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Supporting Information

Synthesis and activation of iron oxide immobilized drug-mimicking reporter under conventional and pulsed X-ray irradiation conditions

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I. General

Thin-layer chromatography was performed on aluminium-backed Merck Kieselgel 60 F_{254} pre-coated plates. Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Bruker 250 spectrometer (250 MHz and 63 MHz) and on a Bruker AV-500 spectrometer (500 MHz and 125 MHz). Chemical shifts for protons are reported in parts per million downfield from tetramethylsilane (TMS) and are referenced to residual proton in the NMR solvent ($CDCl_3$: δ 7.26, CD_3CN: δ 1.94). Chemical shifts for carbon are reported in parts per million downfield from tetramethylsilane and are referenced to the carbon resonance of the solvent ($CDCl_3$: δ 77.16, CD_3CN: δ 118.26). Data are represented as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, dd = double doublet, t = triplet, q = quadruplet, qn = quintuplet, b = broad, m = multiplet), coupling constants in Hertz (Hz). All solvents and inorganic reagents were from commercial sources and used without purification unless otherwise noted. The mass analyzer was an Agilent from ThermoFisher. The capillary tension was 3.5 kV. The cone tension was 24 V. The temperature of the source was 130 °C and the temperature of desolvatation was 350 °C. Data were treated on ThermoQuest.

HPLC analyses were carried out on Waters 515 device with a normal inverse phase column X-Terra® MS C18 (length: 75 mm, diameter: 4.6 mm, stationary phase: 2.5 µm) using a Waters 2487 Dual Absorbance Detector (260-360 nm) and an isocratic system of elution (MeOH-MeCN-H_2O 7-2-1 / H_2O $AcONH_4$ (10 mM) pH 4.6). The volume of injection was 10 µl.

Physico-chemical characterization of the nanomaterial

The iron concentration was determined by a colorimetric assay as described in previous work[1]. The hydrodynamic size and zeta potential of the NPs were investigated by dynamic laser light scattering (DLS), using a Nano-ZS (Red Badge) ZEN 3600 device (Malvern Instruments, Malvern, UK). TEM images were obtained using a FEI Tecnai 12 (Philips), and samples were prepared by depositing a drop of NP suspension on carbon-coated copper grids placed on a filter paper. The grafting of the HMBPyne to the surface of the NPs and compound (1) (1-(2-azidoethyl)-4-(1-((4-(pyren-1-yl)butanoyl)oxy)ethyl)pyridin-1-ium trifluoromethanesulfonate) were studied by Fourier transform infra-red (FTIR) analysis. The FTIR spectra were recorded as thin films on KBr pellets on a Thermo Scientific Nicolet 380 FTIR. Quantification of HMBP-yne coating was evaluated by thermogravimetric analysis (TGA) using a LabsSys evo TG-DTA-DSC 16000 device from Setaram Instrumentation and/or by Energy-dispersive X-ray (EDX) microanalysis using a TM 3000 tabletop microscope equipped with a Swift EDX-ray 3000 microanalysis system (Oxford Instruments). Samples were deposited as powder on a copper surface, and data were collected using a 15 kV accelerating voltage. Quantification of coating was evaluated by studying ratio of iron vs phosphorus and knowing the average number of iron atoms/ particles.
UV spectra were recorded on a Varian Cary 50 Scan UV−vis spectrophotometer. The coupling efficiency of the conjugation onto NP was investigated qualitatively after hydrolysis of pyrene fluorophore using fluorescence measurement recorded on a SpectroFluorimeter Spex FluoroMax (HORIBA Jobin-Yvon, France with a Hamamatsu 98 photomultiplier).

***In vivo* MRI**

A dedicated whole body mouse coil was used for the image acquisitions. A TurboRARE-T2 (RAREmethod) sequence (TE = 9 ms; TR = 3,694,281 ms; NEX = 4 and RARE factor = 8) was used to generate T2 weighted images (Matrix: 256 × 256) with a slice thickness of 1 mm 30 minutes after i.v. USPIO-0.2 injection in B6 mice (at the dose of 200 µmol Fe / kg diluted in 200 µl of vehicle (PBS). Imaging was performed before injection and at different time points after injection (15 min, 45 min, 3h 30 min, and 24 h). The contrast variation C was calculated as $C = (I_t - I_{t=0})/I_{t=0}$ where $I_{t=0}$ is the MRI signal intensity before NP injection and I_t the MRI signal intensity at the time t after injection.

