SUPPORTING INFORMATION

Functional brush poly(2-ethyl-2-oxazine)s: Synthesis by CROP and RAFT, thermoresponsiveness and grafting onto iron oxide nanoparticles

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Experimental Details

Materials

All commercially purchased chemicals were used as received, unless otherwise stated. Methacrylic acid (99%), anhydrous triethylamine (TEA, ≥99%), anhydrous acetonitrile (AN, 99.8%), barium oxide (97%), anhydrous trimethylbromosilane (97%), 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (>97%), 4-(dimethylamino)pyridine (DMAP, ≥99%), *N,N'*-dicyclohexylcarbodiimide (DCC, 99%) were purchased from Sigma Aldrich. Methyl tosylate (MeOTos, 99%) was obtained from Sigma Aldrich and distilled prior to use. 2-Ethyl-2-oxazine (EtOz) was synthesised as described elsewhere.[1] Dimethyl(2-hydroxyethyl) phosphonate (92%) was purchased from Apollo Scientific Limited. 4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid 2-(dimethoxyphosphonyl)-ethyl ester was synthesized according to literature.[2] Dulbecco's modified Eagles Medium (DMEM) Glutamax^{TM} supplemented with 1mM sodium pyruvate, trypan blue solution (0.4%, liquid, sterile-filtered), and 10% v/v and 20% v/v fetal bovine serum and Alamar blue were purchased from ThermoFisher Scientific. Iron oxide nanoparticles (IONPs, 15 nm, spherical) in chloroform were obtained from Ocean NanoTech. Deuterated chloroform (D 99.8%) and methanol (D 99.8%) were purchased from Cambridge Isotope Labs.

Instruments and Methods

Polymer characterization

^{1}H NMR spectra were recorded using a Bruker UltraShield 400 (400.13 MHz) spectrometer running Bruker Topspin, version 1.3 and operating at 400.13 MHz for ^{1}H, 161.96 MHz for ^{31}P and 100.62 MHz for ^{13}C. Deuterated chloroform ($CDCl_3$) or deuterium methanol (MeOH-d_4) were used as solvents. All chemical shifts are reported in ppm (δ) relative to tetramethylsilane, referenced to the chemical shifts of residual solvent resonance ($CDCl_3$ = 7.26 ppm, MeOH-d_4 = 4.87 ppm). MALDI-ToF MS spectra were recorded in reflection mode on a Bruker Daltonics Autoflex II MALDI-ToF mass spectrometer, equipped with a nitrogen LASER delivering 2 ns laser pulses at 337 nm with positive ion ToF detection performed using an accelerating voltage of 25 kV. The matrix solution was prepared by dissolving super-DBH (2,5-dihydroxybenzoic acid + 2-hydroxy-5-methoxybenzoic acid) in MeOH (200 mg mL^{-1}). Sodium iodide was dissolved in MeOH (4 mg mL^{-1}). Polymer samples were dissolved in MeOH (1 to 5 mg mL^{-1}). Samples were prepared by mixing 5 μL of polymer solution, 5 μL of salt solution and 20 μL of matrix solution. Calibration was performed with a poly(ethylene glycol) methyl ether

methacrylate M_w 1100 g mol^{-1} standard. Size exclusion chromatography (SEC) measurements were conducted using an Agilent 1260 GPC-MDS fitted with differential refractive index (DRI), light scattering (LS) and viscometry (VS) detectors equipped with 2 × PLgel 5 mm mixed-D columns (300 × 7.5 mm), 1 × PLgel 5 mm guard column (50 × 7.5 mm) and autosampler. All samples were passed through 0.2 μm nylon filter before analysis. The mobile phase was DMF containing 5 mM NH_4BF_4 with a flow rate of 1.0 mL/min at 50 °C. SEC data was analyzed using Agilent Technologies SEC Software. Calibration curves were produced using Agilent Easi-Vials linear poly(methyl methacrylate) standards (200 - 4.7×10^5 g/mol). DSC spectra were recorded on a Mettler Toledo DSC1. P1b* was measured using a Shimadzu modular system comprising a DGU-12A degasser, an SIL-20AD automatic injector, a 5.0 μm bead-size guard column (50 x 7.8 mm) followed by three KF-805L columns (300 x 8 mm, bead size: 10 μm, pore size maximum: 5000 Å), a SPD-20A ultraviolet detector, and an RID-10A differential refractive index detector. A CTO-20A oven was used to maintain the columns at 40 °C. *N*,*N*-dimethylacetamide (DMAc) with 0.03% w/v LiBr was used as the eluent where samples were run isocratically at 1 mL min^{-1}. Polystyrene standards (0.5 to 2000 kg mol^{-1}) were used for calibration. Analyte samples were filtered through 0.45 μm PTFE filters before injection. Molar mass ($M_{n,\ SEC}$) and dispersity (Đ) values of samples were determined on Shimadzu LabSolutions software.

