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Bioinspired crystallization, sensitized luminescence and cytocompatibility of citrate-functionalized Ca-substituted europium phosphate monohydrate nanophosphors

Jaime Gómez-Morales^{a,*}, Cristóbal Verdugo-Escamilla^a, Raquel Fernández-Penas^a,

Carmen Maria Parra-Milla^a, Christophe Drouet^{b,c}, Michele Iafisco^c, Francesca Oltolina^d, Maria Prat^d, Jorge Fernando Fernández-Sánchez^e

^aLaboratorio de Estudios Cristalográficos, IACT, CSIC-UGR Avda. Las Palmeras, nº 4, E-18100 Armilla (Granada), Spain

^bCIRIMAT, Université de Toulouse, CNRS, Ensiacet, 4 allée Emile Monso, 31030 Toulouse cedex 4, France

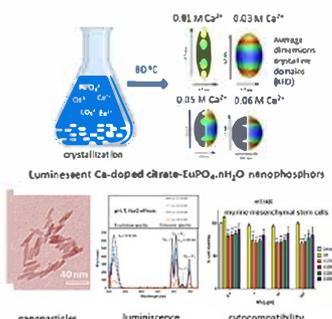
^cInstitute of Science and Technology for Ceramics (ISTEC), National Research Council (CNR), Via Granarolo 64, 48018 Faenza, (RA), Italy

^dDipartimento di Scienze della Salute, Università del Piemonte Orientale, Via Solaroli, 17, 28100 Novara, Italy

^eDepartment of Analytical Chemistry, Faculty of Sciences, University of Granada, Avda. Fuentenueva s/n, 18071 Granada, Spain

GRAPHICAL ABSTRACT

Citrate functionalized calcium doped europium phosphate nanoparticles were crystallized by thermal decomplexing of $\text{Ca}^{2+}/\text{Eu}^{3+}$ /citrate/phosphate/carbonate solutions. The crystallization mechanism, luminescence properties and cytocompatibility of this class of nanoparticles were investigated. These biocompatible nanoparticles appear as promising luminescent probes in the field of bioimaging.



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ABSTRACT

Biocompatible nanosystems exhibiting long lifetime (\sim millisecond) luminescence features are particularly relevant in the field of bioimaging. In this study, citrate functionalized calcium doped europium phosphates nanophosphors of the rhabdophane type were prepared at different synthesis times by a bioinspired crystallization route, consisting in thermal decomplexing of $\text{Ca}^{2+}/\text{Eu}^{3+}$ /citrate/phosphate/carbonate solutions. The general formula of this material is $\text{Ca}_\alpha\text{Eu}_{1-\alpha}(\text{PO}_4)_{1-\alpha}(\text{HPO}_4)_\alpha \cdot n\text{H}_2\text{O}$, with α ranging from 0 to 0.58 and $n \sim 1$. A thorough characterization of the nanoparticles has been carried out by XRD (including data processing with Topas 6.0), HR TEM, TEM, FTIR, TG/DTA, ICP, dynamic light scattering (DLS), electrophoretic mobility, and fluorescence spectroscopy. Based on these results a crystallization mechanism involving the filling of cationic sites with Ca^{2+} ions associated to a concomitant adjustment of the PO_4/HPO_4 ratio was proposed. Upon calcium doping, the aspect ratio of the nanoparticles as well as of the crystalline domains decreased and the relative luminescence intensity (R.L.I.) could be

* Corresponding author.

E-mail address: jaime@lec.csic.es (J. Gómez-Morales).

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modulated. Neither the pH nor the ionic strength, nor the temperature (from 25 to 37 °C) affected significantly the R.L.L. of particles after resuspension in water, leading to rather steady luminescence features usable in a large domain of conditions. This new class of luminescent compounds has been proved to be fully cytocompatible relative to GTL 16 human carcinoma cells and showed an improved cytocompatibility as the Ca²⁺ content increased when contacted with the more sensitive m17. ASC murine mesenchymal stem cells. These biocompatible nanoparticles thus appear as promising new tailorable tools for biomedical applications as luminescent nanopobes.

1. Introduction

Materials at the nano scale possess unique optical, magnetic, and chemical properties which allow the creation of imaging probes with better contrast enhancement, increased sensitivity, improved signal to noise ratio and better spatial and temporal information in comparison to the conventional imaging agents [1]. Among them, luminescent nanoparticles are excellent optical probes that can significantly extend the capabilities of well established fluorophores such as organic dyes and genetically engineered fluorescent proteins [2]. Their advantages include also excellent photostability, tunable and narrow spectra, controllable size, resilience to environmental conditions such as pH and temperature, in combination with a large surface for anchoring targeting biomolecules [2]. Their function is mainly to provide the necessary fluorescent contrast required to visualize specific cellular components and tissues [3]. Several luminescent nanomaterials including quantum dots [4], nanodiamonds [5], gold nanoparticles [6], nanostructures labeled with organic dyes [7], nanocrystalline calcium orthophosphate apatites labeled with lanthanide ions or organic dyes [8–11] and others, have been proposed as fluorophores for these applications. In recent years, lanthanide orthophosphates (LnPO₄) have attracted much attention because they exhibit low solubility in water, high thermal conductivity and high refraction index, thus being candidates for a wide range of applications including phosphors, catalysts, sensors, heat resistant materials [12] and also, as optical probes for bioimaging [13,14]. As imaging agents the favorable features of lanthanide doped nanoparticles include large effective Stokes shift, sharp emission peaks with narrow band width, low photobleaching, stability to photochemical degradation, long luminescence lifetimes, low toxicity and the absence of blinking [10–15].

Lanthanide orthophosphates LnPO₄·nH₂O (n = 0–2) (Ln³⁺ = lanthanide ion) present five polymorphic modifications, i.e. monazite (monoclinic), xenotime (tetragonal), rhabdophane (hexagonal), weinschenkite (monoclinic) and orthorhombic [16]. The hexagonal structure is usually the low temperature phase and it can transform into the monazite structure, while the tetragonal phase maintains its structure after calcination at 900 °C [17,18]. Different methods to prepare LnPO₄·nH₂O nanocrystals with controlled morphologies such as nanorods, nanowires or nanocables have been proposed. Synthetic methods are based on high or low temperature approaches, such as hydrothermal synthesis [19–22], ultrasound irradiation [23], precipitation in oil baths [24], sol gel combined with electrospinning [25], surfactant assisted methods to mediate the slow crystallization of the nanocrystals [26], or else layer by layer deposition of precursors followed by crystallization at room temperature [27].

Among lanthanides ions, trivalent europium, Eu³⁺, has been used to crystallize luminescent compounds such as EuPO₄·nH₂O, or Eu³⁺ doped LnPO₄ of the hexagonal form with improved energy transfer efficiency between the Ln³⁺ and Eu³⁺ [14,27–29]. From another perspective, crystallization of EuPO₄·nH₂O has been also considered as a method to separate Eu³⁺ impurities from phosphoric acid solutions obtained from calcium phosphate apatite rocks

[30,31]. The presence of Eu³⁺ impurities is assumed to result from Eu³⁺ to Ca²⁺ substitution in the apatite structure. Ca substituted EuPO₄·nH₂O crystallizes in such acid solutions due to the presence of Ca²⁺ as main impurity [31].

Luminescent Ln³⁺ doped calcium phosphate (apatite) nanoparticles have been extensively studied as materials for diagnostics and therapeutic applications [10,11,13,14] while Ca substituted EuPO₄·nH₂O has never been proposed for similar topics. Compared to the precipitation of Ln³⁺ doped apatite nanoparticles [32,33], a main advantage of EuPO₄·nH₂O is related to its reduced polymorphism; the hexagonal rhabdophane structure being the crystalline phase that usually precipitates at low temperatures [28]. In contrast, during the calcium phosphate crystallization at low temperature several polymorphs, including monetite, brushite, octacalcium phosphate and apatite can eventually appear [34,35,36], depending on experimental conditions.

In the present work we have prepared Ca doped europium phosphate nanocrystals of the hexagonal phase employing a biospired synthetic route consisting in thermal decomplexing of Eu³⁺/Ca²⁺/citrate/phosphate/carbonate solutions method and assessed their luminescence and cytocompatibility properties. The proposed technique, which was previously used to precipitate calcium phosphate apatite nanoparticles doped with different metallic ions and coated with citrate [37–39], is one pot, straight forward, easily scalable to an industrial level, and respects the principles of green chemistry. The presence of Ca²⁺ in the structure of the nanocrystals as well as of citrate adsorbed on the surface of the constitutive particles (a feature found in bone apatite nanocrystals [40]) are expected to be beneficial from the point of view of the cytocompatibility that is required for bioimaging applications.

2. Materials and methods

2.1. Reagents

Europium chloride hexahydrate (EuCl₃·6H₂O, ACS Reagent, 99.9% pure), calcium chloride dihydrate (CaCl₂·2H₂O, Bioextra, ≥99.0% pure), sodium citrate tribasic dihydrate (Na₃(Cit)·2H₂O where cit = citrate = C₆H₅O₇, ACS reagent, ≥99.0% pure), sodium hydrogenphosphate (Na₂HPO₄, ACS reagent, ≥99.0% pure), sodium carbonate monohydrate (Na₂CO₃·H₂O, ACS reagent, 99.5% pure), and hydrochloric acid (HCl, ACS reagent, 37 wt% in H₂O) were provided by Sigma Aldrich. All solutions were prepared with ultrapure water (0.22 μS, 25 °C, Milli Q, Millipore).

