



(51) International Patent Classification:

A61K 9/19 (2006.01) *A61K 9/51* (2006.01)
A61K 6/00 (2006.01) *A61L 24/00* (2006.01)

(21) International Application Number:

PCT/EP2015/066651

(22) International Filing Date:

21 July 2015 (21.07.2015)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

P 201431091 21 July 2014 (21.07.2014) ES

(71) Applicants: **CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS (CSIC)** [ES/ES]; C/ Serrano, 117, E-28006 Madrid (ES). **CONSIGLIO NAZIONALE DELLE RICERCHE** [IT/IT]; Piazzale Aldo Moro, 7, I-00185 Roma (IT).

(72) Inventors: **DELGADO LÓPEZ, José, Manuel**; Instituto Andaluz De Ciencias De La Tierra (IACT), Avda. de las Palmeras, 4, E-18100 Armilla (Granada) (ES). **GÓMEZ MORALES, Jaime**; Instituto Andaluz De Ciencias De La Tierra (IACT), Avda. de las Palmeras, 4, E-18100 Armilla (Granada) (ES). **FERNÁNDEZ PENAS, Raquel**; Instituto Andaluz De Ciencias De La Tierra (IACT), Avda. de las Palmeras, 4, E-18100 Armilla (Granada) (ES). **IAFISCO, Michele**; Istituto di Scienza e Tecnologia dei Materiali Ceramici (ISTEC), Via Granarolo, 64, I-48018 Faenza (IT). **TAMPIERI, Anna**; Istituto di Scienza e Tecnologia dei Materiali Ceramici (ISTEC), Via Granarolo, 64, I-

48018 Faenza (IT). **PANSERI, Silvia**; Istituto di Scienza e Tecnologia dei Materiali Ceramici (ISTEC), Via Granarolo, 64, I-48018 Faenza (IT).

(74) Agent: **PONS ARIÑO, Ángel**; Glorieta de Rubén Darío, 4, E-28010 Madrid (ES).

(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: PROCESS FOR OBTAINING FLUORIDE-DOPED CITRATE-COATED AMORPHOUS CALCIUM PHOSPHATE NANOPARTICLES

(57) Abstract: Process for obtaining fluoride-doped citrate-coated amorphous calcium phosphate nanoparticles. This material has applications in biomedicine due to its biodegradability and bioactivity; it also promotes cell adhesion and osteogenesis. In dentistry, it may be used in toothpastes, mouthwashes, chewing gums, gels and fluoride varnishes as a remineralising agent of dentine and enamel. It is based on two solutions formed by calcium chloride and sodium citrate on the one hand, and by sodium monohydrogenophosphate and sodium carbonate with a fluoride compound on the other, which are mixed at room temperature. The process is eco-efficient and eco-friendly, as it does not leave any acid residue; it consists of a single stage and it is the first time that an amorphous calcium phosphate coated with citrate and doped with fluoride, which enhances its remineralising action, is obtained.



**PROCESS FOR OBTAINING FLUORIDE-DOPED CITRATE-COATED
AMORPHOUS CALCIUM PHOSPHATE NANOPARTICLES**

5 SECTOR AND OBJECT OF THE INVENTION

Biomaterials of interest in biomedicine (i.e., drug delivery nanocarriers and bone regeneration) and dentistry.

The object of the invention is a process for obtaining amorphous calcium phosphate nanoparticles coated with citrate (a molecule that forms part of the organic phase of the bone) and doped with fluoride. This material has a wide range of applications in the field of biomedicine (i.e. drug delivery nanocarriers and bone regeneration) due to its excellent biodegradability and bioactivity, in addition to promoting cell adhesion and osteogenesis. Likewise, it has multiple applications in dentistry, where it may be used in toothpastes, mouthwashes, fluoride varnishes, chewing gum and gels as a remineralising agent of dentine and enamel.

The process is based on two solutions formed by calcium chloride and sodium citrate on the one hand, and sodium monohydrogenophosphate and sodium carbonate with a fluoride compound on the other, which are mixed at room temperature. With respect to the prior art, it has the advantages that the process is eco-efficient, and eco-friendly since it does not leave any acidic residue (strong acids are not used in the synthesis), it is synthesised in a single stage (on using the sodium citrate as a reactive agent in the synthesis) and it is the first time that a citrate-coated and fluoride-doped amorphous calcium phosphate is obtained, therefore with a stronger remineralising action than amorphous calcium phosphate.