Particle characterization

ATR-FTIR measurements were performed using a Shimadzu IRTracer 100 Fourier transform infrared spectrometer with a GladiATR 10 single reflection ATR accessory. The spectra were obtained in the mid infrared region of 4000-600 cm^{-1} at a resolution of 8 cm^{-1} (512 scans) and analysed using LabSolution IR software. Thermogravimetric analysis measurements were performed using a PerkinElmer Pyris 1 TGA and its corresponding Pyris 1 software measuring at a rate of 20 °C min^{-1} from 25 °C to 700 °C. The weight loss percentage was calculated by the difference between the sample weights at 25 °C and at 700 °C. In order to determine the amount of polymer bound to the NPs surface and calculate the grafting density, only the mass loss recorded between 100 °C and 600 °C or 700 °C was considered.

The grafting density was calculated using the following equation according to Morgese *et al.*[3]

$$\sigma = \frac{\text{total number of ligand molecules grafted onto NPs}}{\text{total surface area of NPs}} = \frac{n_{ligand} \cdot N_A}{N_{NPs} \cdot A_{NPs}}$$

Where n_{ligand} is the moles of the ligand, N_{NPs} the number of nanoparticles, N_A the Avogadro constant and A_{NPs} the surface area of the IONPs. The moles of ligand (n_{ligand}) were determined

from the weight loss detected by TGA by dividing the weight loss by the theoretical molecular weight of the POEtOx$_4$MA and PEtOz$_4$MA brushes calculated from the conversion determined by ^{1}H NMR and SEC. The total number of NPs (N_{NPs}) was calculated by dividing the total mass of the inorganic TGA residue by the mass of a single NP. The latter was calculated by multiplication of the density of iron oxide ($\rho = 5.24\ g \cdot cm^{-3}$) with the volume of one IONP, assuming perfectly spherical nanoparticles with a diameter of 15 nm.

TEM measurements were performed using a Tecnai F20 transmission electron microscope at an accelerating voltage of 200 kV at ambient temperature. An aliquot (5 µL) of 0.2 wt% particle solution was deposited on a Formvar coated copper grid (GSCu100F-50, Proscitech) and was allowed to dry overnight in air and at ambient temperature.

In vitro cytotoxicity assays

Cell Culture

An A549 cell-line was used in this study and tested and cleared for mycoplasma. The cells were maintained in Dulbecco's modified Eagles Medium (DMEM) Glutamax™ supplemented with 1mM sodium pyruvate and 10% v/v fetal bovine serum. Cells were cultured at 37 ºC in a humidified incubator with 5% atmospheric CO_2. Cell counting for passaging was done by adding 0.4% Trypan Blue solution to the cells in medium and using a hemocytometer.

Alamar Blue Cell Viability Assay

NIH 3T3 cells were treated with serial dilutions of polymeric samples (from 1 mg/mL down to 0.00781 mg/mL) and cultured for 24 h at 37 ºC in a humidified incubator with 5% atmospheric CO_2. Each sample was run as a triplicate at each concentration to obtain representative cell viability values. Thereafter, the old medium was removed and a 10% v/v solution of Alamar Blue in Dulbecco's modified Eagles Medium (DMEM) Glutamax™ supplemented with 1mM sodium pyruvate and 10% v/v fetal bovine serum was added to the cells. Afterwards, the cells were incubated for 6 h at 37 ºC in a humidified incubator with 5% atmospheric CO_2. The fluorescence was then measured by using an excitation wavelength of 540-570 nm (peak excitation at 570 nm) and reading the fluorescence emission at 580-610 nm (peak emission at 585 nm). Culture medium without cells was used to calibrate the zero absorbance (blank). Cells in control samples were treated with PBS. The cell viability was calculated according to following equation:

$$\text{Viability}(\%) = \frac{\text{Fluorescence(sample)} - \text{Fluorescence(blank)}}{\text{Fluorescence(control)} - \text{Fluorescence(blank)}} \cdot 100\%$$