2.2. Precipitation method

Nanoparticles were synthesized as follows: a solution of composition (a) 0.12 M Na₂HPO₄, + 0.2 M Na₂CO₃, with pH adjusted to 8.5 with diluted HCl, was poured on a solution of composition (b) xCaCl₂ + yEuCl₃ + 0.4 M Na₃(cit) (with x) being the doping Ca²⁺ concentration 0.01, 0.03, 0.05 and 0.06 M and y = 0.1 · x M (1:1 v/v, 200 mL total) at 4 °C. The mixed solution was introduced

in a 200 mL bottle made of Pyrex® glass, sealed with a screw cap and, without delay, immersed in a water bath at 80 °C and then moved to an oven with circulated forced air at the same temperature. The experiments lasted between few minutes and few days. Once finished, the precipitates were subjected to 6 consecutive cycles of washing by centrifugation with ultrapure water to remove unreacted salts. Afterwards they were freeze dried overnight at 50 °C under vacuum (3 mbar).

2.3. Characterization techniques

The precipitates were characterized by X ray powder diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, scanning and transmission electron microscopy (SEM, TEM), high resolution TEM (HR TEM), and fluorescence spectroscopy. Selected samples were analyzed by dynamic light scattering (DLS), ζ potential and thermogravimetry/differential scanning calorimetry (TG/DTA). Ca, P and Eu contents were quantified by inductively coupled plasma optical emission spectrometry (ICP OES) spectrometer.

The X ray powder diffraction data were collected using a Bruker D8 Advance Vario diffractometer with a Bragg Brentano parafocusing geometry and Cu K $_{\alpha 1}$ radiation (1.5406 Å). Data processing was conducted via the software TOPAS 6.0 [41]. The contribution of the anisotropic peak broadening due to domain size was modeled using the “AnisoCS” TOPAS macro developed by Ectors et al. [42,43] based on the size Voigt approximation, and taking into account the instrumental contribution from a measurement of LaB6 standard (NIST SRM 660c). A biaxial ellipsoid model was selected to fit the experimental data, and the anisotropic sizes obtained are the main radii area weighted in nm.

Fourier transform infrared spectroscopy was used for complementary characterization of the samples. In a first stage, spectral acquisitions were run using a JASCO 6200 FTIR spectrometer equipped with an attenuated total reflectance (ATR) accessory. Subsequently, more in depth analyses were drawn from spectra recorded in transmission mode using a Perkin Elmer Spectrum One FTIR spectrometer using the KBr pellet method. The pellets were prepared by mixing ~1 mg of sample with ~100 mg of anhydrous KBr and then pressed with a hydraulic pump at 10 t into 7 mm diameter discs. Pure KBr pellets were used to record the background. FTIR spectra in transmittance mode were recorded within the wavelength range from 4000 cm⁻¹ to 400 cm⁻¹.

TGA DTA analyses of the samples were carried out in a simultaneous thermal analyzer (STA 449 Jupiter Netzsch Gerätebau, Selb, Germany). About 10 mg of sample was weighted in a platinum crucible and heated from room temperature to 1000 °C under air flow with a heating rate of 10 °C/min.

The chemical composition of the samples was determined dissolving the samples in a diluted HNO₃ solution (~1 wt%) and then analyzed by an ICP OES (Liberty 200, Varian, US) employing wavelengths of 422.673 nm (Ca), 420.504 nm (Eu), and 213.618 nm (P).

Transmission electron microscopy (TEM) observations and selected area electron diffraction (SAED) analysis were performed with a TEM Libra 120 Plus instrument from Carl Zeiss microscope operating at 80 kV. Resolution point point is 0.34 nm and magnification is 8 630,000x. Samples were dispersed in ethanol (absolute, ≥99.8%) using an ultrasound bath. Then a few droplets of the slurry were deposited on conventional copper microgrids with FORMVAR carbon film prior to observation. HR TEM analysis was performed with a TITAN G2 60 300 FEI Instrument operating at 300 kV. The instrument is equipped with EDX Super X detector to perform microanalysis and STEM type HAADF.

The analysis of the particle size distribution and electrophoretic mobility (transformed to ζ potential values) of suspensions was performed in a Zetasizer Nano ZS analyzer (Malvern, UK) using dis-

posable polystyrene cuvettes containing the particles suspended in deionized water (0.5 mg/mL) at 25 °C. Samples were immersed in ultrasonic bath for 5 min prior to DLS analysis. For the measurements of the ζ potential versus pH the MPT 2 autotitrator (Malvern, UK) connected to the analyzer was employed. Diluted HCl and NaOH solutions (0.25 and 0.1 M, respectively) were used as titration agents. No additional electrolytes were added.

2.4. Luminescence spectroscopy

Excitation and emission spectra of the solid samples as well as of aqueous suspensions of the nanoparticles (~0.5 mg/mL) were recorded using a Cary Eclipse Varian Fluorescence Spectrophotometer (Varian Australia, Mulgrave, Australia) with an R928 photomultiplier tube (Hamamatsu, Iwata gun, Shizuoka ken, Japan). For solid nanoparticles λ_{exc} was 394 nm, λ_{em} 614 nm, delay time (t_d) was 0.120 μ s and gate time (t_g) was 5 ms. Spectra were recorded with a photomultiplier voltage of 500 V. The excitation and emission spectra were recorded within the range 250 500 nm and 500 750 nm respectively. For nanoparticles in aqueous suspensions, the following conditions were used: λ_{exc} 230 nm, λ_{em} 614 nm, t_d 0.120 μ s and t_g 5 ms. In this case the photomultiplier voltage was 600 V and the excitation and emission spectra were recorded within the wavelength range 200 550 nm and 400 750 nm respectively.

2.5. Cytocompatibility tests

GTL 16 (a human gastric carcinoma cell line) and m17.ASC (a spontaneously immortalized mouse mesenchymal stem cell clone from subcutaneous adipose tissue) cells (12,000 and 5,000 cells/well in 96 well plates, respectively) were incubated for 24 h and then different concentrations of the differentially doped ($x = 0.01, 0.03, 0.05, 0.06$ M Ca²⁺) particles, ranging from 0.1 to 100 μ g/ml, were added in 100 μ L of fresh medium. After 72 h incubation, cell viability was evaluated by the 3 (4,5 Dimethylthiazol 2 yl) 2,5 diphenyltetrazolium bromide) (MTT, Sigma) colorimetric assay. Briefly, 20 μ L of MTT solution (5 mg/ml in a PBS solution) were added to each well. The plate was then incubated at 37 °C for 3 h. After the removal of the solution, 125 μ L of isopropanol, 0.2 M HCl was added to dissolve formazan crystals. 100 μ L were then removed carefully and the optical density was measured in a multiwell reader (2030 Multilabel Reader Victor TM X4, PerkinElmer) at 570 nm. Viability of parallel cultures of untreated cells was taken as 100% viability, and values obtained from cells undergoing the different treatments were referred to this value. Experiments were performed 4 times using 3 replicates for each sample.

Statistical Analysis. Data are expressed as mean \pm standard deviation of at least three replicates. Statistical analyses were performed using a one way ANOVA with Bonferroni's post test for grouped analyses using GraphPad Prism version 4.03 for Windows, GraphPad Software (GraphPad Prism, San Diego, CA). Differences at $p < 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. Crystallographic, compositional, morphological and spectroscopic characteristics

The XRD patterns of samples precipitated in the presence of increased Ca²⁺ doping concentrations ($x =$ from 0.01 to 0.06 M) at different maturation times are reported in Fig. 1.

The patterns mainly display the distinguishing reflections of the hexagonal phase of EuPO₄·nH₂O (rhabdophane), space group P3₁21 (PDF 20 1044) with lattice constants $a = 6.910$ Å, and $c = 6.340$ Å.