Radiolysis

a) 80 keV X-ray irradiation was performed at the ID17 beamline of the European Synchrotron Radiation Facility (ESRF), Grenoble, France. Irradiated samples were NP suspension (5 µM) in H_2O/EtOH 1/1. At ID17, X-rays produced by a wiggler, are selected in energy using a bent Laue Si(111) X-ray monochromator (bandpass: ~0.8%).[1]

Samples were placed on a remotely controlled sample stage and vertically scanned through the quasi-laminar beam until the delivered dose reached the preset-values (0.3, 3.0, 30.0 Gy). The system was previously calibrated using a standard protocol[2] and the instantaneous dose was monitored by two OKEN gas ionization chambers (OKEN, Japan).

b) A Gamma Cell Biobeam GM8000 facility which is based on Cesium-137 radioactive source was used (BIOBEAM 8000, Gamma-Service Medical GmbH). Prior to NP irradiations, the evaluation of dose profile was determined in specific conditions at oscillation-source mode. Five alanine dosimeters were placed in a tube inside a stainless steel beaker BB13-5 (H x D = 292 x 100 mm) over several heights in the NP irradiation conditions. The aim was to determine the best height to place NP samples to receive dose within the range of ±10%. The measurement of dose rate was 5.86 Gy/min. NPs were irradiated in these conditions at three dose levels: 0.3, 3.0 and 30.0 Gy.

II. Preparation of hydroxyl methylene bisphosphonates (HMBPyne)

(1-Hydroxy-1-phosphonopent-4-ynyl)phosphonic acid (HMBPyne) was synthesized from the condensation of pent-4-ynoyl chloride with tris(trimethylsilyl) phosphite on a gram scale, following a previously describe procedure.[3] Briefly, tris(trimethylsilyl)phosphite (6.7 ml, 20 mmol) was added dropwise at – 20 °C to the previously prepared pent-4-ynoyl chloride (neat) under inert atmosphere. When the addition was completed, the reaction mixture was allowed to stand at room temperature for 4 h. The evolution of the reaction was monitored by ^{31}P NMR. Then, volatile fractions were evaporated under reduced pressure (0.1 Torr) before being hydrolyzed with methanol. After methanol evaporation the product was

dissolved in water at pH 2.3 and lyophilized. The product was then precipitated in a water/ methanol mixture (1 / 9), then the purification was repeated. The sodium salt of HMBPyne was obtained as a white powder (1.9 g, 70 %).

^{1}H NMR (400 MHz, D_2O) δ 2.37 (m, 2H), 2.18 (t, J^{4}_{H-H} = 2.6, 1H), 2.08 (m, 2H). ^{13}C NMR (100.63 MHz, $CDCl_3$) δ 85.8, 73.1 (t, J_{P-CH} = 137.0), 69.2, 32.7, 13.3. ^{31}P NMR (161.98 MHz, $CDCl_3$) δ 17.8. IR (KBr, pH = 7) 3260.8, 2955.4, 2111.2, 1471.7, 1448.0, 1379.0, 1329.7, 1268.6, 1164.1, 1057.7, 945.3, 909.8, 754.6, 738.3, 655.5, 545.1, 450.5 cm^{-1}. HR–MS (ESI-Q Tof) $C_5H_9O_7P_2$: m/z (M–H)-: 242.9824; calc: 242.9830.

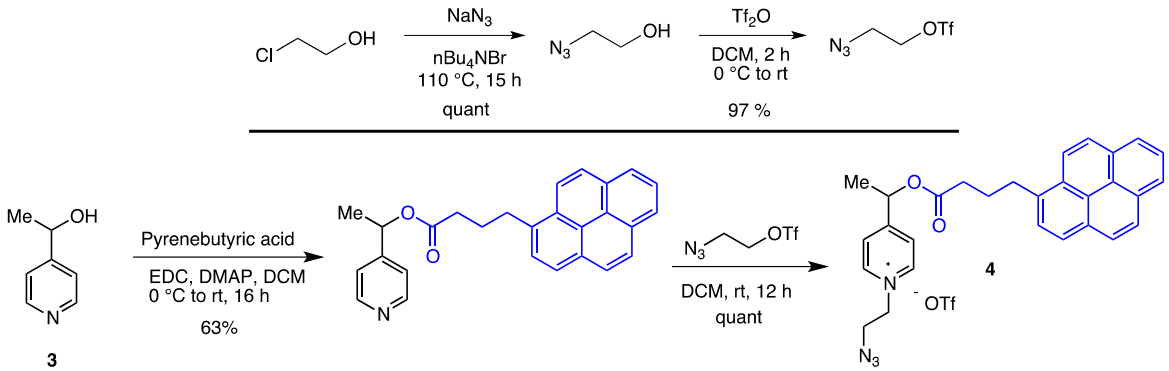
III. Preparation of iron oxide nanoparticles

Iron oxide nanoparticles were synthesised by heating Iron(III) acetylacetonate (1.14 mmol) and benzyl alcohol (10 ml) at 250 °C for 30 min under microwave irradiation.[4] The resulting suspension was separated using a magnet and the precipitate was washed several times with dichloromethane and ethanol. The solid bare nanoparticles were re-suspended in water at pH 2.