STATE OF THE ART

The amorphous phase is a less frequent form of mineral calcium phosphate (CaP) in living organisms. Amorphous calcium phosphate (ACP) has been found in the mitochondria of eukaryotic and prokaryotic cells and is considered the precursor stage in the formation process of the mineral phase of bone, nanocrystalline carbonate-apatite. The surface of this bone apatite has

recently been found to be coated with citrate. ACP also acts as an intermediate phase in the preparation of various CaPs using different methods. This material has a wide range of applications in the field of biomedicine due to its interesting properties such as excellent bioactivity, facilitates cell adhesion and also favours osteoconductivity and osteogenesis. Likewise, it has multiple applications in dentistry, where it may be used in toothpaste, mouthwashes, chewing gum, gels and fluoride varnishes as a remineralising agent of dentine and enamel.

WO98/40406 discloses a product composed of amorphous calcium phosphate stabilised with casein, a phosphoprotein present in milk. This product is currently used as an abrasive material in toothpastes, chewing gum and in tooth whitening post-treatments. However, its efficiency in preventing caries and remineralising damaged varnish has not yet been demonstrated. ACP is also used in compound polymeric materials as a filler material in the preparation of dental pieces. ACP stimulates tooth repair particularly due to the fact that Ca and phosphate ions are released in response to acid pHs generated by bacterial plaque, which are deposited on the tooth structure in the form of hydroxyapatite, regenerating enamel (mainly composed of crystalline hydroxyapatite). Table 1 summarises the main applications of ACP as a biomaterial.

Table 1. Amorphous calcium phosphates used as biomaterials.

Type of material	Application	Effect
Cements	Bone replacement	Hardening agent
	Dentistry	Absorbable with high surface reactivity Source of Ca^{2+} and PO_4^{3-} ions
Coatings	Metallic prostheses	Increases biodegradability and its biological activity
Mineral/organic compounds	Tooth remineralisation	Improves its mechanical properties

	Bone replacement	Release of Ca^{2+} and PO_4^{3-} ions, increasing their biological activity
Aqueous suspension	Release of genes	Absorbable and biocompatible pH-dependent stability

Some of these applications are described in J. Zhao et al., *Amorphous calcium phosphate and its application in dentistry*; Chemistry Central Journal (2011), 5:40 (doi:10.1186/1752-153X-5-40).

As regards the preparation of ACP, it is known in several modalities
 5 obtained from soluble Ca^{2+} and PO_4^{3-} precursors at adequate pHs for precipitation, commonly using soluble precursors whose cation does not give rise to other species that could interfere a posteriori in the composition of the final product, such as $\text{Ca}(\text{OH})_2$, H_3PO_4 , phosphate or ammonium hydrogenophosphate. $\text{Ca}(\text{NO}_3)_2$ is frequently used.

10 The function of citric acid as a Ca^{2+} cation complexer is also known, which is also acceptable from the pharmaceutical viewpoint, in addition to, for example, other polycarboxylic acids such as tartaric acid. For this reason, these acids are also used to stabilise amorphous compositions of ACP. This is set out in the claims of publication WO03059304, where citric acid is proposed, among
 15 other chelate formers with the Ca^{2+} cation, in the proportion 0.1% to 5% by weight in a preparation containing ACP combined with a phosphopeptide.

JP2001169121 proposes the use of citric acid as a stabiliser of ACP already formed by precipitation from phosphoric acid and calcium hydroxide, subjecting it to subsequent milling in the presence of the aforementioned citric
 20 acid.

Therefore, none of these publications mentions a preparation such as the process object of the present invention, in which citrate is added as a reactive agent for ACP precipitation (in a single-stage process) and not as a stabiliser in a phase subsequent to precipitation (two-stage process).