In vitro hemocompatibility studies

Hemolysis studies were conducted according to the following procedure: 1 mL of heparinised rat blood was gently mixed with 49 mL pre-cooled PBS. The blood was pelleted at 3000 rpm, 10 min, and 4 °C. This step was repeated until the supernatant was clear. The supernatant was removed, and the blood was diluted as 0.05% w/w in PBS with pH 7.4. Polymeric samples (10 µL) in PBS with concentrations of 7.5 mg · mL^{-1} and 1.875 mg · mL^{-1} were added into each well containing 140 µL of red bloods cells in PBS to obtain final polymer solutions of 0.5 mg · mL^{-1} and 0.125 mg · mL^{-1} and incubated at 37 °C for 1 h. Experiments were run in triplicates to obtain representative results. The samples and blood were spun down at 3000 rpm for 10 min. The supernatants were transferred to a new plate for UV-absorption reading of haemoglobin release at 540 nm. The negative control was the blood with no sample added in and the positive control was the blood with 2% Triton-100. The hemolysis was calculated using following equation:

$$Hemolysis(\%) = \frac{Absorption(sample, 540\ nm) - Absoprtion(negative\ control, 540\ nm)}{Absoprtion(positive\ control, 540\ nm) - Absorption(negative\ control, 540\ nm)} \cdot 100\%$$

IONP functionalization procedure

200 µL of a freshly sonicated chloroform dispersion of iron oxide nanoparticles (25 mg mL^{-1}) was diluted with 1.8 mL chloroform and further sonicated. To this a solution containing 50 mg of **P1b*** in 700 µL methanol and 2 mL chloroform was added dropwise. After the addition, the solution was sonicated for 15 min and then incubated in a shaker overnight at 37 °C. Afterwards, the suspension was precipitated in liquid nitrogen cooled diethyl ether and centrifuged for 5 min at 4,000 rpm. The precipitate was redissolved in 20 mL Milli-Q water, passed through a 0.45 µm filter and residual polymer brushes removed via centrifugal washing. To this end, *Vivaspin Protein Concentrator Spin* Columns equipped with 100,000 MWCO polyethersulfone membranes were used. The suspension was washed six times by centrifugation (3,400 rpm)-resuspension cycles.

Magnetic resonance imaging experiments

All MR imaging experiments were performed on a 9.4T high field animal MR scanner (Agilent Technologies, Santa Clara, CA, USA), using a 60 mm volume coil. For the measurements, **P1b*** modified IONPs were diluted and immobilized in 1.7% agarose gel to form a concentration range of complexed Fe_2O_3 ranging from 0.0013 – 0.1625 mM. The T1 maps were measured using an inversion recovery spin echo sequence with FOV = 60x60 mm^2, number of TIs = 8, TI = 10, 22.6, 51, 115, 260, 588, 1330, 3000 ms, TR/TE = 4000/8.93 ms, 128x128

matrix, 3 coronal slices, slice thickness = 1 mm, and 4 averages. The T2 maps were acquired with a spin echo sequence with the following parameters: FOV = 60x60 mm^2, matrix = 128x256, TR = 2000 ms, start TE = 14 ms, number of TEs = 8, 3 coronal slices, slice thickness = 1 mm, and 4 averages. The MRI data was processed using the commercial software VnmrJ (Agilent Technologies, Santa Clara, CA, USA, version 4.2) to generate and draw ROIs on the T1, and T2 maps to obtain the T1, and T2 values. The inverse value was plotted across a range of concentrations to determine the relaxivity values: r1 = 1.02 $S^{-1}mM^{-1}$, *r2 = 218.5* r1 $S^{-1}mM^{-1}$.

