$$F = 1 - 10^{-A}$$

Φ_{em} = luminescence quantum yield, F = fraction of light absorbed, I = integrated emission intensities, n = refractive index, A = absorbance of the sample at irradiation wavelength.

Lifetime measurements. For the determination of the lifetimes, the samples were prepared in an air saturated and in a degassed H_2O solution with an absorbance of 0.2 at 450 nm. This solution was irradiated in fluorescence quartz cuvettes (width 1 cm) using a NT342B Nd-YAG pumped optical parametric oscillator (Ekspla) at 450 nm. The emission signal was focused and collected at right angle to the excitation pathway and directed to a Princeton Instruments Acton SP-2300i monochromator. As a detector a R928 photomultiplier tube (Hamamatsu) has been used.

Singlet oxygen measurements

- **Direct evaluation**

The samples were prepared in an air saturated D_2O solution with an absorbance of 0.2 at 450 nm. This solution was irradiated in fluorescence quartz cuvettes (width 1 cm) using a mounted M450LP1 LED (Thorlabs) whose irradiation, centred at 450 nm, has been focused with aspheric condenser lenses. The intensity of the irradiation has been varied using a T-Cube LED Driver (Thorlabs) and measured with an optical power and energy meter. The emission signal was focused and collected at right angle to the excitation pathway and directed to a Princeton Instruments Acton SP-2300i monochromator. A longpass glass filter was placed in front of the monochromator entrance slit to cut off light at wavelengths shorter than 850 nm. As a detector an EO-817L IR-sensitive liquid nitrogen cooled germanium diode detector (North Coast Scientific Corp.) has been used. The singlet oxygen luminesce at 1270 nm was measured by recording spectra from 1100 to 1400 nm. For the data analysis, the singlet oxygen luminescence peaks at different irradiation intensities were integrated. The resulting areas were plotted against the percentage of the irradiation intensity and the slope of the linear regression calculated. The absorbance of the sample was corrected with an absorbance correction factor. As reference for the measurement $[Ru(bipy)_3]Cl_2$ ($\Phi_{Ru(bipy)3Cl2}$=0.22[4]) was used and the singlet oxygen quantum yields were calculated using the following formula:

$\Phi_{sample} = \Phi_{reference} * (S_{sample} / S_{reference}) * (I_{reference} / I_{sample})$

$I = I_0 * (1 – 10^{-A})$

Φ = singlet oxygen quantum yield, S = slope of the linear regression of the plot of the areas of the singlet oxygen luminescence peaks against the irradiation intensity, I = absorbance correction factor, I_0 = light intensity of the irradiation source, A = absorbance of the sample at irradiation wavelength.

- **Indirect evaluation**

The samples were prepared in an air-saturated PBS solution containing the complex with an absorbance of 0.1 at the irradiation wavelength, *N*,*N*-dimethyl-4-nitrosoaniline aniline (RNO, 20 µM) and histidine (10 mM). The samples were irradiated on 96 well plates with an Atlas Photonics LUMOS BIO irradiator for different times. The absorbance of the samples was measured during these time intervals with a SpectraMax M2 Microplate Reader (Molecular Devices). The difference in absorbance (A_0-A) at 440 nm a PBS buffer solution was calculated and plotted against the irradiation times. From the plot the slope of the linear regression was calculated as well as the absorbance correction factor determined. The singlet oxygen quantum yields were calculated using the same formulas as used for the direct evaluation.

Biological evaluation

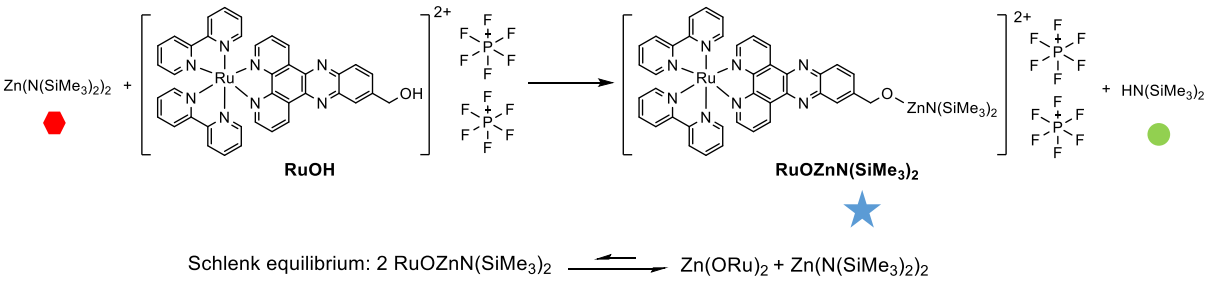
Cell culture experiments. Cells lines were treated in appropriate cell culture media of DMEM (Gibco, LifeTechnologies, USA) supplemented with 10% foetal calf serum for the HeLa cell line (Gibco) and DMEM/F-12 (Gibco) supplemented with 10% foetal calf serum (Gibco) for the RPE-1 cell line. All media was also supplemented with 100 U/ml penicillin-streptomycin mixture (Gibco). Cells were incubated at 37 °C in 5 % CO_2. Cells were passaged when 80% confluency was reached and used within 15 passages from initial purchase.

Cytotoxicity experiments. 96-well plates were seeded with HeLa and RPE-1 cell lines (4000, 2000 and 1000 cells per well for 4, 24 and 48 h time points respectively) in media (DMEM, 100 µl) and incubated overnight. Treatment solutions were made by dilution of compound stock solutions (nanoparticles were stored at a concentration **RuOH** of ~500 µM for **NPs-2,3** and at ~300 µM for **NPs-1,4** in 0.3% w/v PVA water, **RuOH** was stored at 50 mM in DMSO) into the cell media. The concentrations of water and DMSO were kept constant throughout all treatment solutions. The incubation media was removed and replaced with treatment media and the cells incubated in the dark. Following 4, 24 or 48 h, the treatment media was replaced with fresh media and the cells were treated with light (λ_{exc} = 480 nm, 3.21 J cm^{-2}, 10 min) before being incubated in the dark. 48 h post light treatment the cells were treated with resazurin (0.2 mg mL^{-1} final concentration in appropriate media) and incubated a further 4 h. The plates were read by fluorescence plate reader SpectraMax M5 micro plate reader (λ_{ex}, 540 nm; λ_{em}, 590 nm).

ICP-MS cellular uptake. 6-well plates were seeded with HeLa cells (2 x 10^4, 4 x 10^4 and 10 x 10^4 cells per well for 48, 24 and 4 h timepoints respectively) in media (2 ml) and incubated overnight. The next morning the cells were treated with nanoparticles or **RuOH** staining solution in media (5 µM, 2 wells per condition/timepoint) and the cells were incubated with the staining solution for the stated incubation time. Following incubation, the cells were washed (2 x PBS, 2 ml) and trypsinized (300 µl / well). Once detached the cells were washed by centrifugation (PBS, 1 ml) and the pellet suspended (PBS, 1 ml) and the cells counted by haemocytometer. Once counted the cells were pelleted once again before being suspended in HNO_3 (overnight, 60 ^oC) and subsequently diluted into HCl solution (1/10 dilution, 1 % HCl in distilled H_2O). Daily, prior to the analytical sequence, the instrument (sector-field inductively coupled plasma mass spectrometer, HR-ICP-MS Element II, ThermoScientific) was first tuned to produce maximum sensitivity and stability while also maintaining low Uranium oxide formation (UO/U ≤ 5%). Ruthenium stock solution (SCP Science, 1g/L) was diluted several times in 1% distilled hydrochloric acid to obtain standards for the calibration range (from 10 ng/L to 10 µg/L). Then, data were treated as follow: intensities were converted into concentrations using uFREASI (user-FRiendly Elemental dAta proceSsIng)[5] This software,

made for HR-ICP-MS users community, is free and available on http://www.ipgp.fr/~tharaud/uFREASI.

