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Nanocrystals of Zn(Fe)O-based diluted magnetic semi-conductor as potential luminescent and magnetic bimodal bioimaging probes

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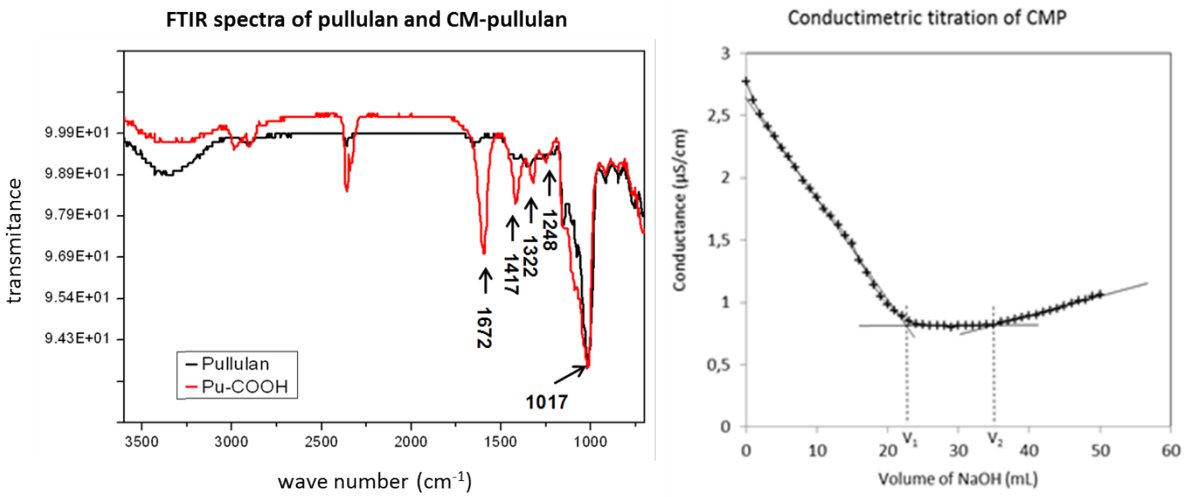
I- Preparation of the nanocrystals

Particle synthesis

NanoDMS were prepared by the polyol method. Typically, the appropriate amount of zinc acetate dihydrate ($Zn(Ac)_2 2H_2O$), iron acetate ($Fe(Ac)_2$) and distilled water were successively introduced into 80 mL of polyol solvent (diethylene glycol (DEG)), sonicated for 30 min. The total amount of (metal Zn and dopant Fe) was fixed at 0.5M. The as-obtained mixture was then heated under reflux of the solvent (245 °C). After 6 h of reaction, the mixture was cooled to room temperature and the precipitate was separated from the supernatant by centrifugation (8000 rpm for 15 min), washed several times with ethanol and acetone, and then dried under vacuum at 50 °C.

Carboxymethylation of pullulan (CMP)

Carboxymethyl Pullulan (CMP) was prepared according to this process: one gram of pullulan (Hayashibara, Japan) was mixed with 1.35 g of powdered sodium hydroxide, followed by the addition of 8 mL propan-2-ol and 2 mL water. It was then mixed with a solution containing 1.5 g monochloroacetic acid in 2 mL propan-2-ol. The carboxymethylation of pullulan occurred under stirring (200 rpm) at room temperature for 4 h. The reaction was then stopped by adding 50 mL 70% ethanol-water (v/v) to the mixture and CMP was separated by filtration, washed four times with 70% ethanol/water (vol/vol) and diethylether before drying overnight at 50 °C under vaccum to give a white powder in a 70 % yield. ESI-fig 1 presents the FTIR spectra of pullulan and CM-pullulan and the conductimetric determination of the content of carboxymethyl groups.



ESI-Figure 1: FTIR spectra of pullulan and CM-pullulan, and conductimetric determination of CM content of CM-pullulan.

FT-IR spectra were recorded on a ThermoNicolet IR (ThermoNicolet, Paris, France). Absorption peaks were evidenced for CMP at 1672 cm^{-1} and 1417 cm^{-1} related to stretching and bending vibrations of the carbonyl of the carboxymethyl groups respectively. Note that stronger absorption bands were also observed at 2850-3000 cm^{-1} corresponding to C-H streching of methylene groups.