Synthesis of macromonomers and polymers

Synthesis of $OEtOz_4MA$ macromonomer (M1)

MeTos (991 µL, $6.545 \cdot 10^{-3}$ mol, 1.00 eq), EtOz (3000 µL, $26.511 \cdot 10^{-3}$ mol, 4.05 eq) and acetonitrile (2.55 mL) were transferred to a pre-dried Schlenk tube under nitrogen atmosphere. The concentration of EtOz was adjusted to 4 mol L^{-1}. The reaction solution was stirred for 7 h at 80 °C. Afterwards, MAA (722 µL, $8.515 \cdot 10^{-3}$ mol, 1.30 eq) was added under nitrogen and the reaction solution stirred for 1 min. Subsequently, anhydrous TEA (1635 µL, $9.820 \cdot 10^{-3}$ mol, 1.50 eq) was added and the reaction solution stirred overnight at 40 °C. Subsequently, the acetonitrile was removed in vacuo, the macromonomer re-dissolved in chloroform and washed three times with saturated aqueous sodium hydrogen carbonate and twice with brine. The aqueous solution was then extracted twice with chloroform. The new organic phase was washed once with saturated sodium hydrogen carbonate solution, once with brine and afterwards combined with the first organic fraction. The combined organic phases were dried over anhydrous magnesium sulphate, filtered and the solvent was removed under reduced pressure to obtain the macromonomer as a yellow viscous oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 6.10 (m, 1H, $=CH_2$), 5.58 (m, 1H, $=CH_2$), 4.16 (m, 2 H, CH_2-COO), 3.30 (m, 14H, N-CH_2-CH_2-CH_2-N), 2.99 (m, 3H, H_3C-N), 2.30 (m, 8H, H_3C-CH_2-CO), 1.95 (m, 3H, CH2-CH-CH_3), 1.74 (m, 8H, N-CH_2-CH_2-CH_2-N), 1.13 (m, 12H, C-CH_3) ppm.

General procedure for the RAFT polymerization of $OEtOz_4MA$

M1, 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid 2-(dimethoxyphosphonyl) ethyl ester and AIBN were dissolved in ethanol ([CTA]:[I] = 4:1) and the mixture was deoxygenated for 20 min with nitrogen. The reaction mixture was stirred at 70 °C and samples were taken under nitrogen over 12 h. The samples were analyzed by ^{1}H NMR spectroscopy and SEC to determine the monomer conversion and molecular weights of the resulting polymers, respectively.

Subsequently, the polymer was separated from unreacted macromonomer by threefold precipitation in liquid nitrogen cooled diethyl ether to be available for further characterization.

Synthesis of P1b* and phosphonate ester cleavage

M1 (1.26 g, 2.28 mmol), 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid 2-(dimethoxyphosphonyl) ethyl ester (29.5 mg, 71.1 µmol) and AIBN (1.17 mg, 7.11 µmol) were dissolved in ethanol (1.77 mL) in a microwave vial, deoxygenated and stirred at 70 °C for 6.5 h. After cooling the solution to room temperature, a sample was taken for ^{1}H NMR analysis and the remaining was diluted with ethanol and precipitated into ice cold diethyl ether. **P1b*** was obtained as a pinkish powder. Conversion: 84%. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 3.92, 3.72, 3.28, 2.98, 2.28, 1.76, 1.10, 0.84 ppm. ^{31}P-NMR (160 MHz, $CDCl_3$): δ = 30.9 ppm.

A solution of **P1b*** in anhydrous DCM (50 mg/mL solution) was degassed and under ice-cooling a solution of trimethylbromosilane (15 eq with regard to polymer) in anhydrous DCM (20 mg/ml solution) was added dropwise. The reaction mixture was stirred for 24 h at room temperature. Afterwards, volatile components were removed under a nitrogen stream. The residue was re-dissolved in methanol (50 mg/mL solution) and stirred for another 24 h at room temperature, after which the solvent was removed under a nitrogen stream. This deprotection-methanolysis cycle was repeated once more to allow for a quantitative cleavage of the phosphonate dimethyl ester groups. The residue was re-dissolved in methanol and three times precipitated in liquid nitrogen cooled diethyl ether to afford the deprotected **P1b*.** ^{1}H-NMR (400 MHz, MeOH-d_4): δ = 3.91, 3.29, 3.22, 3.18, 3.01, 2.90, 2.35, 1.78, 1.01, 0.79 ppm. ^{31}P-NMR (160 MHz, MeOH-d_4): δ = 26.6 ppm.