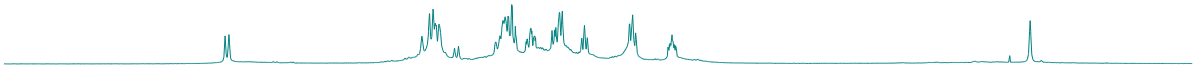
Confocal microscopy. Into 12-well plates were added pre-sterilized 12 mm *Menzel– Gläser* coverslips before HeLa cells were seeded (2 x 10^4, 4 x 10^4 and 10 x 10^4 cells per well for 48, 24 and 4 h timepoints respectively) and incubated overnight. The next morning the cells were treated with nanoparticle or RuOH staining solution in media (50 µM, 2 wells per condition/timepoint) and the cells were incubated. Nucblue (2 drops/ml) was added for the final 20 minutes of stated incubation time. Cells were then washed (PBS X 2) before being fixed (paraformaldehyde, 4 % in PBS, 20 mins) and washed (PBS X 2). Samples used later for indirect immunofluorescence were then incubated in blocking solution (0.2% BSA, 0,05 % Saponin in PBS) for 15 min at RT and incubated with primary anti-LAMP (*BD Biosciences*) antibodies for 1 h at 1:3000 dilution and detected using Alexa 488 conjugated secondary antibodies (*Jackson ImmunoResearch Laboratory*) at 1:400 dilution. All samples were then mounted to microscope slides (*Prolong Glass Antifade Mountant).* The slides were imaged using a Leica SP8 confocal microscope. The ruthenium compounds were excited at 488 nm with emission recorded above 650 nm. Images were recorded at the Cellular and Molecular Imaging Technical Platform, INSERMUMS025–CNRSUMS3612, Faculty of Pharmacy of Paris, Paris Descartes University, Paris, France.



Schlenk equilibrium: 2 $RuOZnN(SiMe_3)_2$ ⇌ $Zn(ORu)_2 + Zn(N(SiMe_3)_2)_2$

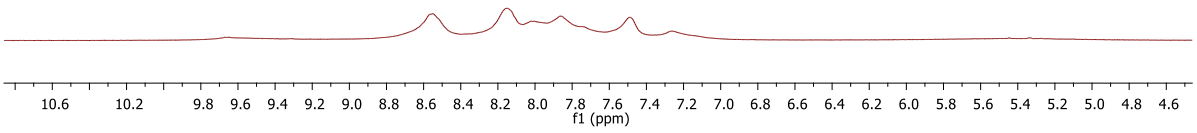
1. Equimolar mixture of **RuOH** and $Zn(N(SiMe_3)_2)_2$
Schlenk equilibrium

2. After 2 hours of reaction



3. After 4 hours of reaction

4. Overnight



1. Equimolar mixture of **RuOH** and $Zn(N(SiMe_3)_2)_2$
   Schlenk equilibrium

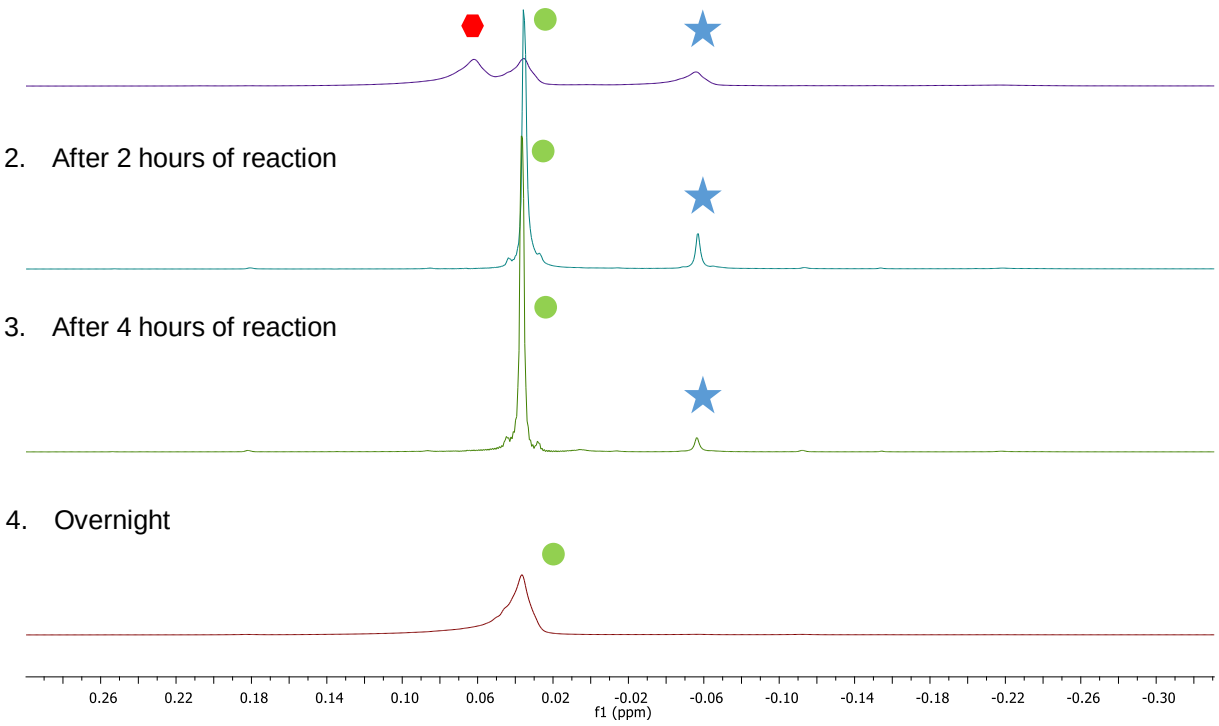


Figure S1. An overlay ^1H NMR spectra of the reaction mixture between equimolar ratio of **RuOH** and $Zn(N(SiMe_3)_2)_2$ in dry CD_3CN and in a J. Young NMR tube at room temperature overtime, 400 MHz.

Figure S2. ^{1}H NMR spectra of **Ru-PLA** prepared from D,L-lactide , * = H_2O.