25 The revisions performed by Dorozhkin S. V. [*Nanosized and nanocrystalline calcium orthophosphates*, Acta Biomaterialia (2010), No. 6 (3),

715-734]; Combes C. and Rey C. [*Amorphous calcium phosphates: synthesis, properties and uses in biomaterials*, Acta Biomaterialia (2010), No.6 (9), 3362-3378] and another revision by Dorozhkin S. V. [*Amorphous calcium phosphates*, Acta Biomaterialia (2010), No.6 (12), 4457-4475] disclose several wet processes, but in which the same conditions, process stages and reactive agents of the process object of the present invention are not applied. In fact, citric acid is often envisaged as a dispersant agent in these preparations and occasionally the carbonate anion, with similar functions.

The publication of J.M. Delgado-López et al. *Crystallization of bioinspired citrate-functionalized nanoapatite with tailored carbonate content* (Acta Biomaterialia (2012) No. 8, page 3491) establishes an apatite and a citrate-coated nanocrystalline carbonate-apatite precipitation process. The substantial differences in the state of the art between the process object of the present invention and this document are as follows:

- (1) Precipitation temperature.
- (2) Precipitation of citrate-coated and fluoride-doped ACP nanoparticles as a stable phase.
- (3) There is no maturity process of the precipitate.
- (4) Apatite or any other crystalline phase of the calcium phosphate is not formed in the precipitate.

BRIEF DESCRIPTION OF THE INVENTION

A first object of the present invention is a process for obtaining fluoride-doped citrate-coated amorphous calcium phosphate (FACP) comprising:

- the preparation of a CaCl_2 solution at a concentration comprised between 0.08 M and 0.12 M and $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ (sodium citrate) at a concentration comprised between 0.35 M and 0.50 M;
- the preparation of a second solution formed by Na_2HPO_4 at a concentration comprised between 0.10 M and 0.15 M with Na_2CO_3 0.2 M and a fluoride compound;
- mixture under stirring of the two solutions prepared in the previous stages in the proportion 1:1 v/v at a pH comprised between 8.3 and 8.7 (adjusted, for example, with HCl) and at room temperature for a time period of less than 2

minutes;

- three successive sedimentation cycles by centrifugation, removal of the supernatant and washing of the precipitate with ultrapure water; and
- freeze-drying of the wet precipitate.

5 In a preferred embodiment of the invention, the concentrations of the reactive agents used in the first solution are 0.1 M for CaCl_2 and 0.4 M for $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ and the concentrations used for the second solution are 0.12 M for Na_2HPO_4 and 0.2 M for Na_2CO_3 .

10 The fluoride compound is selected from among CaF_2 , NaF or KF and is added to a concentration comprised between 0.01 M and 0.1 M. In a preferred embodiment, the fluoride compound is CaF_2 and is added to a concentration of 0.05 M.

15 Another object of the present invention is constituted by fluoride-doped amorphous calcium phosphate nanoparticles obtained by the previous process, having a spherical shape and size comprised between 30 nm and 80 nm, as well as the following Na, Ca, P, citrate, carbonate, fluoride and structural water content comprised:

- between 3.1% and 3.5% by weight of Na
- between 27.0% and 27.4% by weight of Ca
- 20 - between 37.0% and 37.8% by weight of P
- between 3.5% and 5.0% by weight of citrate
- between 5.4% and 7.0% by weight of carbonate
- between 6% and 10% by weight of water
- between 2% and 5% by weight of fluoride

25 The term "water" relates in this aspect of the invention both to adsorbed water and structural water.

 In a third aspect, another object of the invention is the use of nanoparticles in applications such as:

- transport of biomolecules and/or drugs
- 30 - biomaterials in orthopaedic applications
- in dentistry, preferably as a material for preparing cements for filling and/or sealing root Canals and dental repairs, or as a component of toothpastes,

chewing gums, mouthwashes, fluoride varnishes and gels to favoring the remineralisation of enamel through the gradual release of calcium, phosphate and fluoride ions.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 shows transmission electron microscopy (TEM) images of the citrate-coated ACP (a) and FACP (b) nanoparticles. The selected area electron diffraction patterns (SAED) obtained for each of the nanoparticles are also shown as insets. The left inset in A shows a TEM image of a single nanoparticle. Panels c and d represent ACP and FACP X-ray energy dispersive
10 (EDS) spectra, respectively.