Figures and Tables

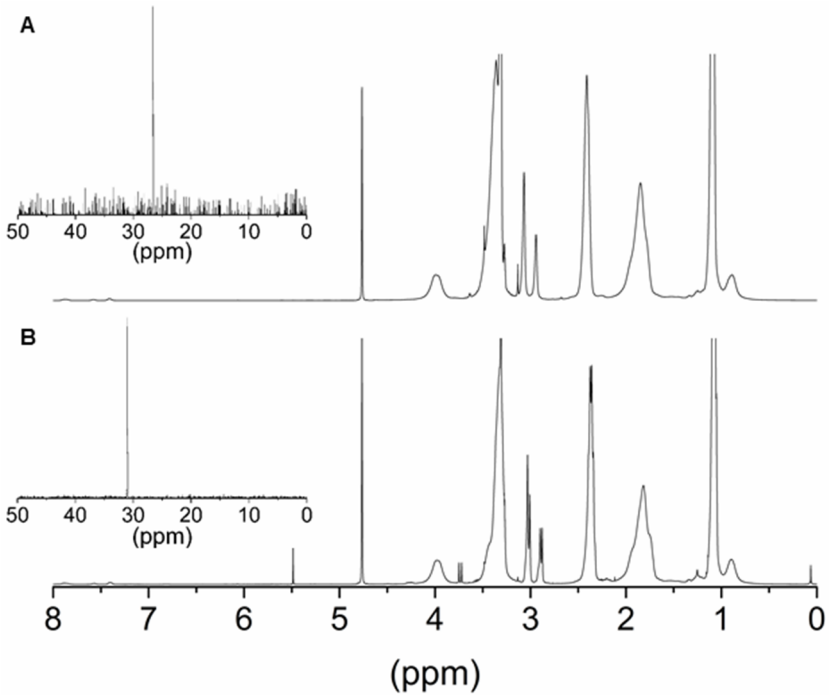

Figure S1. Comparison of ^{1}H and ^{31}P (insets) NMR spectra after (A) and before (B) phosphonate ester cleavage of **P1b***.

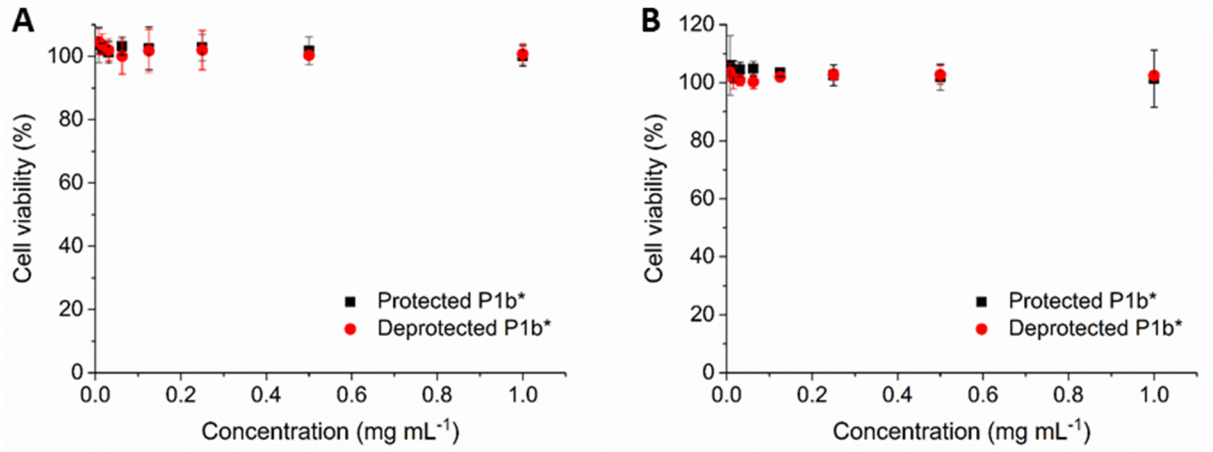

Figure S2. Cell viability of NIH 3T3 cells (A) and N27 cells (B) when exposed to protected and deprotected **P1b***.