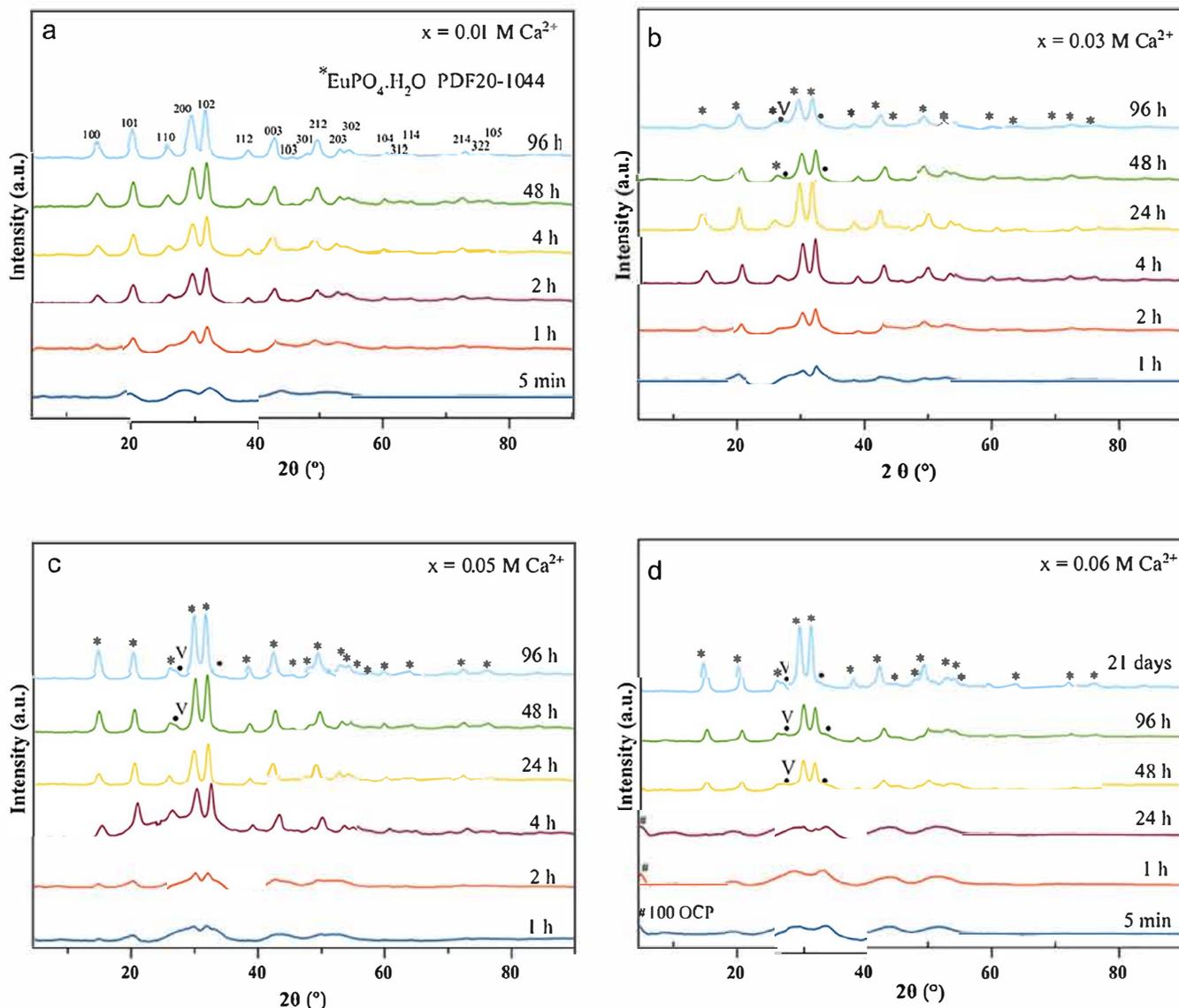


Fig. 1. X-ray diffraction patterns of the samples precipitated using increasing Ca^{2+} doping concentrations: (a) 0.01 M; (b) 0.03 M; (c) 0.05 M and (d) 0.06 M. $^*\text{EuPO}_4 \cdot n\text{H}_2\text{O}$ (PDF 20-1044), # Reflection of (1 0 0) plane of octacalcium phosphate (OCP, PDF 44-0778), • Reflections (1 0 1) and (1 1 4) of CaCO_3 vaterite (V, PDF 33-0268).

The main reflections of this phase are situated at 2θ 14.67° (1 0 0), 20.35° (1 0 1), 25.80° (1 1 0), 29.77° (2 0 0), 31.95° (1 0 2), 38.44° (1 1 2) 42.61° (0 0 3) and 49.46° (2 1 2). The diffraction peaks at the shorter maturation times are broad and poorly defined suggesting a relatively lower degree of crystallinity and nanodimensions. The evolution with maturation time of the full width at half maximum (FWHM) of the two main reflections at 29.77° and 31.95° indicates an increase in size or crystallinity up to 48 h for $x = 0.010 \text{ M Ca}^{2+}$, 24 h for $x = 0.030 \text{ M Ca}^{2+}$, 96 h for $x = 0.050 \text{ M Ca}^{2+}$ and 21 days for $x = 0.06 \text{ M Ca}^{2+}$. Only a small decrease in size or crystallinity can be deduced after 24 h maturation for $x = 0.03 \text{ M Ca}^{2+}$. No peaks assignable to other crystalline phase were identified when using the 0.01 M Ca^{2+} doping concentrations even for early precipitation times (Fig. 1a). On the contrary, the samples precipitated using the 0.06 M Ca^{2+} concentration show a different phase evolution (Fig. 1d). At maturation times of 5 min, 1 h and 24 h the main reflection at $2\theta = 4.74^\circ$ assignable to (1 0 0) plane of octacalcium phosphate (OCP, PDF 44 0778) is present. The broad width of this reflection along with the bulging of the baseline in the 2θ range from 22° to 38° suggests the nanocrystalline character of the OCP phase. The peak at 4.74° was

not observed in the samples matured for more than 24 h, which in turn, shows mainly the reflections of the hexagonal phase of $\text{EuPO}_4 \cdot n\text{H}_2\text{O}$. Thus the formation of OCP in early stages was only transient until final ion reorganization of the system took place.

For Ca^{2+} doping concentrations $x = 0.03, 0.05$ and 0.06 M , traces of an additional phase start to appear at high maturation time, which is especially visible at around $2\theta \sim 27.0$ and 32.8° . These additional peaks detected for high calcium concentrations are attributed to the reflections (1 0 1) and (1 1 4) of CaCO_3 vaterite (PDF 33 0268), eventually doped with europium, which becomes stable in the calcium rich medium. According to ICP OES data (see below) an amount close to 0.4, 10 and 14 wt% respectively for 0.03, 0.05 and 0.06 M was evaluated for 96 h of maturation, besides the europium phosphate phase. In contrast, no vaterite was detected for the lowest Ca^{2+} concentration ($x = 0.01 \text{ M}$) or for low maturation times, where the samples consist solely in single phased Ca doped $\text{EuPO}_4 \cdot n\text{H}_2\text{O}$. Vaterite is a highly biocompatible allotropic form of calcium carbonate that is used in the biomaterials field, e.g. for the setup of bone cement formulations [44]. The eventuality of traces of this minor phase is thus not problematic *per se* for biomedical applications; in any case, we show here

the possibility to avoid its presence if needed, by adequately tailoring the precipitation conditions such as calcium doping concentration.

The microstructural study of samples matured for 96 h, analyzing the full XRD pattern of the Ca doped $\text{EuPO}_4 \cdot n\text{H}_2\text{O}$ with TOPAS 6.0 software (Fig. 2 and Table 1), reveals a trend from elongated to more isometric shapes of the single crystal domains when increasing the Ca^{2+} doping concentration. The coherent length (L) of the domains (Table 1 and insets in Fig. 3) evolves from 13.3 ± 0.1 nm for the less Ca doped sample to 6.9 ± 0.2 nm for the highest Ca^{2+} concentrations. Similarly, the aspect ratio switches from 3.6 to 1.1. The sample obtained for $x = 0.05$ M Ca^{2+} displayed a coherent length and width almost doubled of that obtained for $x = 0.03$ M Ca^{2+} in spite of similar aspect ratios. Note that in all panels of Fig. 2, τ is slightly higher than 1.6, which indicates that the population of single crystal domains is polydisperse.

TEM observations (Fig. 3a-d) allowed visualizing the morphology and average dimensions of the nanoparticles, which were found about 3 times larger than the sizes determined by Rietveld refinement. It is important to remark that TEM and XRD allow evaluating two different features of the material (observed particles size vs coherent size of crystal domains) therefore only the general trend of data can be compared.

The average dimensions of the particles determined by TEM (Table 1) typically varied from 40 ± 7 nm in length with the aspect ratio (length/width) of 5 for the less Ca doped sample (1.3 wt% Ca) to 19 ± 7 nm in length with the aspect ratio of 2.54 for the sample with the highest Ca content (14.2 wt% Ca). It is thus observed that increasing the Ca^{2+} content the nanoparticles are in general more isometric with ill defined shape. In the case of $x = 0.05$ M Ca^{2+} we found a mixture of elongated and ill defined isometric nanoparticles, therefore this doping point can represent the transition between these two morphologies.

Fig. 3e and f show the STEM images in bright field (BF) and high angle annular dark field (HAADF) of the less Ca doped sample. The indexed SAED pattern (Inset of Fig. 3f) of this sample shows the crystallographic planes of the hexagonal rhabdophane phase, i.e. (1 0 1), (1 1 0), (2 0 0), (1 0 2), (1 1 2), (2 1 2), (3 0 0) and (3 2 2). HRTEM images of the same sample (Fig. 3g and h) reveal the lattice fringes of the nanoparticles, mostly structured as single crystal domains, whose d spacings correspond to planes (2 0 0) and (1 0 2) as also revealed in the FFT images (insets of Fig. 3g and h). The elemental mappings of the less Ca doped nanoparticles (Fig. 3i-l) show a homogeneous distribution of Ca, Eu, O and P.

TGA DTA analyses (see SI, Fig. S1) revealed a rather comparable behavior for all samples, and the data are very close to those