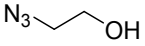
IV. Preparation of nanoparticles coated with HMBPyne

HMBPyne ligand was added to the solution of bare nanoparticles at pH 2 with a mass-ratio HMByne to nanoparticles equal to 2; under these conditions, nanoparticles are positively charged and bisphosphonates are negatively charged. Surface functionalization was realized by stirring the mixture at room temperature for 2 h. Then the pH of the solution solution was set to neutral with NaOH and the excess of free ligands was eliminated by repeating 3 times the ultrafiltration by using DI water.

V. Preparation of the redox probe



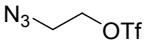
2-Azidoethan-1-ol



A mixture of 2-chloroethanol (2.0 g, 24.8 mmol, 1 eq.), NaN_3 (2.1 g, 32.2 mmol, 1.3 eq.) and $n$$Bu_4NBr$ (200 mg, 0.62 mmol, 0.025 eq.) was stirred for 15 h at 110 °C. After cooling, the product was taken up with Et_2O (20 ml) and the precipitate of NaCl, remaining NaN_3 and the phase transfer catalyst was filtered off. The salts were washed with Et_2O (20 ml). The solvent was then removed under reduced pressure and the crude was obtained as a colorless liquid (2.15 g, 24.7 mmol, quant.).

^{1}H NMR ($CDCl_3$, 250MHz): δ 3.80 (2H, t, *J* = 4.75 Hz), 3.46 (2H, t, *J* = 5.25 Hz), 2.30 (1H, bs). ^{13}C NMR ($CDCl_3$, 63MHz): δ 61.4, 53.4. MS (ESI): *m/z* =88.0 $[M+H]^+$.

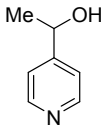
2-Azidoethyl trifluoromethanesulfonate



Triflic anhydride (1.56 g, 5.52 mmol, 1.2 eq) was added at 0 °C to a solution of 2-azidoethan-1-ol (0.40 g, 4.6 mmol, 1 eq) in DCM (4 ml). The mixture was stirred for 2 h at rt then washed with water (3 x 15 ml). The organic layer was washed with brine (10 mL), dried on Na_2SO_4 and concentrated (0.97g, 4.47 mmol, yield 97%).

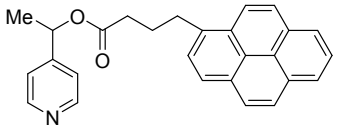
^{1}H NMR ($CDCl_3$, 500MHz): δ 4.63 (2H, t, *J* = 3.5 Hz), 3.71 (2H, t, *J* = 5 Hz). ^{13}C NMR ($CDCl_3$, 63MHz): δ 61.7, 49.1.

1-(Pyridin-4-yl)ethan-1-ol (3)[5]



In a two neck–round bottom flask under argon the corresponding pyridine carboxaldehyde (4 mmol) was dissolved in anhydrous THF. At 0 °C methylmagnesium bromide (3.0 M in diethyl ether, 3 eq.) was slowly added and the mixture was then stirred at rt for 4 hours. After quenching by adding a saturated solution of NH_4Cl under cooling with ice, the reaction solution was extracted with DCM, the organic layer was washed with brine and dried over anhydrous sodium sulfate. The crude product was purified by column chromatography on silica gel (Cyclohexane/EtOAc: 4/1) to obtain **3** as a yellow oil (420 mg, 85%).
^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.54 (2H, d, *J* = 6.6 Hz), 7.31 (2H, d, *J* = 3.8 Hz), 4.92 (1H, m, *J* = 3.8 Hz), 1.59 (3H, d, *J* = 3.8 Hz).