The degree of substitution (DS) of carboxymethylated groups (CM), which is the relative number of CM per anhydroglucose unit of pullulan, was obtained by conductimetry (Eutech Instruments, Thermo Fisher, France). Briefly, about 0.1 g of CMP acutely weighed was dissolved in 100 mL of 0.05 M HCl and the pH was adjusted to about 2.0 with diluted NaOH. The solution was then titrated with 0.030 M NaOH. The typical titration curve is shown in ESI-Fig. 2. The first part of the curve (below V_1) corresponds to the neutralization of H^+ ions coming from the strong acid initially added, the plateau observed just after (V_1 to V_2) is due to the neutralization of the carboxylic groups, while the final increase in conductivity (above V_2) corresponds to an excess amount of NaOH. From V_2-V_1. The average amount of CM from 6 titrations was 3.62±0.08 $mmol.g^{-1}$ corresponding to a DS(CM) 0.84±0.05.

Carboxymethyl pullulan coated diluted semi-conductor (CMP-nanoDMS)

The nanocrystals were coated with CMP (MW=75,000 $g.mol^{-1}$). CMP-nanoDMS were carried out using 0.25 g of nanoDMS and 0.50 g of CMP. The precursors were introduced into 50 mL of distilled water solvent and the mixture was heated to 60°C. After 2 h of reaction, the as prepared nanohybrides were separated from the supernatant by centrifugation (8000 rpm for 15 min), washed several times with distilled water, and then dried under vacuum at 50 °C.

Colloid preparation

Nanocrystals were weighed to prepare a suspension of 0.625 mg/mL in PBS by sonication for 1 hour. The pH was adjusted to 2.5 with a few drops of 1M HCl. After 24h, the suspension was neutralized with 1M NaOH. The colloidal suspension was kept at 4°C.

II- Characterization of nanocrystals

Chemical analysis

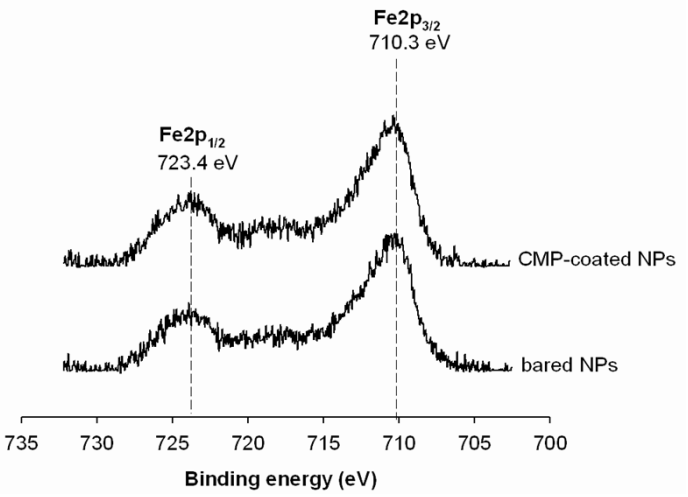
The chemical composition of the powders was obtained from suspension (0.5M) by atomic absorption spectroscopy (AAS) with a Perkin-Elmer Analyst 100 apparatus (Prekin-Elmer, USA) after degradation of the nanocrystals in boiling HCl (35%).

X-ray photoelectron spectroscopy (XPS)

The particles were also characterized by X-ray photoelectron spectroscopy (XPS - PHI 5600-ci spectrometer, Physical Electronics U.S.A., MN, U.S.A.) using a standard X-ray source for survey spectra (0−1400 eV), while high resolution spectra of C1s, O1s, Fe2p and Zn2p were obtained by using a standard magnesium X-ray source (1253.6 eV). For neither analysis was applied charge neutralization. The detection was performed at 45° with respect to the surface normal and the analyzed area was 0.016 cm^2. The spectrometer work function was adjusted to give 285.0 eV for the main C (1s) peak. Curve fittings for both the survey and the high resolution C (1s) peaks were determined by means of least squares minimization procedure employing Gaussian-Lorentzian functions and a Shirley-type background.

	%C1s [285.0 eV C-C/C-H] (286.5 eV C-O) {288.6 eV O-C=O}	%O1s [529.5 eV O-Metal] (531.3 eV Metal-OH or CO-Metal) {532.9 eV O-C}	%Fe2p3 [710.2 eV Fe^{2+}]	%Zn2p3 [1021.1 eVZnO]	C/O	Fe/Zn	O-Metal /(Fe+Zn)
DEG-nanoDMS	20.7 [11.0%] (4.5%) {5.2%}	52.0 [29.4%] (17.4%) {5.2%}	9.6 [9.6%]	17.7 [17.7%]	0.398	0.542	1.077
CMP-nanoDMS	22.2 [11.1%] (6.5%) {4.6%}	52.3 [26.9%] (23.4%) {2.0%}	9.1 [9.1%]	16.4 [16.4%]	0.424	0.555	1.055

ESI-Table 1: Nanocrystals composition deduced from XPS analyses.