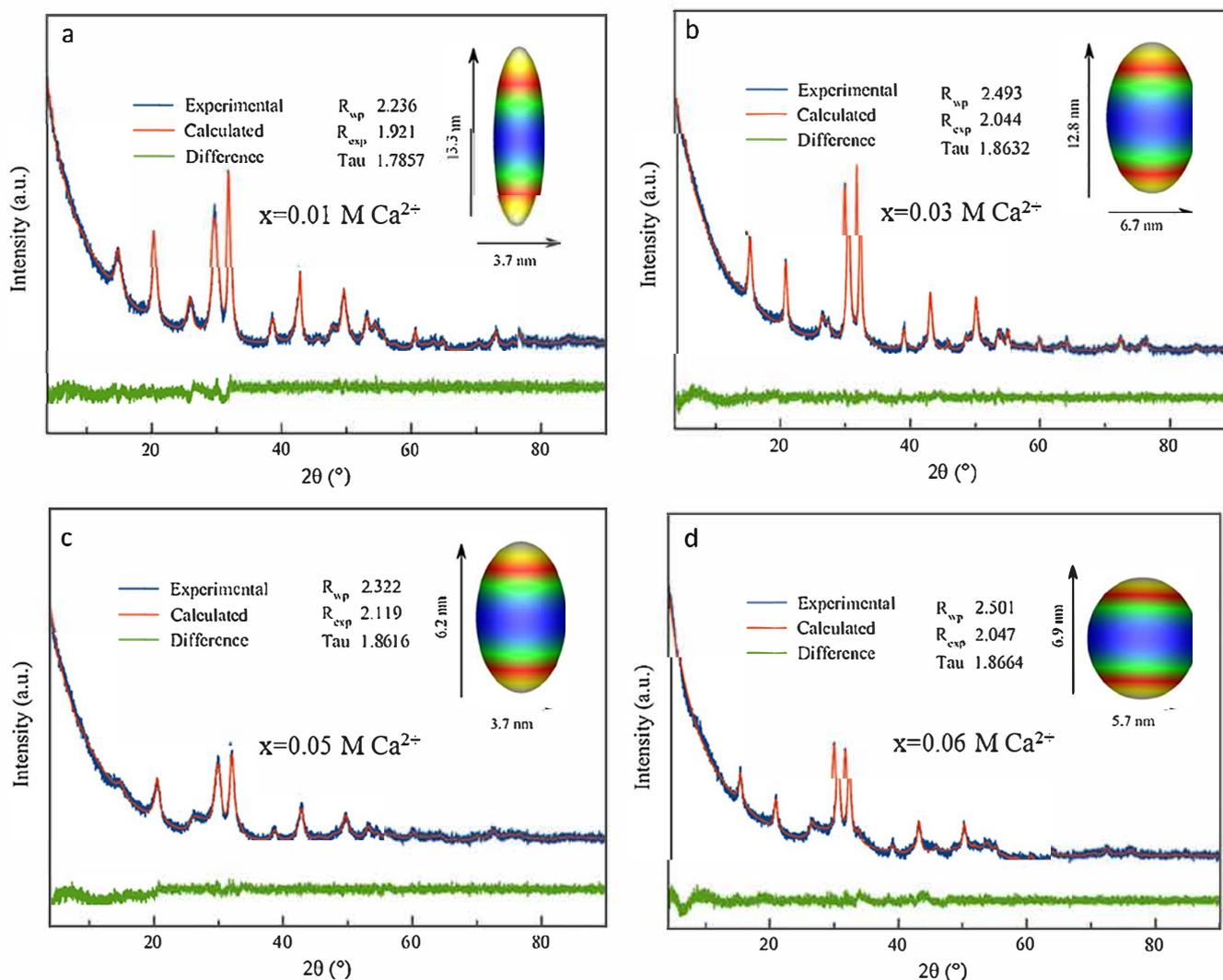


Fig. 2. Microstructural evolution of the single crystal domains of Ca-doped $\text{EuPO}_4 \cdot n\text{H}_2\text{O}$ nanoparticles of samples precipitated at 96 h using the Ca^{2+} doping concentrations: (a) 0.01 M; (b) 0.03 M; (c) 0.05 M and (d) 0.06 M. The two XRD vaterite reflections are excluded. R_{wp} and R_{exp} are agreement factors in % for the refinement. τ is a polydispersity factor ($1 \leq \tau \leq 2$), being 2 the theoretical limit for the Gaussian part of the size Voigt approximation used.

reported for similar materials [45–48]. The main weight losses occurred in the range $T_{\text{room}} - 700\text{ }^{\circ}\text{C}$ corresponding to the loss of adsorbed and structural water, while the weight loss between 700 and $1000\text{ }^{\circ}\text{C}$, that is more evident in the samples prepared

using the higher Ca^{2+} doping concentrations, is associated to the loss of CO_2 from the carbonate decomposition of vaterite. In details, the first weight loss of about 2–6 wt%, attributed to the residual adsorbed water on the powder surface due to the storage

Table 1

Quantitative compositional, elemental chemical analysis and average size (length and width) of Ca-doped cit- $\text{EuPO}_4 \cdot n\text{H}_2\text{O}$ samples prepared with Ca^{2+} doping concentration x from 0 to 0.06 M, at 96 h.

Ca^{2+} mol/L	Value ^g of α (± 0.05)	Analyzed Elements (wt.%)					Calculated oxide components (wt.%) ^e			Total ^f (wt.%)
		Eu ^a	Ca ^a	P ^a	H_2O Adsorbed ^b	H_2O Structural ^b	Eu_2O_3	CaO	P_2O_5	
0	0	53.0 ± 3.0	0	10.6 ± 0.6	6.0 ± 0.6	5.8 ± 0.7	61.4 ± 1.2	0	24.3 ± 1.0	100.2 ± 3.1
0.01	0.09	51.0 ± 2.0	1.3 ± 0.1	11.9 ± 0.4	2.1 ± 0.2	5.4 ± 0.5	61.7 ± 1.2	1.9 ± 0.1	28.5 ± 1.0	100.0 ± 3.1
0.03	0.30	43.7 ± 0.3	4.9 ± 0.1	12.6 ± 0.1	3.7 ± 0.4	6.2 ± 0.6	52.4 ± 0.3	7.1 ± 0.1	29.9 ± 0.2	100.3 ± 1.7
0.05	0.49	31.5 ± 0.1	11.6 ± 0.1	12.6 ± 0.1	4.3 ± 0.4	6.6 ± 0.7	37.5 ± 0.1	16.7 ± 0.1	29.6 ± 0.2	100.5 ± 1.7
0.06	0.58	26.2 ± 0.3	14.2 ± 0.1	12.6 ± 0.2	5.6 ± 0.6	6.4 ± 0.6	30.8 ± 0.3	20.2 ± 0.2	29.3 ± 0.4	100.1 ± 2.3
Rietveld Refinement ^c (TOPAS 6.0)							TEM ^d			
		Length (L, nm)	Width (W, nm)		R_1 (L/W) aspect ratio		L, nm	W, nm		R_2 (L/W) aspect ratio
0.01		13.3 ± 0.1	3.7 ± 0.3		3.6		39.5 ± 7.1	7.9 ± 1.6		5.0
0.03		6.2 ± 0.2	3.7 ± 0.3		1.7		19.7 ± 3.2	6.9 ± 2.6		2.8
0.05		12.8 ± 0.1	6.7 ± 0.2		1.9		41.1 ± 7.1	16.4 ± 5.7		2.5
0.06		6.9 ± 0.2	5.7 ± 0.2		1.1		19.3 ± 6.9	7.6 ± 2.0		2.5

^a Measured by ICP-OES;

^b measured by TGA-DTA

^c average dimensions (coherent length and width) of the single crystal domains determined by Rietveld refinement of the full diffraction pattern with TOPAS 6.0;

^d average dimensions of the nanoparticles determined by TEM;

^e without mentioning here H_2O , citrate and carbonate ions;

^f counting H_2O , citrate and carbonate (vaterite) ions;

^g value of α in the chemical formula $\text{Eu}_{1-\alpha}\text{Ca}_\alpha(\text{PO}_4)_1-\alpha(\text{HPO}_4)_\alpha \cdot n\text{H}_2\text{O}$

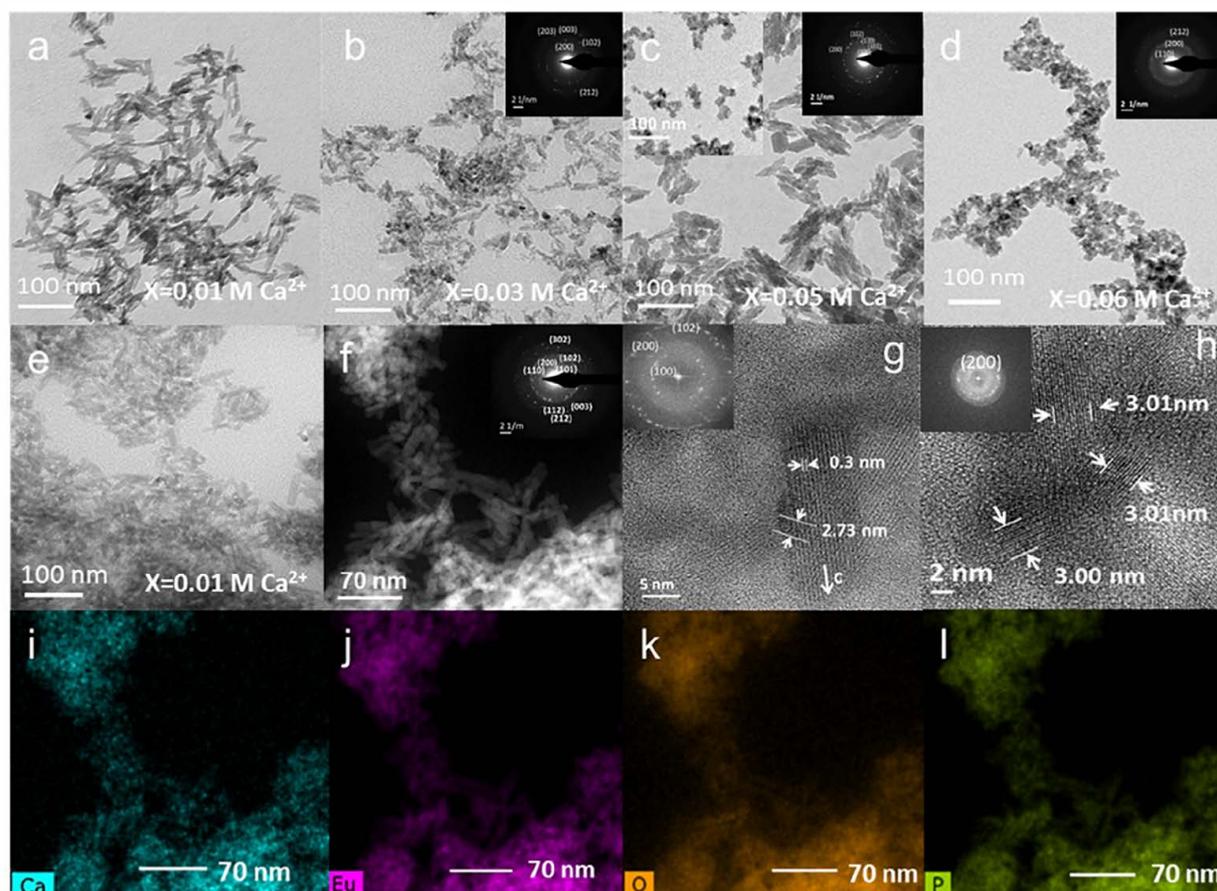


Fig. 3. (a–d) TEM micrographs of Ca-doped cit- $\text{EuPO}_4 \cdot n\text{H}_2\text{O}$ nanoparticles prepared with different Ca^{2+} doping concentrations at 96 h: (a) 0.01 M; (b) 0.03 M; (c) 0.05 M and (d) 0.06 M. Insets show the indexed SAED patterns of the nanoparticles. (e, f) BF HAADF-STEM micrographs of the sample prepared with the Ca^{2+} doping concentration of 0.01 M. Inset in (f) shows the indexed SAED pattern. (g, h) HR-TEM images showing some lattice fringes and d-spacing of the sample prepared with the Ca^{2+} doping concentration of 0.01 M. Insets show the corresponding FFT images displaying the (1 0 0), (2 0 0) and (1 0 2) planes. (i–l) EDX Element mapping analysis of the sample displayed in panel f.