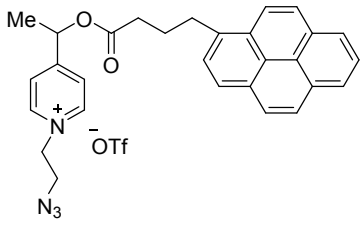
1-(Pyridin-4-yl)ethyl 4-(pyren-1-yl)butanoate



In a two-necked flask under argon, 1-(pyridin-4-yl)ethan-1-ol (0.20 g, 1.22 mmol, 1.1 eq.) and 4-(1-pyrenyl)butyric acid (320 mg, 1.10 mmol, 1 eq.) were dissolved in DCM (4 ml). At 0 °C EDC·HCl (0.42 g, 2.2 mmol, 2 eq.), and DMAP (cat) were added and the reaction mixture was allowed to stir for 24 h. The solvent was removed via rotary evaporation and the crude was purified on silica gel column (DCM / MeOH, 9 / 1) to obtain 1-(pyridin-4-yl)ethyl 4-(pyren-1-yl)butanoate (0.27 g, 0.7 mmol, yield: 63%).

^{1}H NMR ($CDCl_3$, 500MHz): δ 8.57 (2H, dd, *J* = 4.5, 1.5 Hz), 8.28-8.25 (1H, m), 8.17 (2H, d, *J* = 8 Hz), 8.10 (2H, t, *J* = 8 Hz), 8.03 (2H, s), 8.00 (1H, t, *J* = 7.5 Hz), 7.85 (1H, m), 7.23-7.22 (2H, m), 5.86 (1H, q, *J* = 6.5 Hz), 3.40 (2H, t, *J* = 7.5 Hz), 2.54-2.50 (2H, m), 2.22 (2H, qn, *J* = 6.5 Hz), 1.52 (3H, d, *J* = 6.5 Hz). ^{13}C NMR ($CDCl_3$, 125MHz): δ 172.6, 150.6, 150.3, 150.2, 135.6, 131.6, 131.0, 130.2, 128.9, 127.6, 127.5, 127.0, 126.0, 125.3, 125.1, 125.0, 123.3, 122.0, 120.8, 70.9, 34.1, 32.8, 26.8, 26.7, 22.11. MS (ESI): *m/z* =394.1 $[M+H]^+$. HRMS (ESI): m/z calcd for $[C_{27}H_{23}O_2N]^+$ 393.1729, found 393.1735

1-(2-Azidoethyl)-4-(1-((4-(pyren-1-yl)butanoyl)oxy)ethyl)pyridin-1-ium trifluoromethanesulfonate (4)



1-(Pyridin-4-yl)ethyl 4-(pyren-1-yl)butanoate (0.2 g, 0.5 mmol, 1 eq) and 2-azidoethyl trifluoromethanesulfonate (0.14 g, 0.66 mmol, 1.3 eq) were dissolved in DCM (1 ml) and stirred at rt for 16 h (shielded from light). After evaporation to dryness the crude product was purified on column of silica gel (DCM/MeOH 95/5) to obtain **4** (0.24 g, 0.53 mmol, quant).

^{1}H NMR ($CDCl_3$, 500MHz): δ 8.69 (2H, d, *J* = 6.5 Hz), 8.25 (1H, d, *J* = 9 Hz), 8.17 (2H, d, *J* = 8 Hz), 8.11-8.09 (2H, m), 8.03-7.98 (3H, m), 7.84 (1H, d, *J* = 8 Hz), 7.64 (2H, d, *J* = 6.5 Hz), 5.7 (1H, q, *J* = 6.8 Hz), 4.69-4.66 (2H, m), 3.93 (2H, t, *J* = 5.5 Hz), 3.48-3.40 (2H, m), 2.55 (2H, t, *J* = 7.5 Hz), 2.24 (2H, qn, *J* = 7.5 Hz), 1.65-1.55 (2H, m), 1.46 (3H, d, *J* = 6.5 Hz). ^{13}C NMR ($CDCl_3$, 125MHz): δ 172.2, 161.9, 145.2, 135.2, 131.5, 131.0, 130.2, 128.9, 128.0, 127.7, 127.6, 127.0, 126.2, 125.7, 125.2, 125.1, 125.0, 124.6, 123.3, 69.9, 60.5, 50.7, 33.8, 32.7, 26.4, 21.7. MS (ESI): *m/z* =463.4. HRMS (ESI): m/z calcd for $[C_{29}H_{27}O_2N_4]^+$ 463.2129, found 463.2137

Compound **1** was characterized by IR spectroscopy (KBr pellets). Distinctive peaks were assigned as the azide stretching (2150 cm^{-1}), ester bond stretching (1750 cm^{-1}), and pyrene

aromatics (845 cm^{-1}), respectively. The UV spectra of **1** measured in acetonitrile / H_2O (1 / 1) solution, shows a first absorption maximum situated at 338 nm, attributed to the presence of pyrene, with molar extinction ε^{max} = 32,560 $M^{-1}cm^{-1}$.