Figure 2 shows nanoparticles X-ray diffractograms (a) and Raman spectra (b).

Figure 3 shows MTT cell proliferation assays conducted on human osteoblasts incubated for 1, 3 and 7 days with ACP nanoparticles (100 µg/mL,
15 500 µg/mL, 1,000 µg/mL). *p ≤ 0:05; ***p ≤ 0:001; n =3.

EMBODIMENT OF THE INVENTION

ACP nanoparticles were obtained by a precipitation process by mixing two solutions containing:

- (i) 0.1 M CaCl_2 + 0.4 M $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ and
- 20 (ii) 0.12 M Na_2HPO_4 + 0.2M Na_2CO_3

in the proportion 1:1 v/v, a total of 200 ml and adjusting the pH to 8.5 with HCl at ambient temperature.

When the mixture takes on a milky appearance (approximately 30 s after mixing), the particles are subjected to three successive sedimentation cycles by
25 centrifugation, removal of the supernatant and washing of the precipitate with ultrapure water (MilliQ®, Millipore). Subsequently, the wet precipitate is freeze-dried and the particles are subsequently characterised.

In order to obtain these fluoride-doped particles, CaF_2 0.05 M is added to the solution (ii).

30 Characterisation techniques

The nanoparticles were analysed using a Scanning Transmission Electron Microscope (STEM Philips CM 20) operating at 80 kV. This equipment

also allowed the acquisition of selected area electron diffraction (SAED) patterns and X-ray energy dispersive (EDS) spectra. For these analyses, the freeze-dried samples were dispersed in ultrapure water and then a few drops of this suspension were deposited on conventional copper gratings.

5 The amount of Ca and P was quantified using optical emission spectroscopy (ICP-OES) using a Liberty 200 (Varian, Australia) spectrometer. To this end, the freeze-dried samples were dissolved in concentrated ultrapure nitric acid (1% v/v).

10 The thermogravimetric analysis (TGA) was performed using a SDT Q 600 system (TA Instruments, New Castle, DE, USA) under a constant flow of nitrogen ($100 \text{ mL}\cdot\text{min}^{-1}$) and increasing the temperature up to $1,200^{\circ}\text{C}$ at intervals of $10^{\circ}\text{C}\cdot\text{min}^{-1}$.

15 The X-ray diffraction patterns were acquired using an X-Pert PRO (PANalytical) diffractometer equipped with a PIXcel detector operating at 45 kV and 40 mA, with incident Cu $K\alpha$ radiation ($\lambda = 1,5418 \text{ \AA}$). Variable spectral bandwidths (anti-scatter) having a radiation length of 10 mm were used. The 2θ range was varied from 5° to 70° with increments in 2θ of 0.039 .

20 The Raman spectra were obtained using a LabRAMHR spectrometer (Jobin-Yvon, Horiba, Japan). This unit is equipped with a diode laser as an excitation source ($\lambda=532 \text{ nm}$) and a Peltier-cooled CCD detector (1026×256 pixels). The spectra were obtained with a spectral resolution of 3 cm^{-1} .

25 The quantity of fluoride in the samples was quantified by X-ray fluorescence spectroscopy (XRF) using a PHILIPS Magix Pro (PW-2440) spectrometer. Additionally, the fluoride content was also determined by spectrophotometry complexed with zirconyl chloride and eriochrome cyanine R and measuring the absorbance of the complex at 570 nm .

Analysis of in vitro cell culture

30 The biological response of the nanoparticles was evaluated using human osteoblast cell lines (MG-63, Lonza, Italy). The cells were cultured in DMEM/F12 medium (PAA, Austria), containing 10% of fetal bovine serum (FBS) and streptomycin-penicillin (100 U/mL - $100\mu\text{g/mL}$) at 37°C and in a CO_2 atmosphere (5%). Subsequently, the cells were separated from their medium by

trypsinisation and then centrifuged and resuspended. The Trypan blue exclusion test was used to count the live cells (cell viability test). The cells were deposited on 96-well plates with a density of 3.0×10^3 cells per well. Twenty-four hours later, three different concentrations of citrate-coated ACP nanoparticles were added to the cell culture (100 $\mu\text{g/mL}$, 500 $\mu\text{g/mL}$, 1,000 $\mu\text{g/mL}$), previously sterilized by 25 kGy γ radiation. The cells were incubated under standard conditions (37°C, 5% CO_2) for 1, 3 and 7 days. The culture medium was renewed every three days. All these assays were conducted in a laminar flow cabinet.