conditions in air, was found up to 120 °C with a broad endothermic peak in the range 50–100 °C in the DTA curve. The second weight loss began at about 120 °C and finished at about 700 °C. It corresponds to the dehydration (release of structural water) of the hexagonal phase. This step proceeded in two sequences. An important weight loss was registered up to about 350 °C and then it decreased slowly to 700 °C. This event was accompanied by a broad endothermic effect. The exothermic peak in the range 750–950 °C represents the phase transformation of the lanthanide orthophosphate from hexagonal to monoclinic [28].

Table 1 gathers the compositional information relative to the samples. In particular two interesting findings can be pointed out: (i) while the P wt% remained almost constant in all samples, the Ca wt% increased and the content of Eu accordingly decreased; (ii) the weight loss corresponding to the structural H₂O accounts for about 5–7 wt%, which is equivalent to a hydration content *n* of about 1 mol of H₂O (Table 1).

On the basis of these analyses, it is interesting to examine in further details the chemical composition of the samples. Phosphate groups are large ionic entities ensuring, as in many phosphate compounds such as apatites, the stability of the crystallographic structure. Therefore, the existence of phosphate vacancies is very improbable as this would most likely destabilize the whole structure. Also, the carbonation of LnPO₄ phases has not been reported to our knowledge. Yet, since the crystal electroneutrality has to be

preserved upon Ca for Eu substitution, the replacement of trivalent Eu³⁺ cations by divalent Ca²⁺ should be undergone with simultaneous protonation of an equivalent number of PO₄³⁻ ions into HPO₄²⁻. This presence of HPO₄²⁻ ions in calcium doped europium phosphate has already been considered in the literature.³¹ This leads for the samples generated in the present study, and excluding citrate for simplicity of writing, to the general formula Eu_{1- α} Ca _{α} (PO₄)_{1- α} (HPO₄) _{α} nH₂O with 0 ≤ α ≤ 1. From ICP data, the estimated value of “ α ” ranges between 0 and 0.58 for α varying from 0 to 0.06 M Ca²⁺. For samples corresponding to α = 0.03, 0.05 and 0.06 M, an additional amount of vaterite is then also present, reaching 0.01, 0.22 and 0.30 mol per mole of europium phosphate, respectively.

For complementary characterization of the samples, FTIR spectroscopy was also applied, and Fig. 4a–d show the spectra of the samples obtained upon increasing Ca²⁺ doping concentration. They display similar spectroscopic features and show the expected global shape of a rhabdophane phase [45,48] with a broad band in the range 1000–1100 cm⁻¹, related to the asymmetric stretching mode of PO₄³⁻ groups (ν_3 PO₄), and two intense bands at ~620 and 535 cm⁻¹ due to the bending mode of PO₄³⁻ ions (ν_4 PO₄). The broad band observed between 3600 cm⁻¹ and 2600 cm⁻¹ corresponds to the O–H stretching from the associated water. Several spectral contributions also point to the presence of citrate molecular ions, with contributions at 2926 and 2856 cm⁻¹ assignable to the

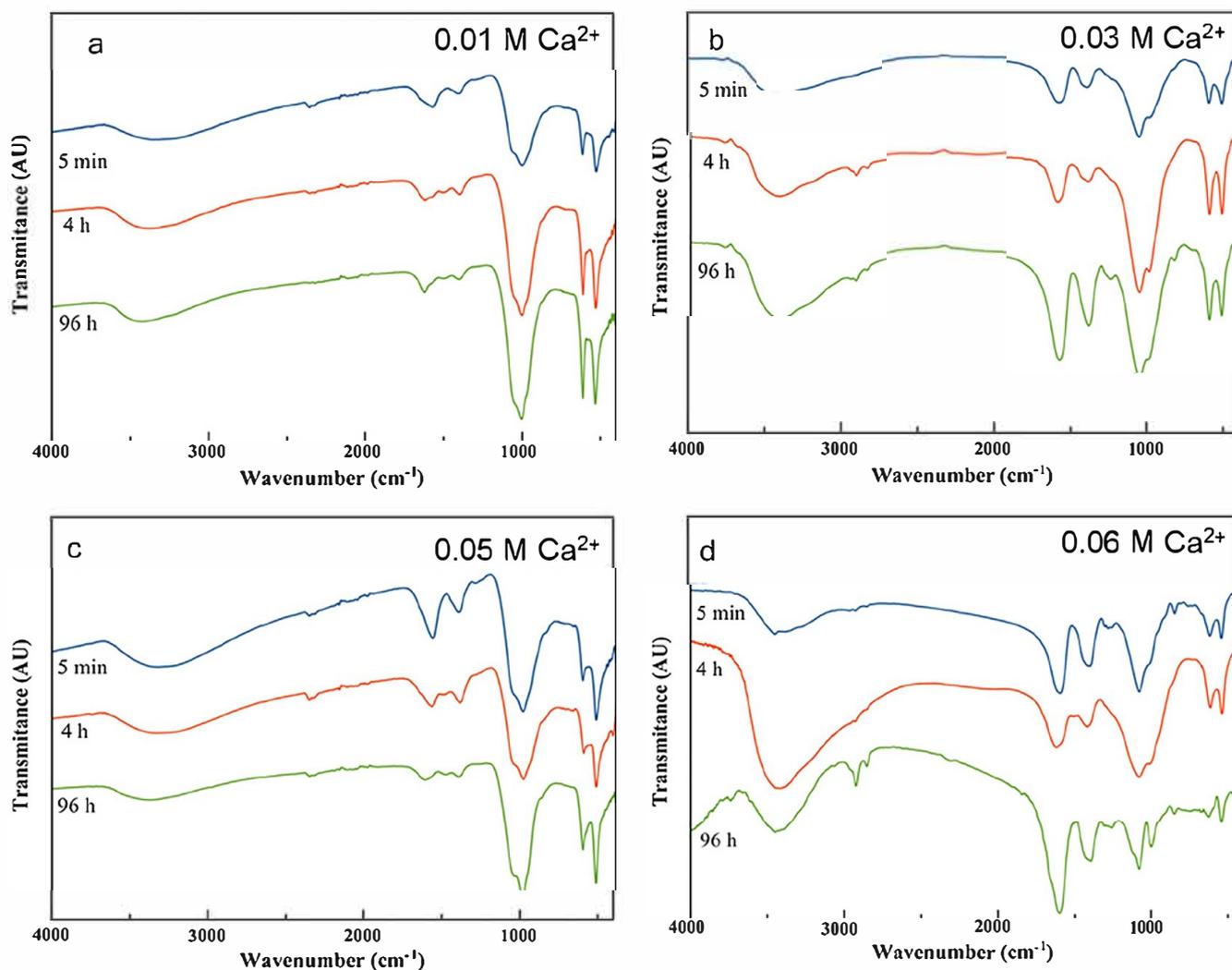


Fig. 4. (a–d) FTIR spectra of samples synthesized at Ca²⁺ doping concentrations 0.01, 0.03, 0.05 and 0.06 M at different maturation times.

stretching vibration of CH_2 groups [49], a small band detected here around 867 cm^{-1} which can be related to the bending mode of COO^- groups [50], and broad signals at about 1590 cm^{-1} and 1402 cm^{-1} assignable to the antisymmetric and symmetric stretching of carboxylate ($-\text{COO}^-$) groups of the adsorbed citrate, respectively [51].

Traces of CaCO_3 vaterite have been revealed previously from XRD analysis for $x = 0.003, 0.05$ and 0.06 M Ca^{2+} , at least at high maturation times. The presence of this secondary phase explains the modifications observed in the $830\text{--}890\text{ cm}^{-1}$ and $1250\text{--}1550\text{ cm}^{-1}$ ranges (e.g. for 96 h of maturation, see ES, Fig.S2). In these regions are located not only COO^- vibrations from citrates, as mentioned above, but also of carbonate ions when present (respectively their $\nu_2\text{CO}_3$ and $\nu_3\text{CO}_3$ vibration modes). The contributions initially present at 867 and 1402 cm^{-1} for low Ca doping and single phased systems are shifted to around 872 and 1412 cm^{-1} in the case of samples $x = 0.05$ and 0.06 M Ca^{2+} , which can be explained by the co presence of traces of vaterite [52] known to lead to vibration bands at 870 and 1420 cm^{-1} . It may also be noted that the presence of HPO_4^{2-} ions, which is expected to give a band around $870\text{--}880\text{ cm}^{-1}$, cannot be detected in this work due to the presence of other vibration due to citrates and carbonates in the same domain.