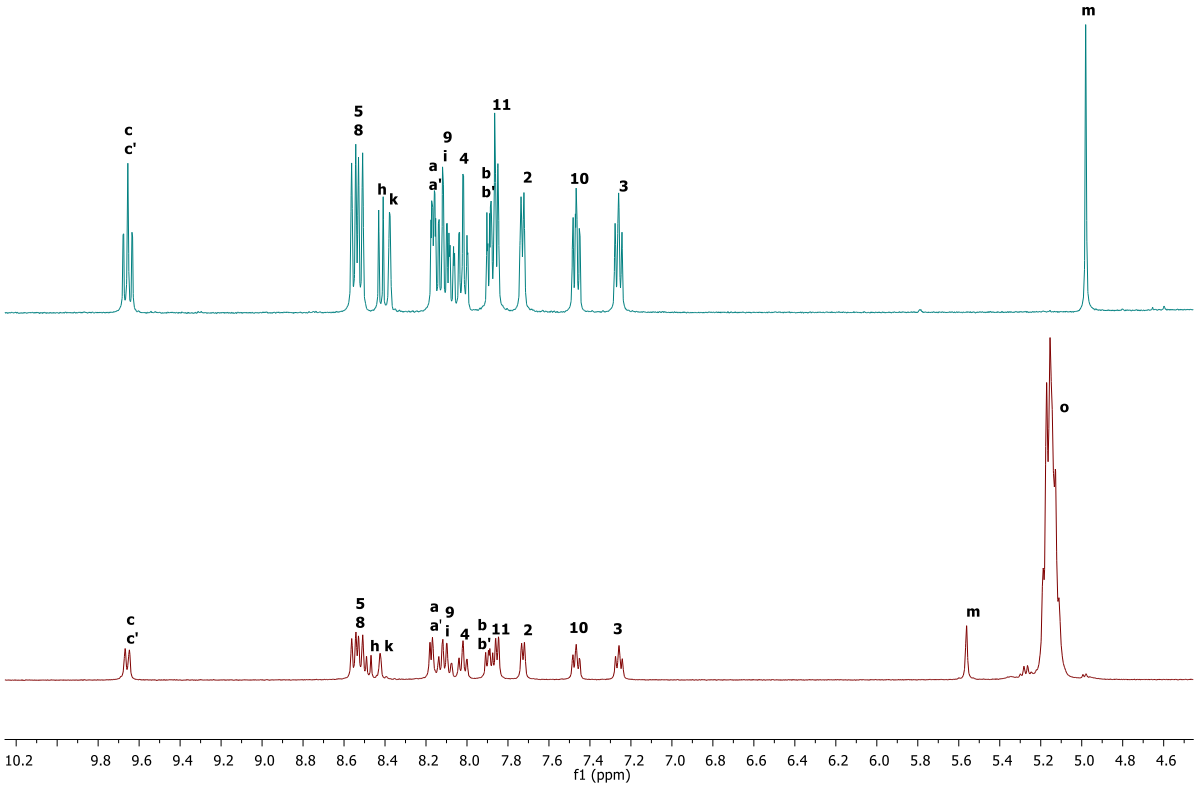


Figure S3. An overlay of ^{1}H NMR spectra of **RuOH** (green) and **Ru-PLA** (red).

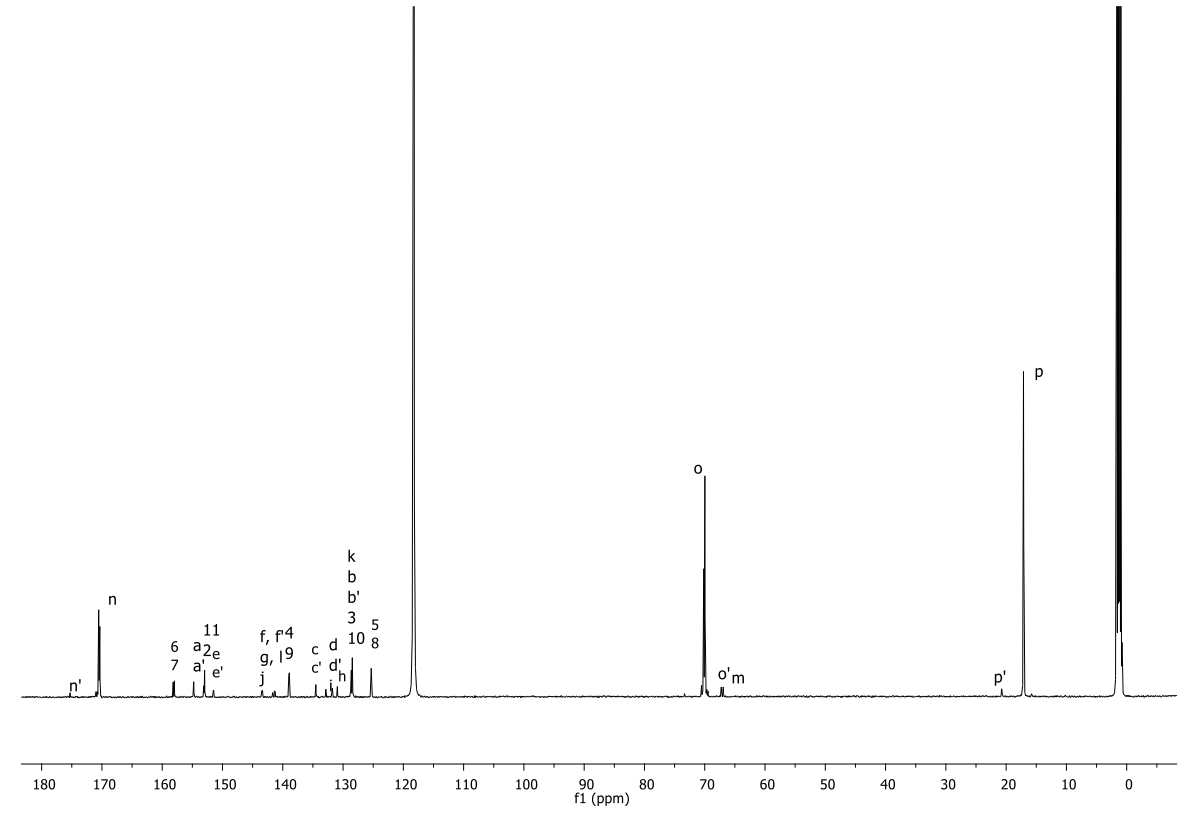
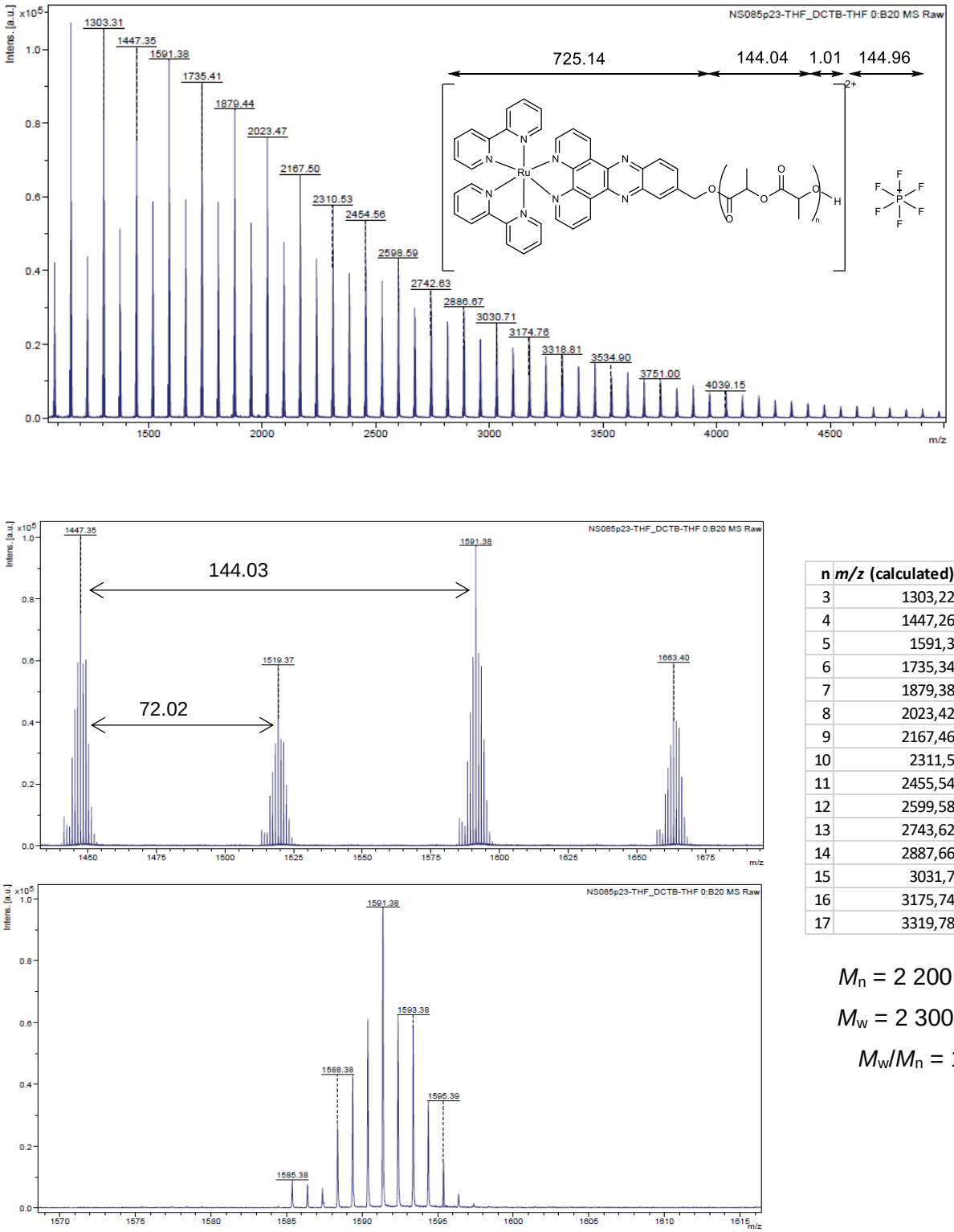