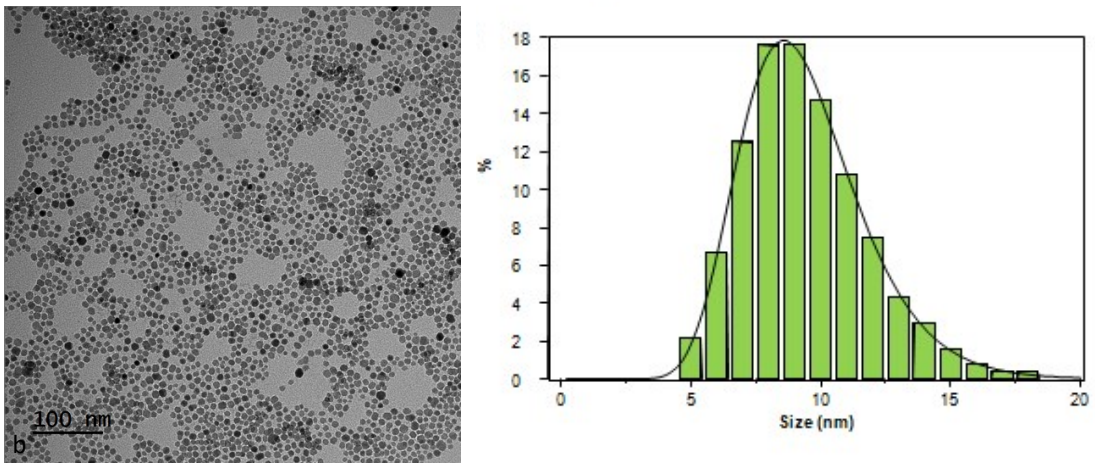
VI. Coupling of the redox probe (4) with HMBPyne-coated iron oxide nanoparticles

The synthesis of the immobilized compound (**4**) was realized *via* copper(I)-catalyzed azide/alkyne cycloaddition (CuAAC), in the presence of a catalytic amount of CuI (0.1 eq.) at rt for 24 h. The reaction was performed in acetonitrile / water (1/1) mixture, in a 0.2 molar ratio of azide to alkyne in a way that 1 mg/ml solution of HMBPyne-coated iron oxide nanoparticles (1 equiv.) was reacted with 0.2 equiv. of azide. The azide was used in sub-stoichiometric amount in order to avoid saturation and subsequent particle aggregations that may be driven by the π–π interaction of the pyrene. Reaction mixtures were stirred at rt for 24 h in the dark. The obtained NPs were washed several times with water (deionized) and then re-dispersed at pH 7.4 in water.

VII. Evaluation of the average number of pyrene butyric acid per nanoparticles

The average number of pyrene per nanoparticle was determined saponification of the coupled product using alkaline medium (pH 12, 24 h). Particles were separated from the supernatant by magnet-sorting. The pH of the supernatant was adjusted to 7.4 and the pyrene concentration was correlated by using fluorescence calibration curve (λ_{ex} = 341 nm and λ_{em} = 376 nm, Figure SI6).