3.2. Crystal size distribution and electrokinetic properties (ζ potential) by DLS

The analysis of crystal size distribution (CSD) and surface ζ potential is fundamental to evaluate the potential applications of nanoparticles as drug delivery vehicles or as luminescent probes in medical imaging [53]. In the present study it was decided to plot the volume based frequency distribution of the different Ca^{2+} doped cit- $\text{EuPO}_4 \cdot \text{H}_2\text{O}$ samples because they clearly show the percentiles of the distribution D_{10} and D_{50} (Fig. 5a and S3 of SI). These percentiles represent the percentage of cumulative volume under size distribution (percentage of the population smaller than the size indicated). Thus, D_{10} , which is the percentile more close to the size of the fine individual particles, is always within the nanometer size range while D_{50} , which represent the median diameter of the CSD, is here partially influenced by particle aggregation. D_{50} (not shown) is fully influenced by the aggregation of nanocrystals.

It is worth mentioning that median diameters for samples prepared with $x = 0.01$ ($D_{50} = 44\text{ nm}$), 0.05 ($D_{50} = 164\text{ nm}$) and 0.06 M

Ca^{2+} ($D_{50} = 80\text{ nm}$) are quite close to those of D_{10} (28, 38 and 52 nm, respectively) and that D_{10} defines well the size of individual nanoparticles, particularly for $x = 0.01$ and 0.05 M Ca^{2+} (L measured by TEM of 39.5 and 41.1 nm, respectively). This fact indicates that aggregation is very low below the median diameter of the CSD and negligible below D_{10} . Considering that ζ potential of the suspension affects the aggregation state of the nanoparticles, i.e. the lower the absolute value of the ζ potential the greater the trend to aggregation, it is deduced that the interactions among nanoparticles are very weak. The aggregates are broken only after 5 min sonication, in spite of the low values of the ζ potential at any of the pHs analyzed (Fig. 5b). Only for $x = 0.03\text{ M Ca}^{2+}$, with D_{50} 955 nm and D_{10} 68 nm the aggregation process is more important. For this sample the ζ potential within the pH range from 4 to 10 is closer to zero. We have not found a general trend relating the evolution of the CSD with the Ca^{2+} doping concentration nor with the maturation time of the precipitates (see Fig. S3a d).

The above findings confirm that we succeeded in preparing Ca doped cit- $\text{EuPO}_4 \cdot \text{nH}_2\text{O}$ nanoparticles of the hexagonal rhabdophane phase with a tailored Ca^{2+} content and adsorbed citrate on their surface, a chemical feature which is also exhibited by bone apatites [40]. The aspect ratio of both the single crystal domains and the observed nanoparticles decreased as the Ca^{2+} content increases. To explain these results an important issue is the analysis of the growth behavior of the lanthanide orthophosphates. In general, those crystals with hexagonal rhabdophane type structure grow along the $[001]$ direction resulting in the formation of nanowires or nanorods. The growth morphology, however, results from the interplay between the growth rates of the outermost crystallographic planes of the nanocrystals and the inhibiting/promoting effect of the additives or templates in solution. Therefore, the first key influence to consider when analyzing the morphology is that of the crystalline structure.

The overall rhabdophane structure has been described as columns built up of alternate lanthanide ions, with eight fold coordination, and tetrahedral orthophosphate ions [12]. These columns, or periodic bond chains, are extended along the c axis, each one linked to four neighboring columns, consequently forming open channels that run through the structure along the c axis. These large channels incorporate structural H_2O molecules, between 0.5 and 2 molecules per formula unit, which might stabilize the hexagonal crystal structure [28,54]. From a thermodynamic point of view the activation energy for crystal growth along the c axis must be lower than that in perpendicular directions along the a or

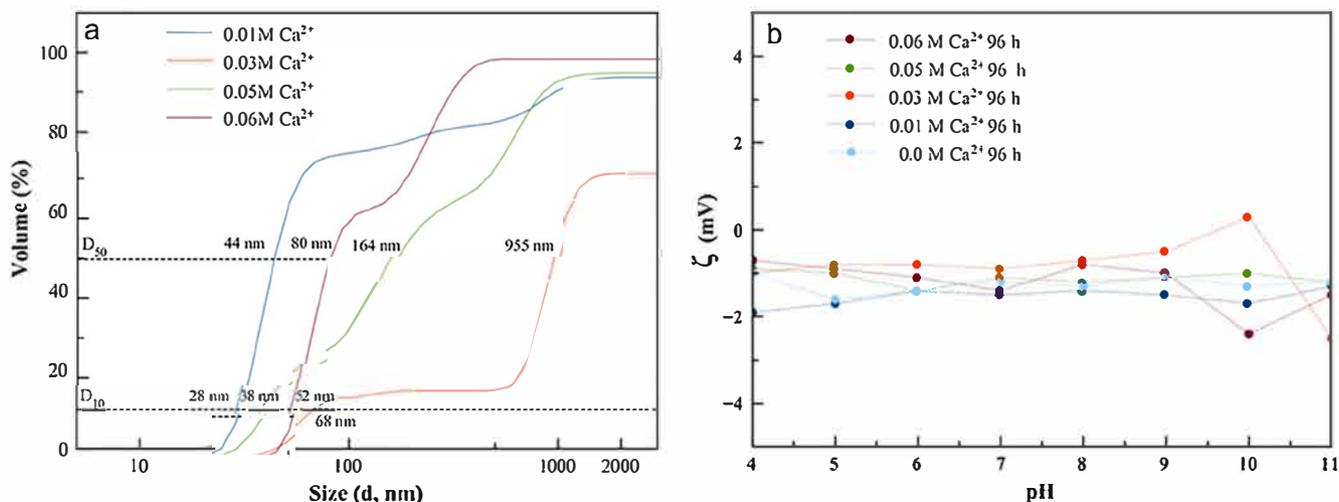


Fig. 5. (a) Cumulative volume oversize distribution of the Ca-doped cit- $\text{EuPO}_4 \cdot \text{nH}_2\text{O}$ nanocrystals prepared at 96 h. (b) ζ -potential vs pH of cit- $\text{EuPO}_4 \cdot \text{nH}_2\text{O}$ and Ca-doped cit- $\text{EuPO}_4 \cdot \text{nH}_2\text{O}$ nanocrystals prepared at 96 h.

b axis, thus indicating that [0 0 1] will be the preferential growth direction.

A second key influential factor is linked to the presence of additives in the solution. Citrate ions are expected to play here two different roles during the precipitation of the nanoparticles: (i) it is a medium strength $M^{2+/3+}$ ligand that enables the preparation of metastable Ca^{2+}/Eu^{3+} /citrate/phosphate/carbonate solutions at slightly basic pH; these complexes can be easily destabilized by increasing the temperature, thus releasing the $M^{2+/3+}$ ions and leading to the precipitation of the Ca^{2+} doped nanoparticles. (ii) After the nucleation event the embryonic nanoparticles, initially amorphous, quickly transformed to crystalline nanophases, which may also be influenced by citrate ions. Taking into account their 3 peripheral carboxylic acid heads, free citrate ions may selectively adsorb on specific outermost planes of forming nanophases, slowing down in particular the growth along the [0 0 1] direction, thus leading to the formation of nanorods instead of long nanowires with high aspect ratios. Most probably these planes are {*h*00} and {*h*01} since faces (1 0 0) and (1 0 1) are displayed on the growth morphology (Fig. 3g).

The trend to lower aspect ratios and formation of ill defined morphologies in the nanoparticles described in this work is thus attributed not only to the effect of incorporating Ca^{2+} in their crystal structure, which affects the type of periodic bond chains, but also to the above effect of citrate. The formation of nanoparticles with lower aspect ratios was also found in other doped nanoparticles in carboxylate free solutions, i.e. doped apatite [55], thus probing that metal ions also contribute to the inhibiting effect of crystal growth along the [0 0 1] direction.

It is also worth noting the behavior of the nanoparticles in water suspensions. We have found that in some of the samples the trend to aggregate is low below the median diameter of the CSD and negligible below D_{10} .

In all samples the ζ potential values are slightly negative at pH 7 and 5 (Fig. 5b). These pH values are close to the physiological pH in blood circulation and to the more acidic pH in the endosome lysosome intracellular compartment, respectively. These low negative values of the ζ potential can be explained on the basis of the balance between the interactions of the negatively charged free carboxylate groups ($-COO^-$) of the adsorbed citrate molecules with the aqueous medium and those with the solid phase. The former should be weaker than the latter, i.e. electrostatic interaction of the $-COO^-$ groups with the partially charged surface groups $>Eu^{3+} > Ca^{2+} > PO_4^{3-}$ of the outermost layer of the nanoparticle surfaces, thus exposing less ionic charge toward the medium. This detail indicates that interactions between nanocrystals within the nanosized range are very weak, so that dispersion is easily achieved by simple agitation, sonication, or else, by a subsequent functionalization of the nanoparticles with a biocompatible ligand.

3.3. Luminescence properties of Ca doped cit $EuPO_4 \cdot nH_2O$ nanoparticles

3.3.1. Luminescence in solid state

It is well known that some lanthanides, especially europium (III) and terbium (III), form highly fluorescent chelates with many different organic ligands [56]. The sensitized fluorescence results from the ligand absorbing light, the energy of which is then transferred to the chelated metal ion. In fact, $Eu(III)$ emits the energy as narrow banded, line type fluorescence with a long Stokes shift (over 250 nm) and an exceptionally long fluorescence decay time (up to 1 ms) [56]. Because of the long fluorescence decay time (over 10 times longer than the average background fluorescence) of $Eu(III)$, a delay time (t_d) and a gate time (t_g) can be used during the measuring, remarkably reducing the background fluorescence.