Figure S4. ^{13}C NMR of **Ru-PLA** prepared from D,L-lactide.



n	*m/z* (calculated)	*m/z* MALDI
3	1303,22	1303,31
4	1447,26	1447,35
5	1591,3	1591,38
6	1735,34	1735,41
7	1879,38	1879,44
8	2023,42	2023,47
9	2167,46	2167,5
10	2311,5	2310,53
11	2455,54	2454,56
12	2599,58	2598,59
13	2743,62	2742,63
14	2887,66	2886,67
15	3031,7	3030,71
16	3175,74	3174,76
17	3319,78	3318,81

M_n = 2 200 g/mol

M_w = 2 300 g/mol

M_w/M_n = 1.07

Figure S5. MALDI-TOF MS analysis of a Ru-conjugate

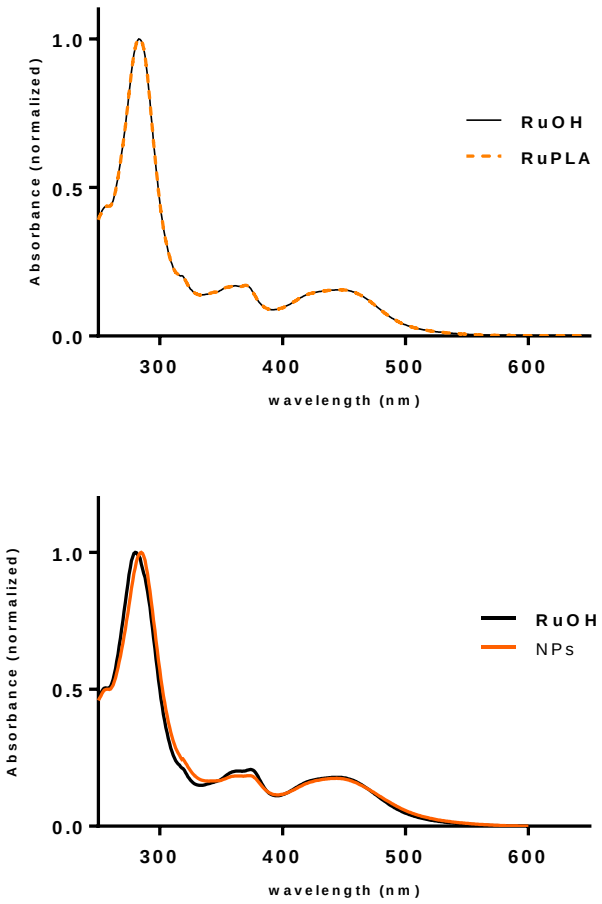


Figure S6. UV-Vis spectra of **RuOH** and **Ru-PLA** in CH_3CN (top) and in water (bottom).

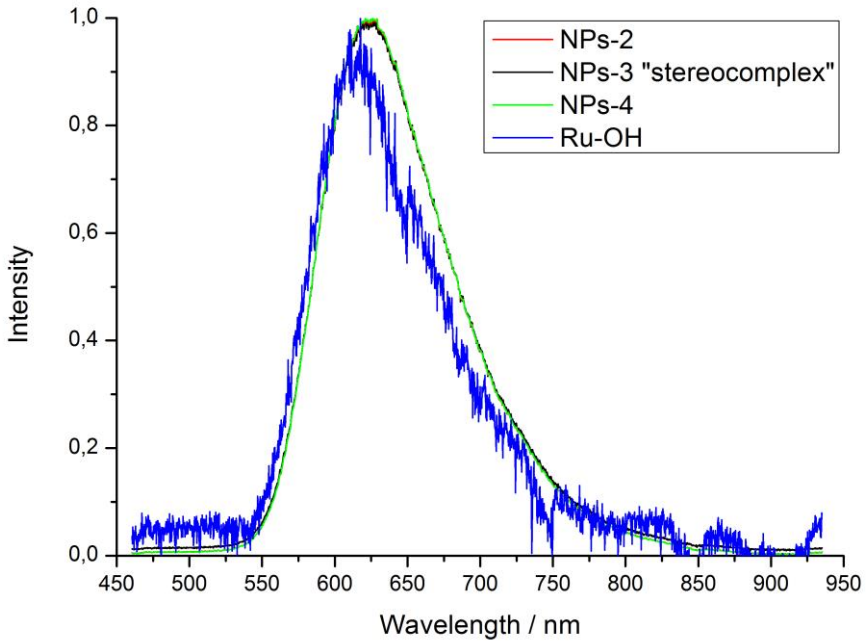


Figure S7. Emission spectra of **RuOH** and **NPs-2,3,4** in H_2O.

Table S1. Photophysical properties of **RuOH** in comparison to **NPs-2**,**3,4** in H_2O. [a)] contains 1% DMSO.