ESI-Figure 2 : High resolution spectra of Fe2p3 on bared and CMP-coated Nps.

X-ray diffraction

The powders were analyzed by X-ray diffraction on a Panalytical X'pert Pro diffractometer (INEL, Paris, France) equiped with a multichannel X'celerator detector, using Cu Kα radiation (k = 1.54187 angstrom) in the 2θ range 10–100.

Transmission electron microscopy (TEM)

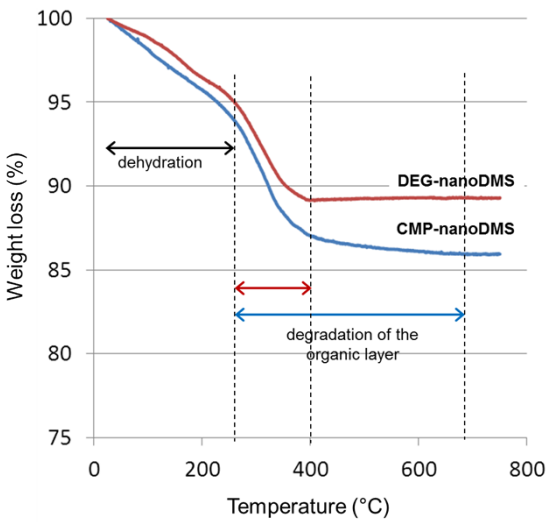
The morphology of the nanocrystlas was studied by transmission electron microscopy (TEM) performed on a JEOL 2100F microscope (JEOL, Paris, France) operating at 200 kV. A drop of a suspension of the powder in ethanol was deposited on a carbon grid. The particle size was obtained from the recorded TEM images using a digital camera and the SAISAM software (Microvision Instruments, Paris, France) by calculating the surface average particle diameter through a statistical analysis from about 200 particles considered to be spherical.

Particle size analysis and isoelectric point analysis

The hydrodynamic diameter of particles in PBS (0.5M Zn) and the zeta potentials were measured by dynamic light scattering (DLS), using a Malvern DTS Nano zetasizer 173° (Malvern). Zeta potential dependence on pH was obtained by measuring the zeta potential in aqueous solution by adjusting the pH value by the addition of HCl and NaOH (1 M).

Thermogravimetric analysis (TGA)

The mass fraction of organic coating on the crystals was measured by thermogravimetric analysis (TGA) in the range of temperature 20–800 °C, using a Mettler Toledo TGA 851 apparatus (Mettler Toledo, Canada) (nitrogen flow: 40 mL/min; heating rate: 10 °C min-1, from 25 °C to 800 °C). DEG and CMP-coated nanoDMS exhibited a two steps overall weight loss of 10% and 15% respectively which can be ascribed to the departure of the adsorbed water (up to about 260°C) and to the decomposition of the organic polyol or polymer adsorbed on the surface (up to 400°C and 700°C for nanoDMS and CMP-nanoDMS respectively).



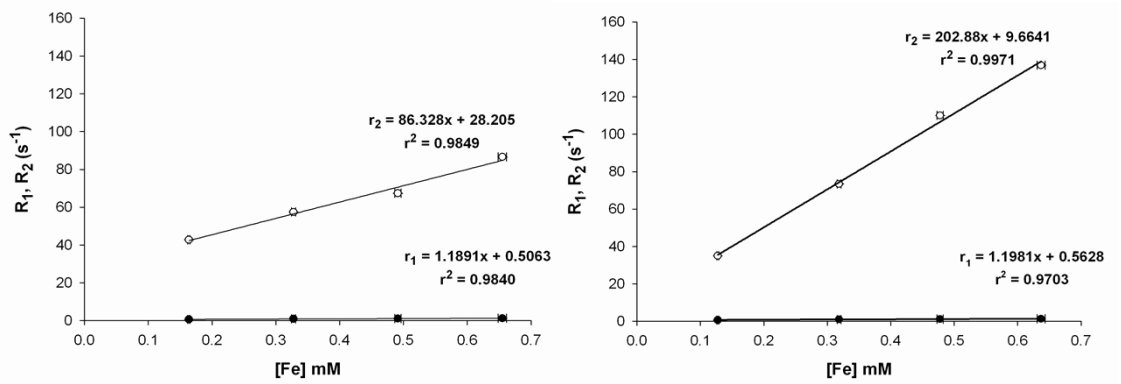
ESI-Figure 3: Thermogravimetry (TG) curves of CMP-nanoDMS by comparison with the DEG-coated ones. The red and blue double arrows depict the weight loss domains of DEG and CMP-coated nanocrystals respectively.