The luminescence properties of solid Ca doped cit $EuPO_4 \cdot nH_2O$ samples are practically the same as those depicted in Fig. 6 which correspond to $x = 0.03$ M Ca^{2+} ; neither the maturation time nor the Ca^{2+} doping concentrations affect the excitation and emission wavelengths.

The observed excitation wavelengths for the powder were 319, 362, 379, 394 and 465 nm which correspond to the Eu^{3+} ion ${}^7F_0 \rightarrow {}^5H_6$, ${}^7F_0 \rightarrow {}^5D_4$, ${}^7F_0 \rightarrow {}^5I_7$, ${}^7F_0 \rightarrow {}^5L_6$, and ${}^7F_0 \rightarrow {}^5D_2$ transitions, and the emission wavelengths were 577, 590, 614, 650 and 699 nm, which correspond to the Eu^{3+} ion ${}^5D_0 \rightarrow {}^7F_0$, ${}^5D_0 \rightarrow {}^7F_1$, ${}^5D_0 \rightarrow {}^7F_2$, ${}^5D_0 \rightarrow {}^7F_3$ and ${}^5D_0 \rightarrow {}^7F_4$ transitions, respectively [57]. This was expected because the electronic transitions of *f* orbitals are not affected by crystal's field.

The emission wavelength corresponding to the hypersensitive transition (${}^5D_0 \rightarrow {}^7F_2$, 614 nm) produces the highest relative luminescence intensity. This is in agreement with the literature because this emission dominates the spectrum for nanosized particles [28]. Therefore, the optimum excitation and emission wavelengths of solid Ca doped cit $EuPO_4 \cdot nH_2O$ were 394 nm and 614 nm, respectively.

Figure S4 (see SI) shows the effects of Eu (wt%), adsorbed water (wt%) and adsorbed citrate (wt%) from Table 1 on the relative luminescence intensity (R.L.I.). We deduce that the R.L.I. determined in solid state is not affected by only one of these parameters individually. We can see, for instance, that the variation of R.L.I. with adsorbed water is abnormal, because water is a well known quencher of the luminescence. In the same way, we cannot establish a linear relationship between R.L.I. and adsorbed citrate. Most likely several phenomena must overlap and compete (quenching, auto bleaching, energy transfer, effect of chemical structure and so on) that make complex the interpretation of the luminescent behavior in solid state. Anyway, as the main objective of these materials is to be employed for imaging applications, therefore dispersed in aqueous media, a deeper discussion of these aspects affecting the luminescence in solid state are out of the scope of this piece of work.

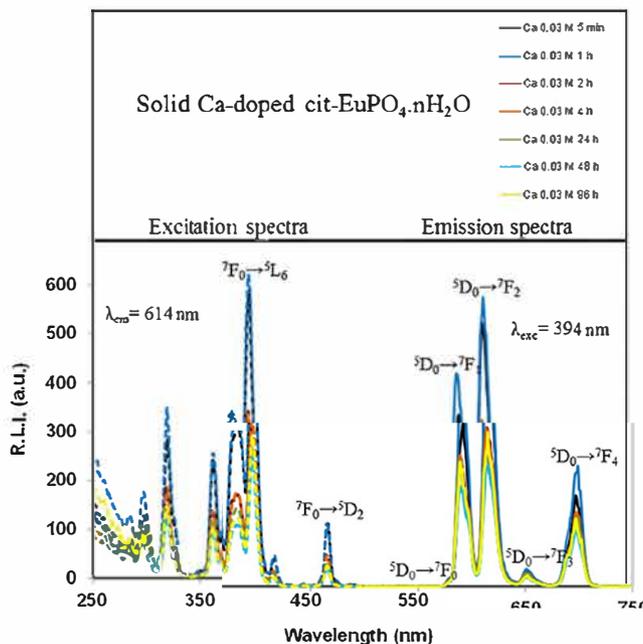


Fig. 6. Excitation (dashed lines) and emission (solid lines) uncorrected spectra of Ca-doped cit- $EuPO_4 \cdot nH_2O$ prepared with $x = 0.03$ M Ca^{2+} at maturation times of 5 min, 1 h, 2 h, 4 h, 24 h, 48 h and 96 h. Slit-widths_{exc/em} = 5/5 nm, $t_d = 120$ μ s and $t_g = 5$ ms.

An important parameter in luminescent complexes is the luminescence lifetime (τ). The decrease of the R.L.I. versus time for pure cit $\text{EuPO}_4 \cdot n\text{H}_2\text{O}$ ($x = 0 \text{ M Ca}$) and for samples prepared with doping concentrations $x = 0.01, 0.03, 0.05$ and 0.06 M at 96 h is shown in Fig. S5 of SI. For each case, the decay profile was analyzed as a single exponential component ($R.L.I. = A e^{-t/\tau} + C$) obtaining τ of 904, 1029, 1119, 1294 and 1083 μs , respectively. It is possible to deduce that the luminescence lifetime for all materials is almost the same, of the order of the millisecond. This order of magnitude is similar to that of colloidal apatite nanoparticles stabilized with a phospholipid moiety, AEP [58], and points out a long luminescence lifetime as compared to the auto fluorescence of biological matter (of the order of the nanosecond), thus allowing us to envision applications as biomedical probes.

3.3.2. Luminescence in aqueous suspension

The luminescence properties of Ca doped cit $\text{EuPO}_4 \cdot n\text{H}_2\text{O}$ samples suspended in water are depicted in Fig. 7, which shows the

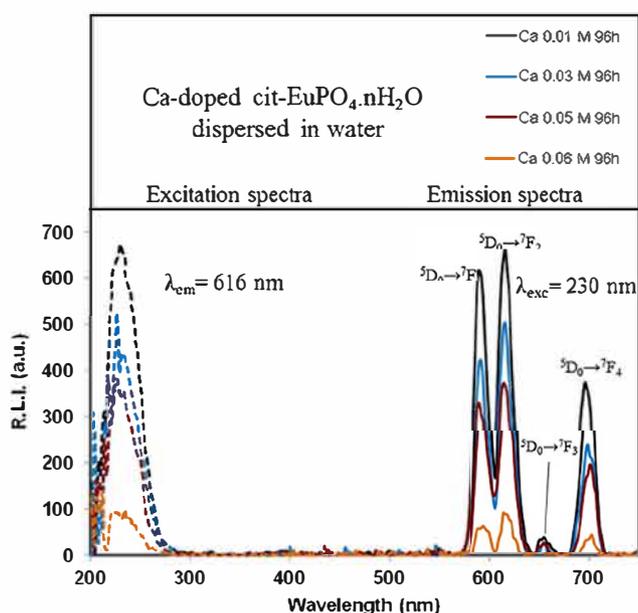


Fig. 7. Excitation (dashed line) and emission (solid line) spectra of Ca-doped cit- $\text{EuPO}_4 \cdot n\text{H}_2\text{O}$ samples prepared with different Ca^{2+} doping concentrations at 96 h suspended in water at 25 °C and pH = 7.0. Slit-widths $_{exc/em} = 5/5 \text{ nm}$, $t_d = 120 \mu\text{s}$ and $t_g = 5 \text{ ms}$.

uncorrected excitation and emission spectra. The maximum excitation wavelength observed was 230 nm. This transition corresponds to an electron that is transferred from the oxygen 2p orbital to an empty 4f orbital of the Europium, which is ascribed as a charge transfer state band [59–61].

The observed emission wavelengths were 590, 616, 647 and 696 nm, which correspond to the Eu^{3+} ion ${}^5\text{D}_0 \rightarrow {}^7\text{F}_1$, ${}^5\text{D}_0 \rightarrow {}^7\text{F}_2$, ${}^5\text{D}_0 \rightarrow {}^7\text{F}_3$ and ${}^5\text{D}_0 \rightarrow {}^7\text{F}_4$ transitions, respectively [57]. The emission wavelength corresponding to the hypersensitive transition (${}^5\text{D}_0 \rightarrow {}^7\text{F}_2$, 616 nm) produces the highest relative luminescence intensity, which once again agrees with the nanosized character of the material [28]. Therefore, the optimum excitation and emission wavelengths of the Ca doped cit $\text{EuPO}_4 \cdot n\text{H}_2\text{O}$ materials dispersed in water were 230 nm and 616 nm, respectively.

On the other hand, it is also possible to observe that an increase on the Ca^{2+} doping concentration (which is related with a decrease of Eu^{3+} content in the material) decreases the luminescence of the materials suspended in water. Analyzing the variation of the luminescence versus the Eu wt% according to Table 1, it is possible to establish a positive linear relationship (see Fig. 8a). Then, in this case, the luminescence variations are basically attributed to the amount of Eu in the materials and, therefore, all the effects that perturb the luminescence on solid state must be attenuated in the aqueous medium.

It is well known that the media affect the luminescence intensities of luminophores [62]. To obtain a deeper understanding, the effects of pH, ionic strength and temperature were evaluated.

The influence of pH on the luminescence intensity was studied over the range 5.0–7.4 (Fig. 8b) and the effect of ionic strength was evaluated by suspending the particles in 0, 25, 50, 75 and 100 mM NaCl solutions (see SI, Fig. S6). It is possible to deduce that neither the pH nor the ionic strength affect significantly the R.L.I. of the suspended materials, which is important for the final applications of these nanoparticles.