Compound	Emission maximum	Luminescence Quantum Yield	Lifetime / ns	
			degassed	air saturated
RuOH[a)]	618	>0.1%	950	202
NPs-2	626	1.3%	998	220
NPs-3 “stereocomplex”	626	1.4%	932	231
NPs-4	626	1.4%	1040	207

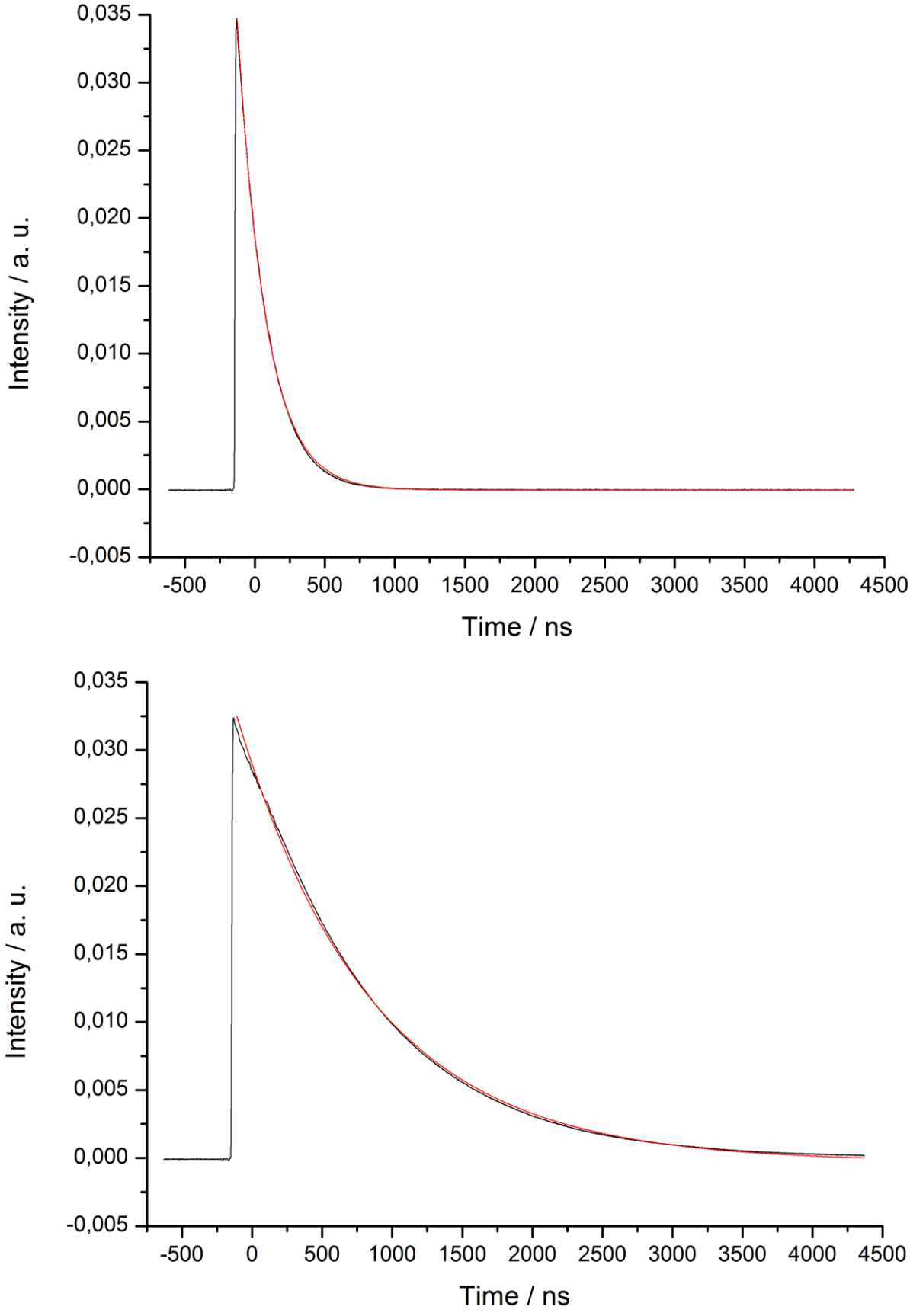


Figure S8. Lifetime spectra of **RuOH** in aerated (above) and degassed (below) H_2O.

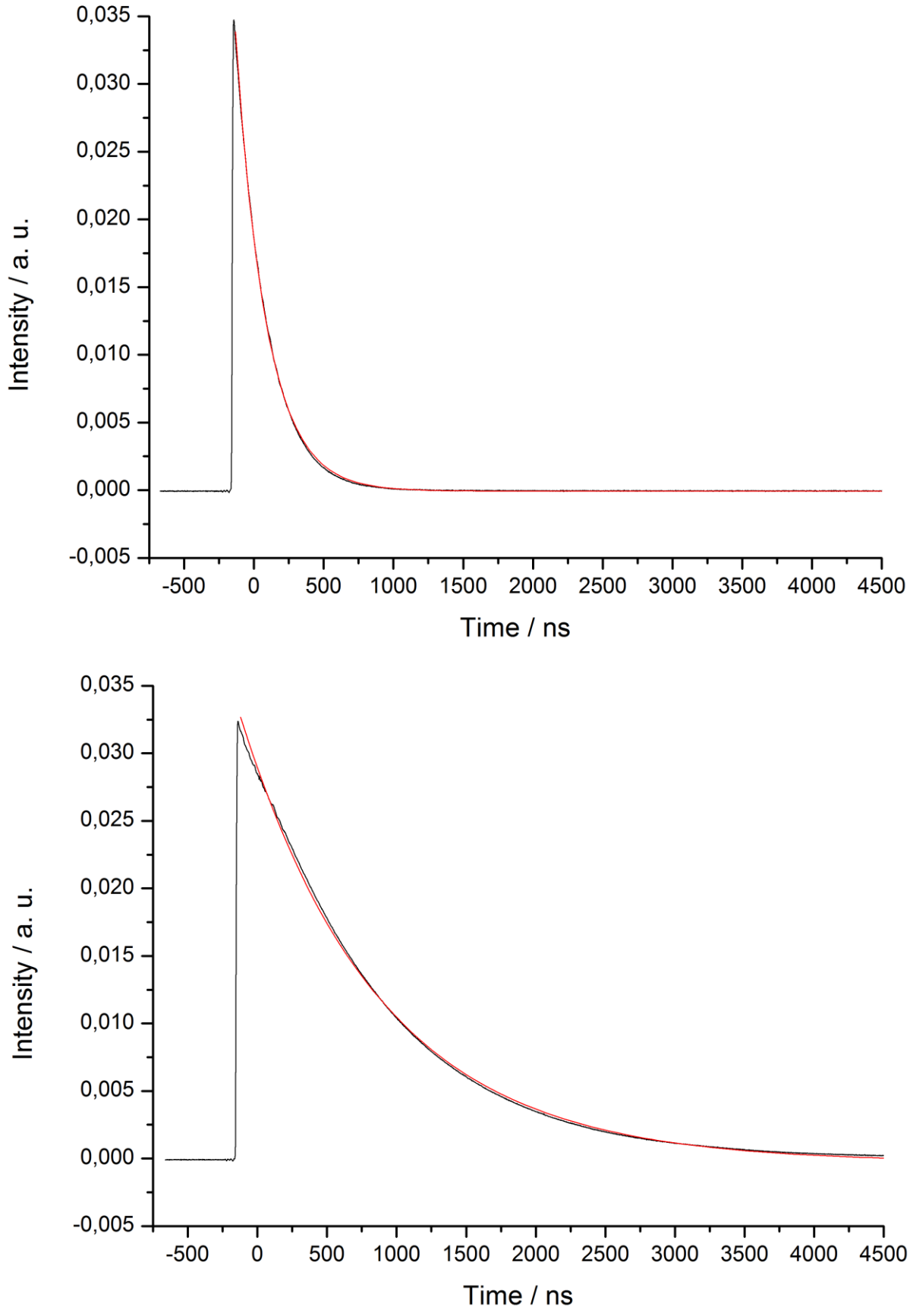


Figure S9. Lifetime spectra of **NPs-2** in aerated (above) and degassed (below) H_2O.