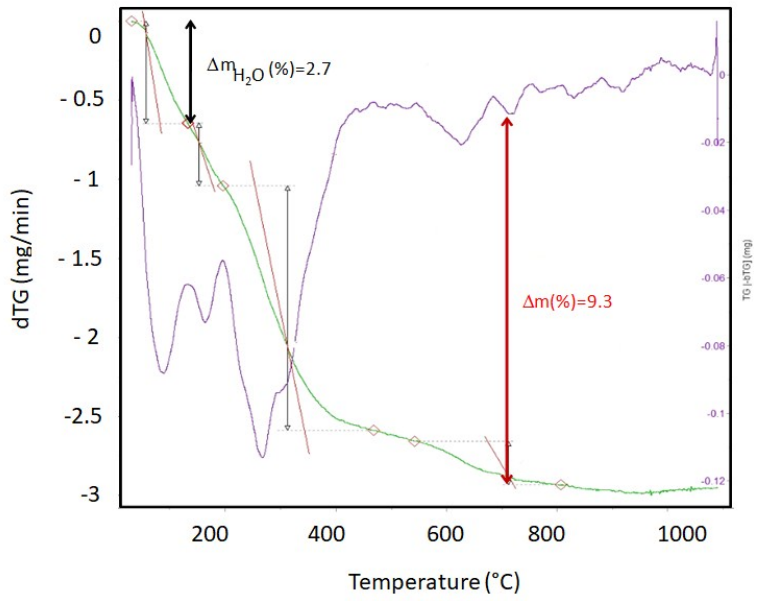
VIII. Figures and table



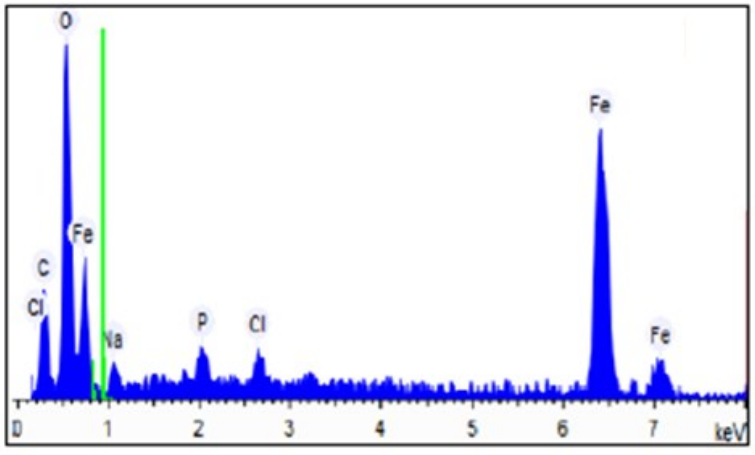
Figures SI1. TEM size distribution histogram counted on 500 nanoparticles (green bare) and corresponding log-normal fit (black curve).

	D_H (nm)	Zeta potential (mV)
USPIO–yne NPs	17	- 30
USPIO 0.2 NPs	18	- 29

Table SI1. Hydrodynamic size and zeta potential of USPIO-yne NPs before and after coupling with 0.2 equiv. of compound **1**. D_H: Hydrodynamic diameter.



A



B

Figure SI2: The TGA (A) and EDX analysis (B) of USPIO-yne NPs

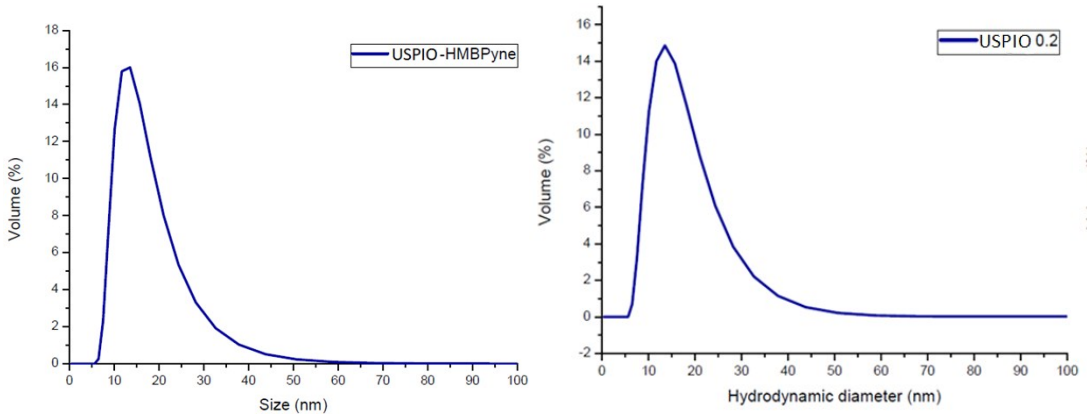


Figure SI3. Size distributions (in volume) of USPIO-yne and USPIO-0.2

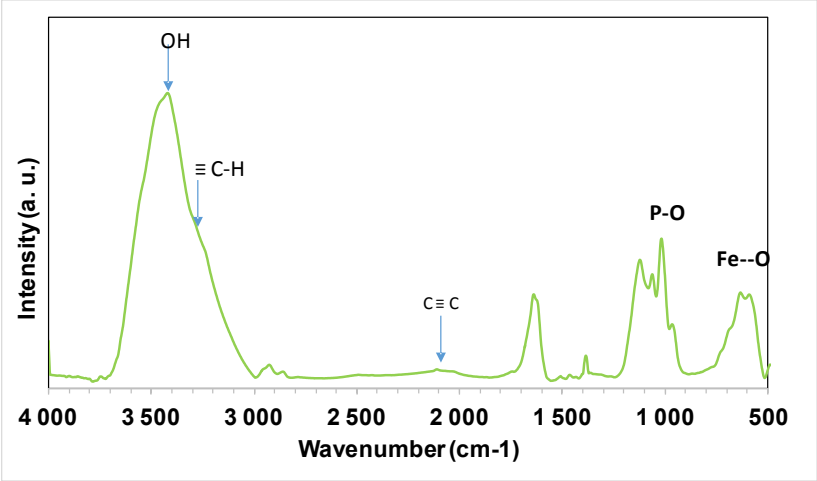


Figure SI4. FTIR spectrum of USPIO-yne NPs (KBr). The C-H stretch of the akyne is observed at the 3260-3330 cm^{-1} region and the C-C triple bond (weak) at 2100-2260 cm^{-1}.