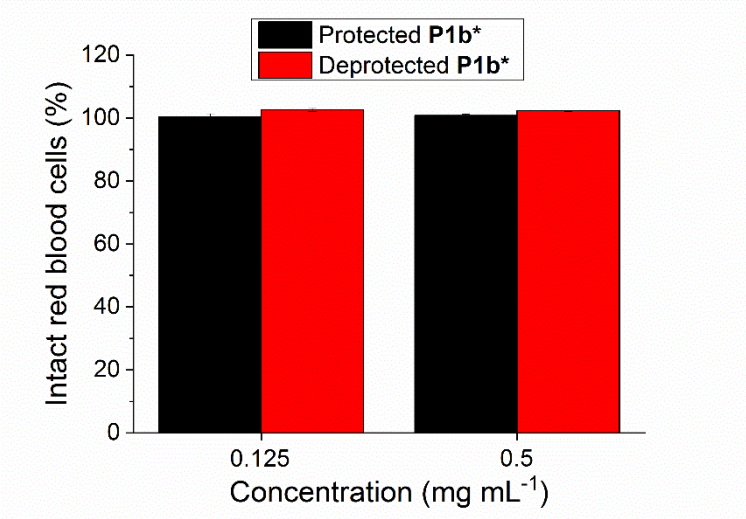

Figure S3. Hemolytic activity of protected and deprotected **P1b***.

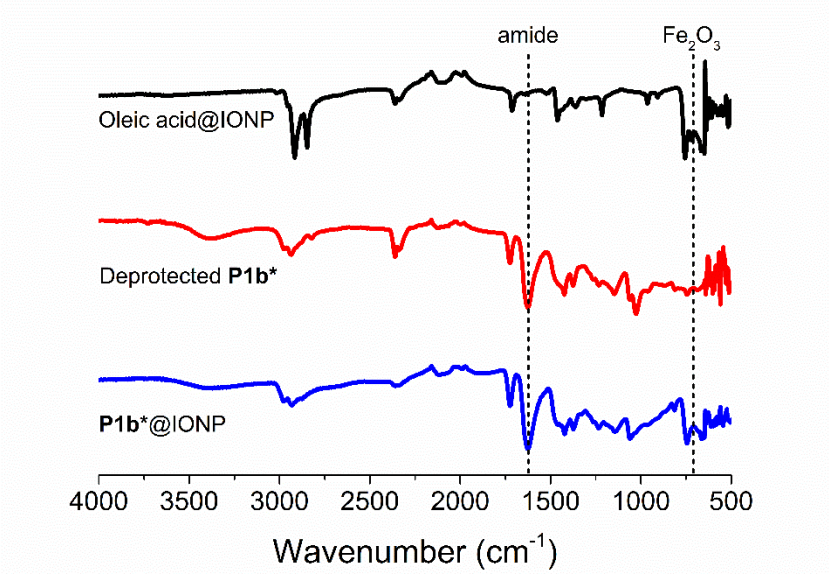

Figure S4. ATR-FTIR spectra of IONP, deprotected **P1b*** and **P1b***@IONPs.

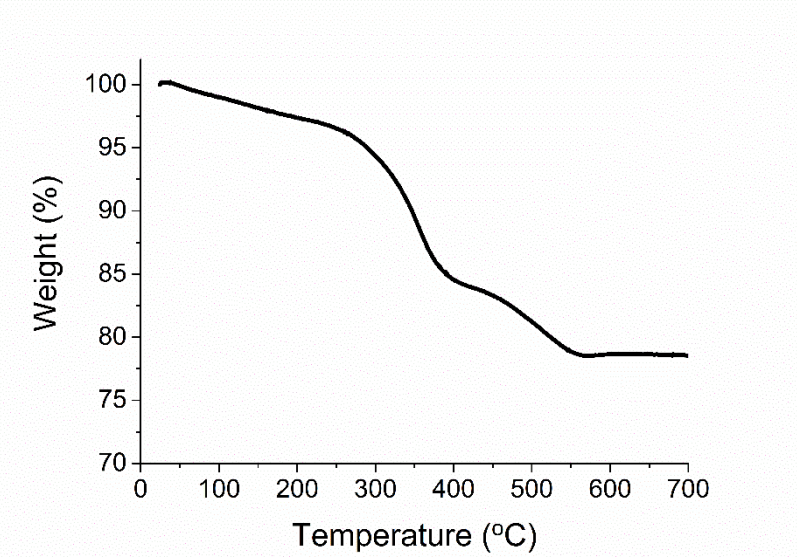

Figure S5. TGA spectrum of **P1b*** @IONPs.

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