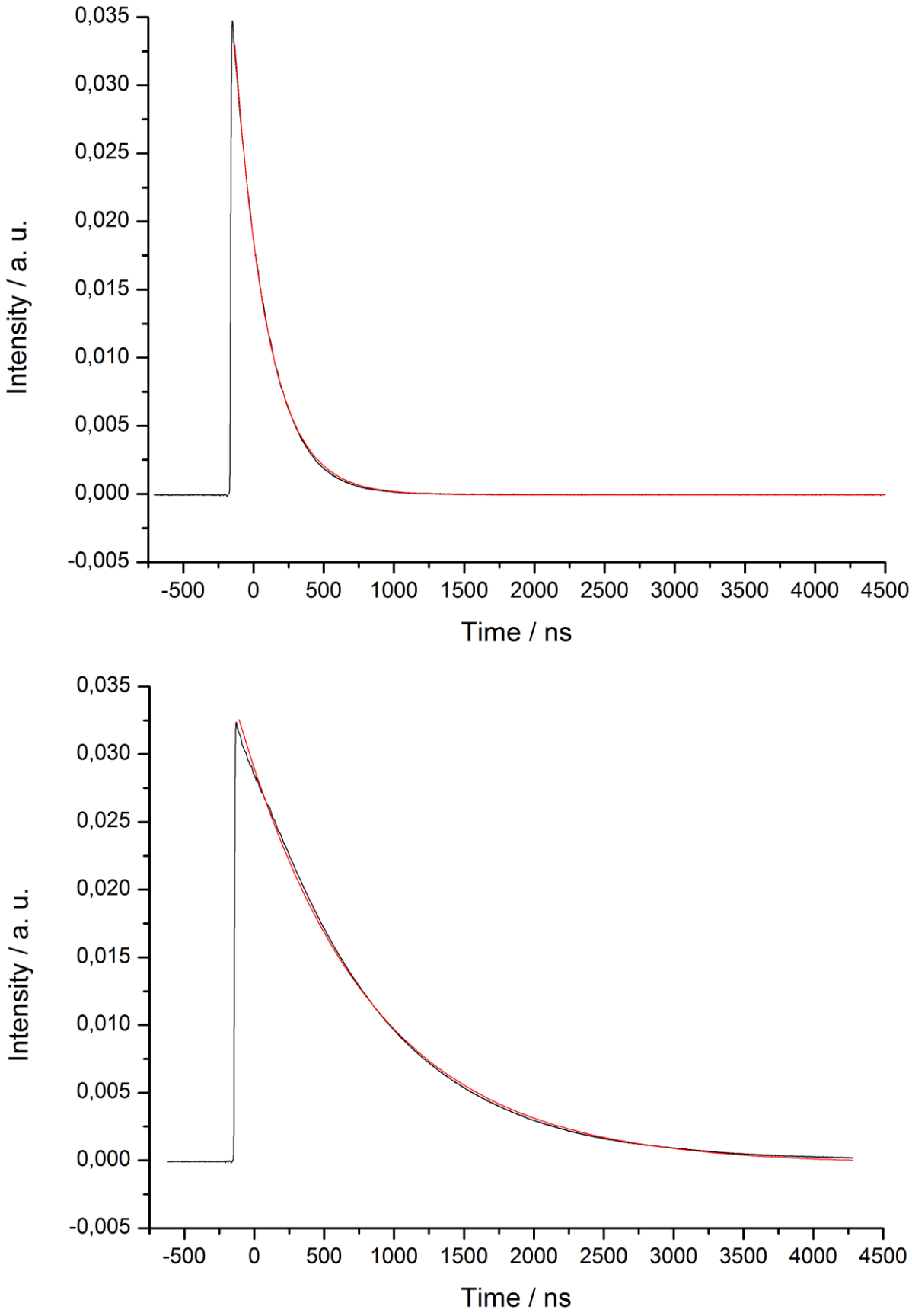


Figure S10. Lifetime spectra of the complex **NPs-3** in aerated (above) and degassed (below) H_2O.

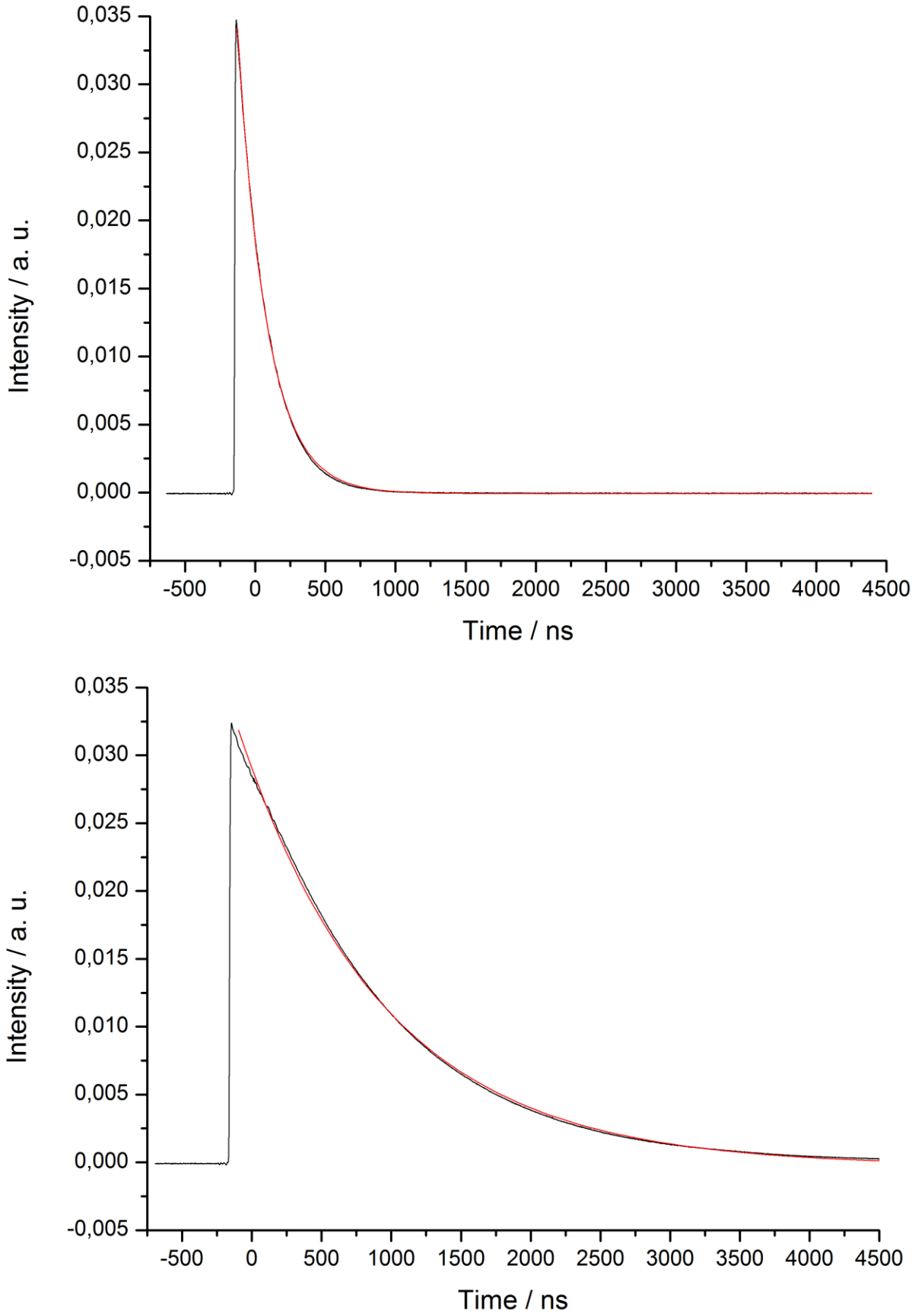


Figure S11. Lifetime spectra of the complex **NPs-4** in aerated (above) and degassed (below) H_2O.