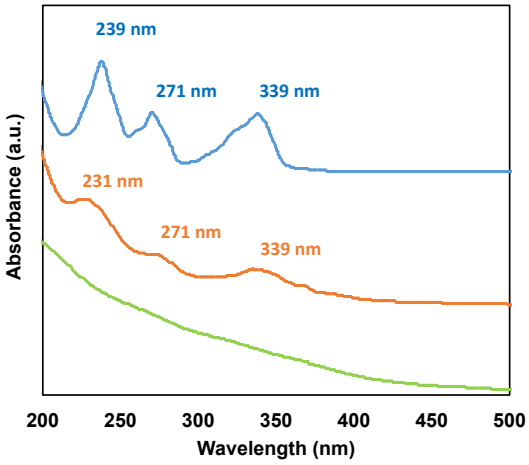


Figure SI5. UV spectra of USPIO-yne NPs (green) and USPIO-0.2 (orange) in water at pH 7 and of picolinium azide, **4**, measured in acetonitrile / H_2O (1/1) solution (blue). The three absorption maxima at 239, 271 and 339 nm of picolinium azide originate from the localized excitation of the pyrene.

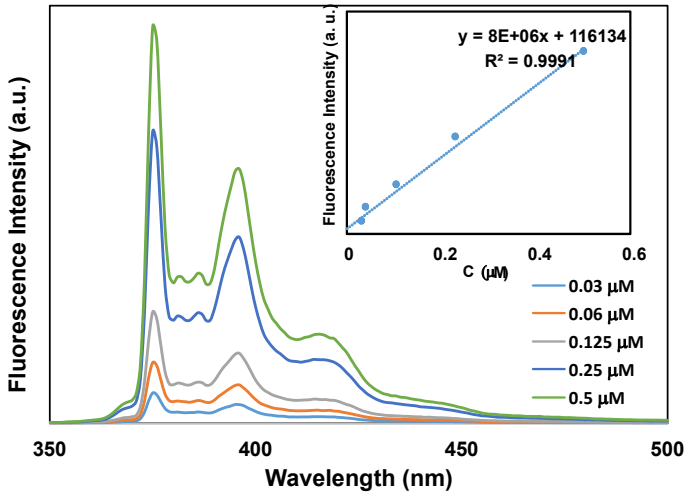