Optical measurements

The optical properties of nanocrystals were investigated at room temperature. The excitation source at 366 nm was provided by a YAG:Nd quadrupled frequency laser. The emission was analyzed by using a HR250 monochromator (Jobin-Yvon, Longjumeau, France) coupled with a UV-enhanced intensified charge coupled device (Roper scientific, Lisses, France). Under pulsed laser excitation, luminescence spectra were recorded in a pseudo CW mode with a continuous integration of the intensity during 300 ms corresponding to three full illumination pulses. The excitation power and the geometrical arrangement of the set-up were the same for all samples.

^{1}H relaxometric measurements

Dilutions of DEG and CMP-nanoDMS were prepared in PBS (100, 75, 50, 25% v/v) and distributed in NMR tubes. Then, longitudinal and transverse relaxation times (T1 and T2) were measured at 60 MHz (1.41 T) using a BrukerMiniSpecrelaxometer (Bruker, Germany). The temperature was set to 37°Cfor all measurements. Finally, the relaxation rates (1/T1 and 1/T2) were plotted against Fe concentration values (from GF-AAS), and relaxivities (r1 and r2) were calculated from the slope of the graphs.



ESI-Figure 4: Relaxometric properties of DEG-nanoDMS (left) and CMP-nanoDMS (right)

In vitro contrast media MRI studies

Dilutions of DEG and CMP-nanoDMS were prepared in PBS (75, 50, 25, 10, 7.5, 5, 2.5% v/v) and distributed in 500 µl-tubes immersed in water. The tubes were inserted in a 60-mm RF coil and imaged at 25oC with a 1 T small-animal MRI system (M2M, Aspect Imaging, Netanya, Israel). *T1*-weighted 2D spin-echo acquisitions were performed with the following parameters: TE = 10.8 ms; TR = 400ms; fα=90°; FOV= 70 mm; 1.9 mm slices with 0.1 mm gap; dwell time = 16 µs, matrix: 200 X 200;

3 exc. *T2*-weighted 2D spin echo acquisitions were also performed (TE = 75 ms; TR = 2500 ms; fα=90°; 1 exc.).

Magnetization measurement

The magnetization curves of the nanocrystals were determined using a homemade vibrating sample magnetometer (VSM) under an applied magnetic field up to 0.93 T = 9300 Gauss.

Cytotoxicity

Cytotoxicity assays were performed on Human Umbilical Vein Endothelial Cells (HUVEC) cultured for 24h. The 24-well cell culture clusters were plate with cell suspension of a 2×10^4 Cells/well. After 24h of incubation, plates were washed with 1mL/well PBS and the cells were treated with various concentration of nanoparticles prepared in 5% PBS (0, 1, 10, 20, 50, 100 μg mL^{-1}). Incubation was performed at 37°C in a 5% CO_2 humidified incubator for 24h. Replicate wells were used for each control and test concentration per plate. Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method according to manufacturer's instructions. The culture medium was removed and 0.5 $mg.mL^{-1}$ of MTT reagent was added quickly (100 μL/well) and kept at room temperature for 3 h. At the end of the period, the medium was replaced by 300 μL of isopropanol and the sample was placed at 4 °C for additional 24 h. Absorbance was measured at 540 nm with a standard microplate reader Biotek Instrument EL800 (Biotek, Colmar, France). As the yellow MTT is reduced to purple formazan by cellular enzymes in living cells, quantity of formazan measured at 540 nm is directly proportional to the number of living cells in the culture.

All toxicity (viability and kinetics of proliferation) experiments were conducted in triplicate (three independent experiments). Statistical analyses were carried out using JMP software (JMP software, Paris, France) and a statistical significance was accepted at $p \leq 0.05$ for all tests.

After 6 days of incubation in presence of nanocrystals, cells were washed two times with PBS, fixed with 4 % of paraformaldehyde in PBS at 4 °C for 30 min and permeabilized with 0.1 % Triton-9100 for 5 min at room temperature. Then, the cells were rinsed with PBS and were incubated with PBS 1 % BSA for 20 min. The F-actin was stained with red phalloidin FluoProbes B-607 (B-607, Paris, France) diluted to 20 % in PBS for 30 min. All samples were thoroughly washed three times with PBS before inspection with a confocal microscope (Zeiss LSM 510 META, Paris, France).

The confocal microscope is an inverted microscope Zeiss LSM 510 META (Axiovert, Zeiss, Paris, France). It is controlled by the acquisition software LSM510 (LSM510, Paris, France). It has three laser lines: Argon Laser (488 nm/Green), Laser HeNe (543 nm/red) and Laser 633 nm (far red). Statistical analyses were performed using SPSS 14.0 data analysis software (SPSS, Chicago, USA).