Table S2. Singlet oxygen quantum yields in H_2O upon irradiation at 450 nm. Average of three independent measurements. n.d. = not detectable. [a)] contains 1% DMSO.

	Direct D_2O	Indirect PBS
RuOH[a)]	n.d.	3%
NPs-2	n.d.	11%
NPs-3 stereocomplex	n.d.	11%
NPs-4	n.d.	12%

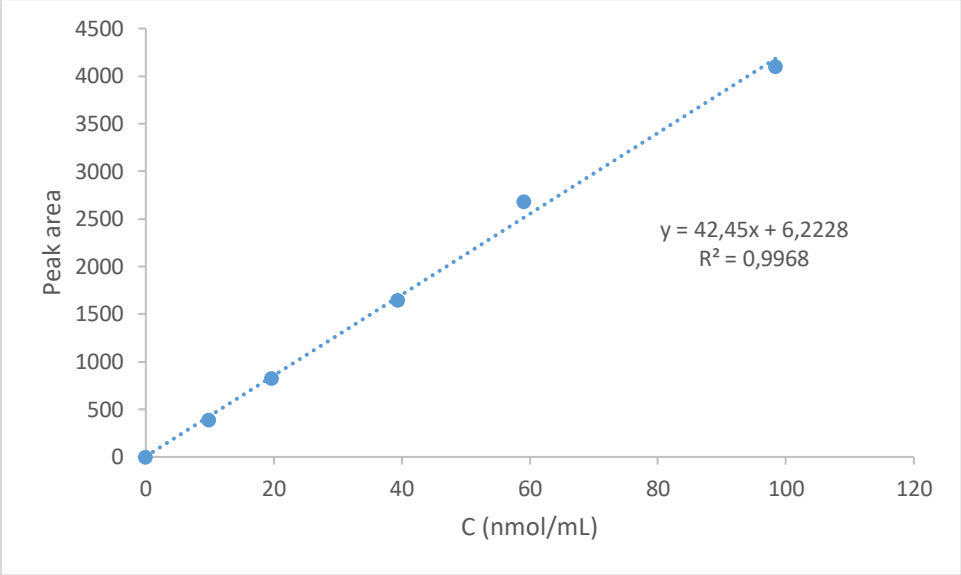


Figure S12. Standard curve of **RuOH**

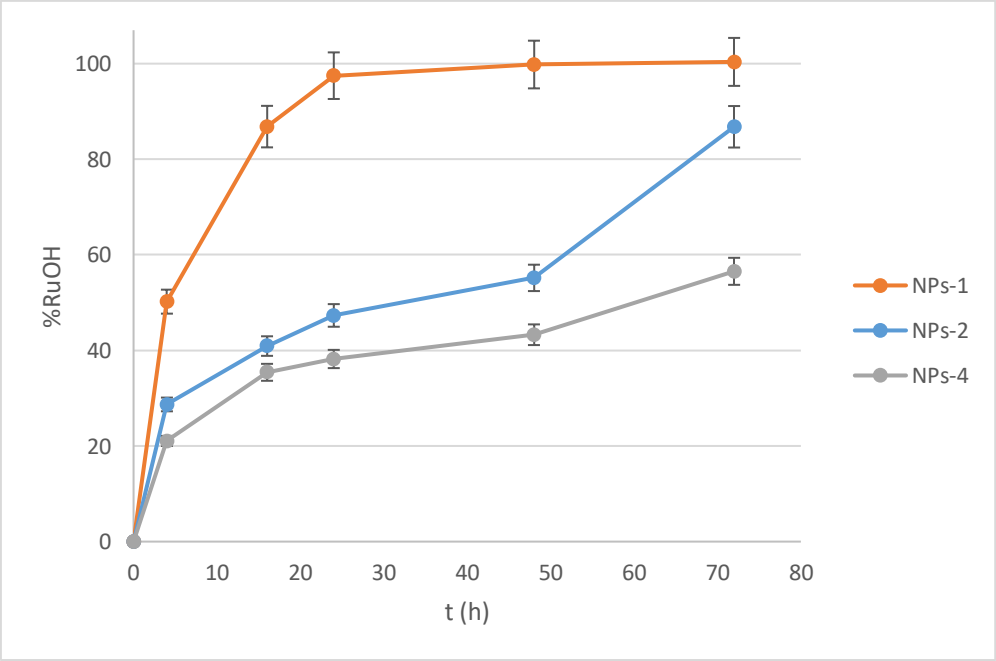


Figure S13. RuOH release kinetics from **NPs-1,2,4** in PBS (1 X, pH = 7.0 – 7.2) at 37 °C overtime, with an estimated measurement error of 5 %.

Table S3. Cytotoxicity data[a] for **NPs-1,2,3,4** and **RuOH** (μM) in HeLa and RPE-1 cells. Light treatment at 480 nm (10 mins, 3.21 J cm^{-2})

	HeLa 4 hrs			RPE-1 4 hrs			HeLa 24 hrs			RPE-1 24 hrs			HeLa 48 hrs			RPE-1 4 8hrs		
	Light	Dark	PI[b]	Light	Dark	PI[b]	Light	Dark	PI[b]	Light	Dark	PI[b]	Light	Dark	PI[b]	Light	Dark	PI[b]
NPs-1	28.0 ± 3.2	> 100	3.6				18.7 ± 3.6	> 100	5.9				12.7 ± 3.3	43.4 ± 17.8	3.4			
NPs-2	34.2 ± 17.4	> 100	2.9	> 100	> 100		14.5 ± 6.3	> 100	6.9	98.2	> 100		23.4 ± 3.8	61.4 ± 17.9	2.6	53.6 ± 17.6	> 100	1.9
NPs-3	41.3 ± 4.5	> 100	2.5	> 100	> 100		9.5 ± 1.1	> 100	11.25	> 100	> 100		8.4 ± 4.3	62.9 ± 13.4	7.5	76.6 ± 17.5	>100	1.3
NPs-4	16.7 ± 4.3	> 100	6				14.5 ± 6.3	81.3 ± 9.1	10.9				4.4 ± 0.8	31.8 ± 7.1	7.5			
RuOH	> 500	> 500		> 500	> 500		274.4 ± 70.1	> 500	1.8	465.7 ± 85.6	> 500		99.1 ± 12.7	248.6 ± 37.7	2.5	114.5 ± 11.5	234.4 ± 18.7	

[a] IC_{50} values were an average of three measurements. [b]PI refers to the phototoxicity index, which is the ratio between the IC_{50} values in the dark and the ones upon light irradiation.