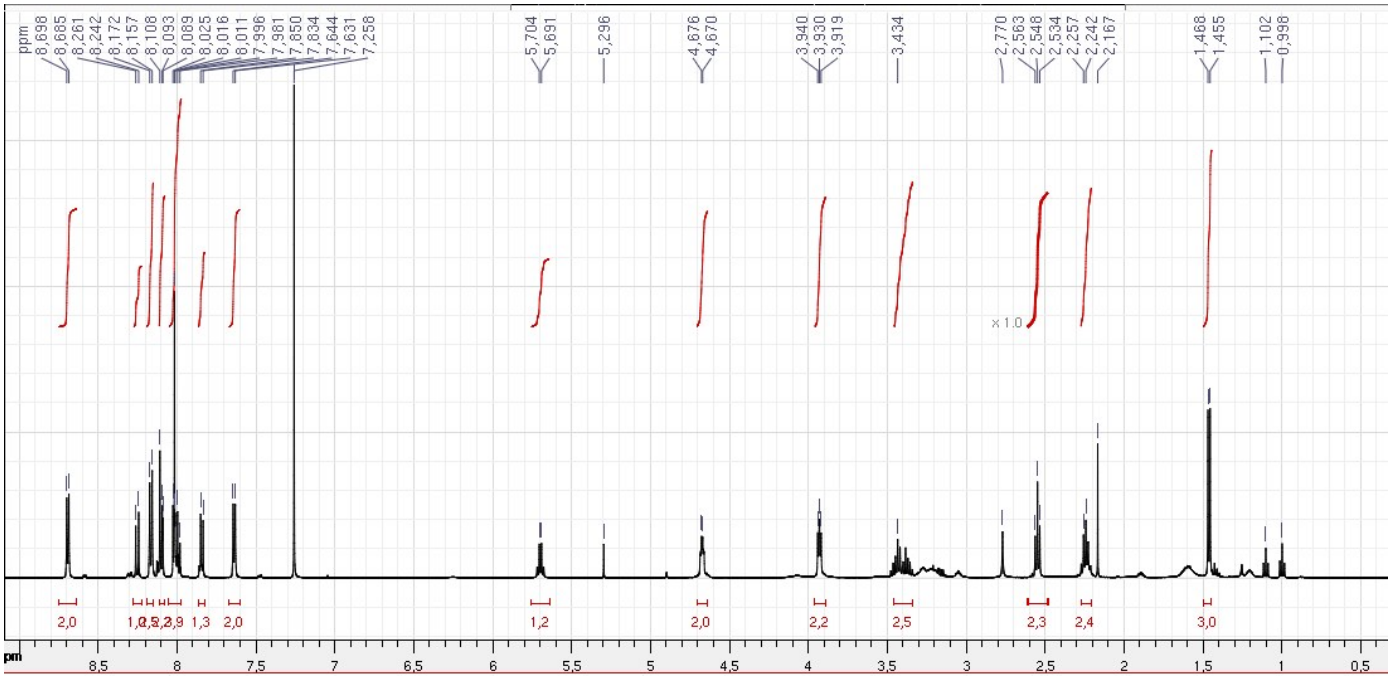
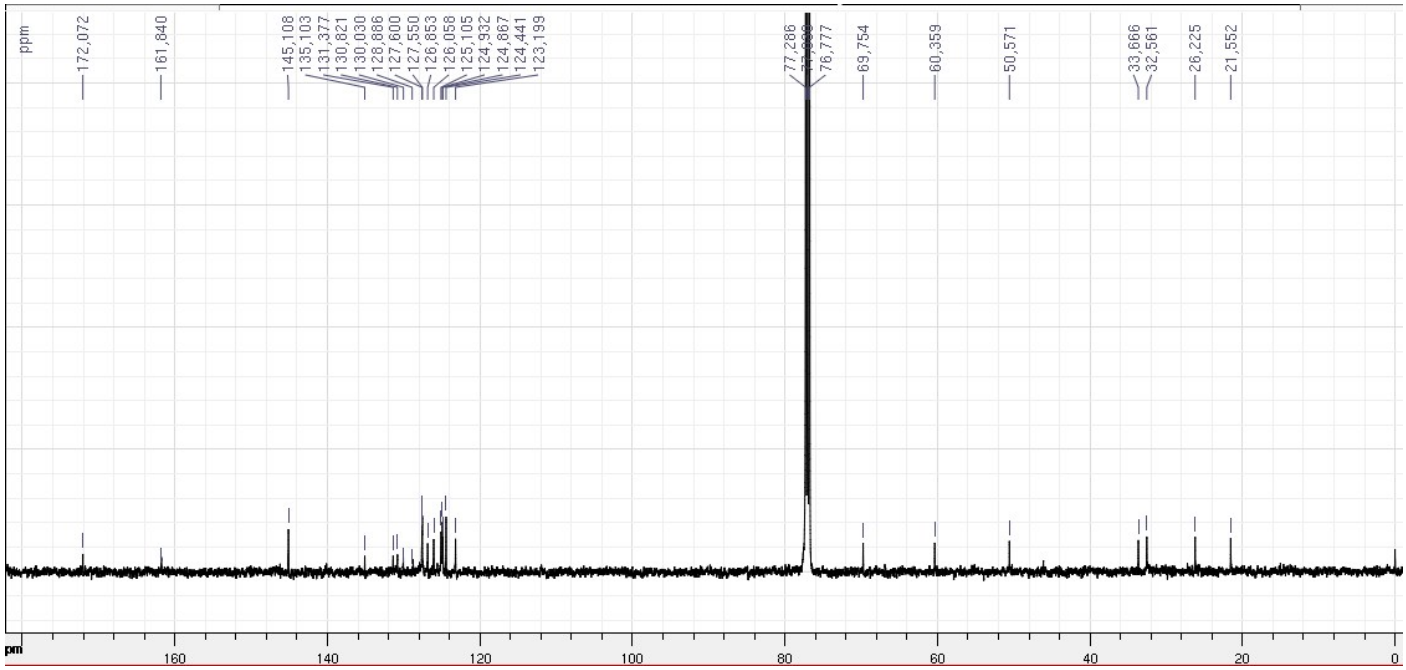


Figure SI6. Fluorescence emission spectra of pyrene butyric acid at various concentrations in water (λ_{ex} = 341 nm, pH 7), insert calibration curve using 376 nm emission band.

IX. NMR spectra of 1





X. References

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