The temperature may also theoretically affect the luminescence by quenching of the excited states; i.e. increasing T increases the molecular motion and collisions, and hence decreases the luminescence emission by increasing encounters probabilities [63]. Fig. S7 of SI shows the experimental results. To sum up, increasing from 25 to 37 °C does not affect the luminescence emission of all the tested materials in aqueous suspension (0.05 and 0.01 M Ca^{2+} , maturation time of 96 h). The change in fluorescence intensity is normally 1% per degree Celsius [63] and the decreases for these materials were 0.6 and 1.0% per degree Celsius, respectively. This is very important in view of medical and biomedical imaging ($\sim 37.4 \text{ }^\circ\text{C}$).

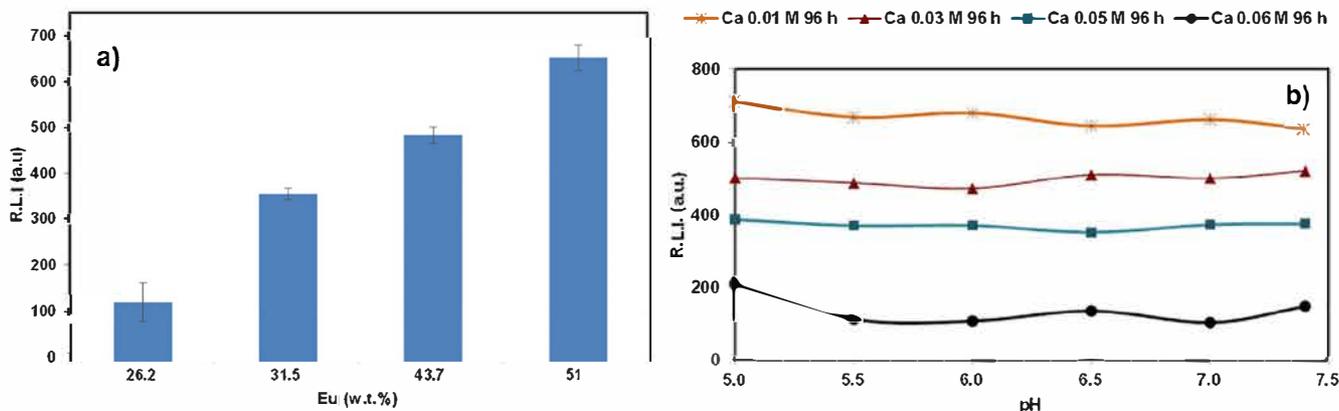


Fig. 8. Variation of R.L.I. of Ca-doped cit- $\text{EuPO}_4 \cdot n\text{H}_2\text{O}$ samples dispersed in aqueous solution at 25 °C: (a) with wt % Eu at pH = 7 and (b) with the pH. $\lambda_{exc/em} = 230/614 \text{ nm}$, slit-widths $_{exc/em} = 5/5 \text{ nm}$, $t_d = 120 \mu\text{s}$ and $t_g = 5 \text{ ms}$.

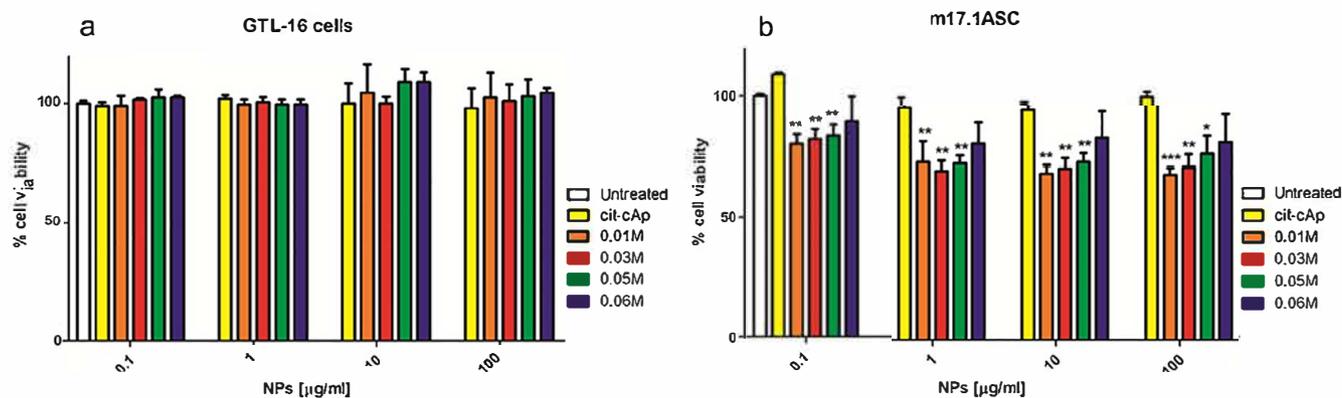


Fig. 9. Viability of GTL-16 cells (a) and of m17.1ASC cells (b) incubated with Ca-doped cit-EuPO₄·nH₂O particles doped with different concentration of Ca²⁺ for three days. Viability was assessed in MTT assays. Data represent means ± sd of three independent experiments performed in quadruplicate and statistical analyses were carried on using One-way ANOVA, with Bonferroni comparison test. For statistical analysis all data were compared to untreated samples (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

3.4. Cytocompatibility of Ca doped cit EuPO₄ nH₂O

The cytocompatibility of cit EuPO₄ nH₂O samples doped with different concentrations of Ca²⁺ was tested in a MTT assay on the GTL 16 human carcinoma cells and on the m17.1ASC murine mesenchymal stem cells, after incubation at concentrations ranging from 0.1 to 100 µg/ml. No toxicity was observed on GTL 16 cells at any nanoparticle concentration (Fig. 9a), since in all cases full cell viability was observed. In the case of mesenchymal stem cells, low levels of Eu³⁺ were fully compatible with cell viability (Fig. 9b). However, if nanoparticles were doped with higher concentrations of Eu³⁺ a certain level of toxicity was observed. In these cases, the addition of doping Ca²⁺ in samples resulted in higher cell viability in a dose response manner. In no case viability was lower than 70%, which is the cut off indicated by ISO 10993 5:2009 [64].

The different behavior of the two cell lines is not unexpected, since it is largely reported from the literature that cell lines can differ in their sensitivity to external stimuli [65,66] and indeed m17.1ASC cells were already found to be less resistant than GTL 16 cells to the stress possibly induced by the presence of nanoparticles [39]. On the other side, both cell types were sensitive to the toxic activity of doxorubicin, which reduced their viability to less than 40%. This *in vitro* evaluation thus pointed out the good cytocompatibility of the Ca doped cit EuPO₄ nH₂O nanoparticles.

4. Conclusions

This work was aimed to prepare luminescent EuPO₄ nH₂O nanophosphors doped with Ca²⁺ and coated with citrate since the presence of both components in the constitutive nanoparticles should be beneficial regarding the cytocompatibility that is required for bioimaging applications.

By applying the bioinspired crystallization route, consisting in thermal decomplexing of Ca²⁺/Eu³⁺/citrate/phosphate/carbonate solutions, we succeeded in preparing Ca doped citrate coated EuPO₄ nH₂O nanoparticles of the hexagonal rhabdophane phase. The general formula of this material, excluding citrate, is Ca_αEu_{1-α}(PO₄)_{1-α}(HPO₄)_α nH₂O, with α ranging from 0 to 0.58 and n ~ 1. Their average dimensions (length and width) are below ~40 nm and ~8 nm respectively, and their aspect ratio decreases from 5.0 to 2.5 when the Ca²⁺ content increases, thus leading to more isotropic and irregular morphologies. Similar trend was found for the dimensions of the single crystal domains, whose coherent length and width are below ~13 and ~4 nm and the aspect ratio decreased from 3.6 to 1.1. In aqueous suspensions, these materials presented a low degree of aggregation below the median size (D₅₀), which become negligible below the percentile 10 (D₁₀), and very

low ζ potential values (mostly between 3 and 0.5 mV) in the pH range from 11 to 4.

Concerning the luminescence properties of these nanoparticles, neither the maturation time nor the concentration of doping agent affected significantly the excitation and emission wavelengths in solid state and aqueous suspensions. In solid state the observed excitation wavelengths were 319, 362, 379, 394 and 465 nm while the emission wavelengths were 577, 590, 614, 650 and 699 nm. In aqueous suspensions the maximum excitation wavelength observed was 230 nm and the emission wavelengths were 590, 616, 647 and 696 nm. In aqueous suspensions it was found that relative luminescence intensity (R.L.I.) is regulated by the Ca²⁺ level doping the structure, decreasing it by increasing the Ca²⁺ content (or decreasing Eu³⁺ amount) of the nanoparticles. Neither the pH (from 5 to 7.5) nor the ionic strength (from 0 to 100 mM NaCl), nor the temperature (from 25 to 37 °C) affected significantly the R.L.I.

This class of luminescent labeling agents is fully cytocompatible when in contact with GTL 16 cells (a human gastric carcinoma cell line) and shows an improved cytocompatibility as the Ca²⁺ content increases, when in contact with the more sensitive m17.1ASC cell line (a spontaneously immortalized mouse mesenchymal stem cell clone from subcutaneous adipose tissue). On the basis of these analysis and the data reported by other authors regarding *in vivo* biocompatibility of Eu doped calcium phosphates nanoparticles [67,68], it is expected that also these Ca²⁺ doped EuPO₄ nH₂O nanoparticles would be biocompatible *in vivo*. They can thus appear as promising new tailorable tools in view of biomedical applications as luminescent nanoprobe. As an example, they could also be the starting blocks to develop multifunctional nanoparticles for efficient drug delivery, once coupled with therapeutic and targeting molecules [34].

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jcis.2018.11.083>.

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