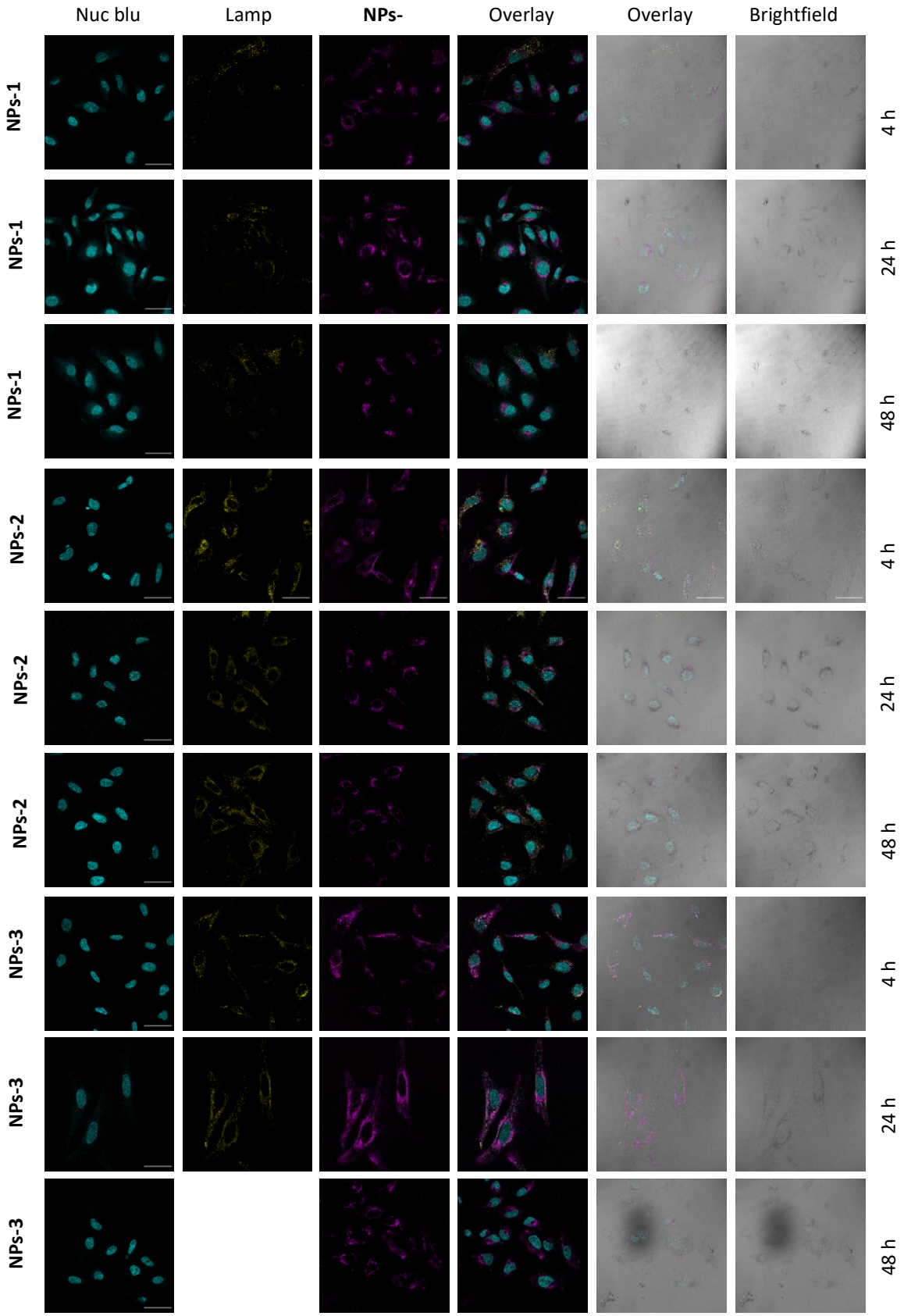


Figure S14a. Confocal microscopy images of HeLa cells incubated with **NPs-** (50 μM, 37 °C, 4, 24, 48 hr). **NPs** co-stained with the nuclear stain, NucBlue, and the lysosomal specific antibody LAMP. Cells were imaged on a LeicaSP8 confocal microscope. Scale equivalent in all images (scale bar 50 μm).

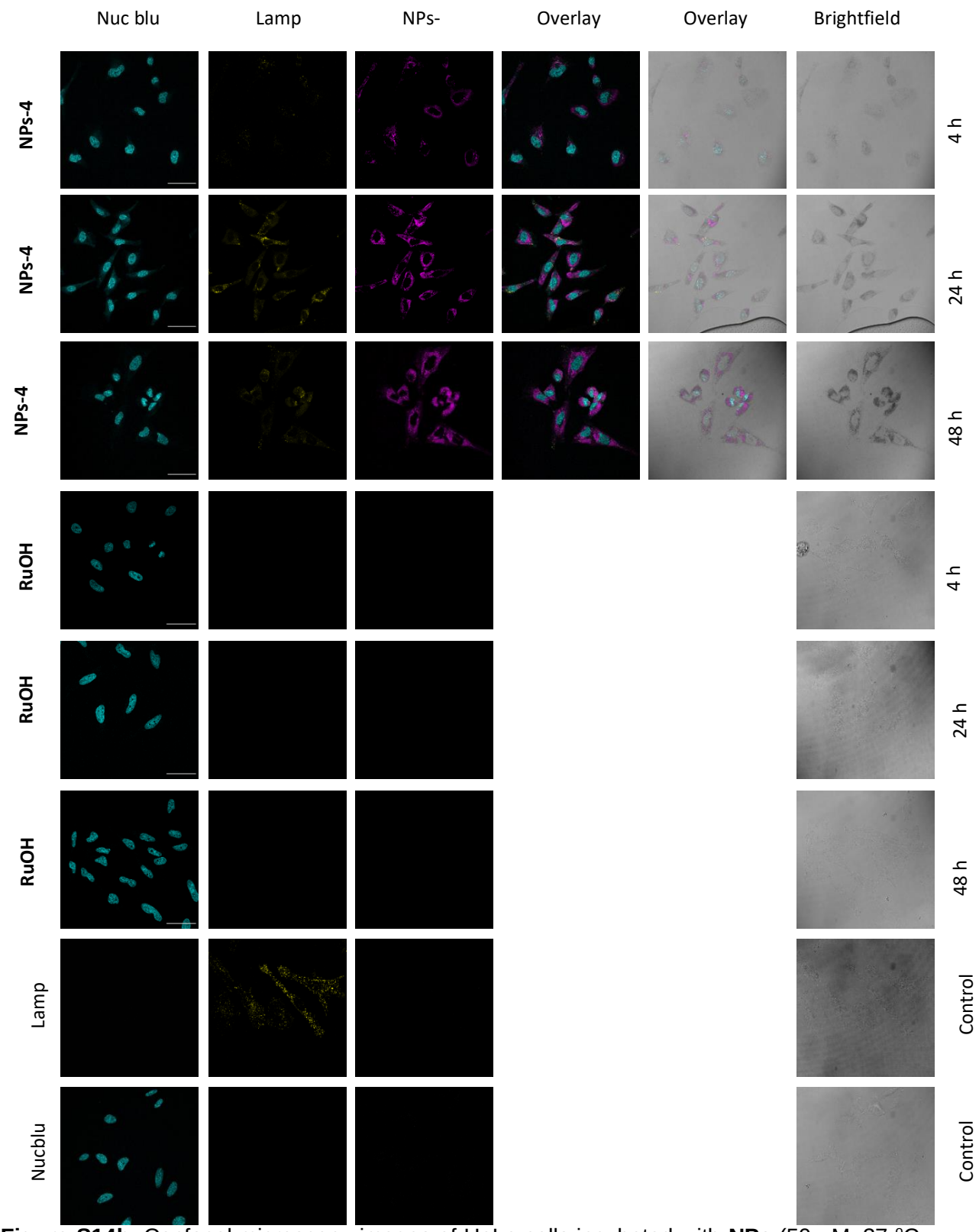


Figure S14b. Confocal microscopy images of HeLa cells incubated with **NPs** (50 μM, 37 ºC, 4, 24, 48 hr). **NPs** co-stained with the nuclear stain, NucBlue, and the lysosomal specific antibody LAMP. Cells were imaged on a LeicaSP8 confocal microscope. Scale equivalent in all images (scale bar 50 μm).

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