10 **MTT cytotoxicity and cell viability**

The MTT method [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was used to determine the possible toxic effect of the nanoparticles. This assay is based on the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by the mitochondrial enzyme succinate dehydrogenase in a blue-coloured compound (formazan), whose concentration may be colorimetrically determined, making it possible to determine the mitochondrial functionality of the treated cells.

The cells, after being in contact with the nanoparticles for 1, 3 and 7 days, were incubated in MTT dissolved in PBS (5 mg mL^{-1}) in the proportion 1:10 for 2 hours at 37°C. The cells were then incubated with 200 μL of dimethyl sulfoxide (Sigma) for 15 min to dissolve the formazan crystals. A Multiskan FC Microplate (Thermo Scientific) spectrometer was used to measure absorbance, which is directly proportional to the number of metabolically active cells, at 570 nm. Three samples were analysed for each of the time intervals studied (1, 3 and 7 days).

Results

TEM images (Fig. 1) indicate that both the non-doped samples, ACP (A), and doped samples, FCAP (B), are spherical nanoparticles with sizes comprised between 30 nm and 80 nm. Also, the absence of diffraction points in the SAED patterns evidences their amorphous nature. In turn, the EDS spectra confirm that they are composed only of Ca and P. The F peak in the doped particle spectrum that should appear around 0.68 KeV is not observed, possibly

because it is overlapped by the oxygen peak (0.2 KeV), which is by far more intense. The absence of peaks in the X-ray diffraction patterns confirms the amorphous nature of these materials (Fig. 2A). Raman spectra are also typical of amorphous calcium phosphates, as the main peak appears at 952 cm^{-1} ,
5 slightly shifted with respect to the main crystalline hydroxyapatite peak (961 cm^{-1}). The chemical composition of the ACP and FACP materials obtained by TGA, ICP and X-ray fluorescence has already been described earlier.

The biological response of the nanoparticles was studied using osteoblast cells (MG-63). Three different nanoparticle concentrations (100, 500
10 and $1,000\text{ }\mu\text{g/ml}$) were added to the culture medium and, after a certain incubation period (1, 3 or 7 days), the number of metabolically active cells was quantified by MTT assays (Fig. 3). An increase in cell proliferation was observed in all cases (even for the highest concentration) after 1 to 7 days of incubation. Also, for the lowest concentration studied, cell growth is comparable
15 to that observed by the cells in the absence of nanoparticles (control). However, increasing the concentration, cell growth is much less significant than in the control, possibly due to the fact that they are excessively high nanoparticle concentrations. Despite this, the cell viability and morphology assays (not shown) obtained very similar results for all the concentrations studied. These
20 results clearly indicate that the nanoparticles are completely biocompatible in contact with this human osteoblast cell line.

CLAIMS

1. A process for obtaining fluoride-doped citrate-coated amorphous calcium phosphate nanoparticles comprising:
 - 5 - the preparation of a CaCl_2 solution at a concentration comprised between 0.08 M and 0.12 M and $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ at a concentration comprised between 0.35 M and 0.50 M;
 - the preparation of a second solution formed by Na_2HPO_4 at a concentration comprised between 0.10 M and 0.15 M with Na_2CO_3 0.2 M and a
10 fluoride compound;
 - mixture under stirring of the two solutions prepared in the previous stages in the proportion 1:1 v/v at a pH comprised between 8.3 and 8.7 at ambient temperature for a time period of less than 2 minutes;
 - three successive sedimentation cycles by centrifugation, removal of the
15 supernatant and washing of the precipitate using ultrapure water; and
 - freeze-drying of the wet precipitate.
2. A process, according to claim 1, characterised in that the concentrations of the reactive agents used for the first solution are 0.1 M for CaCl_2 and 0.4 M
20 for $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$.
3. A process, according to any one of claims 1 and 2, characterised in that the concentrations used for the second solution are 0.12 M for Na_2HPO_4 and 0.2 M for Na_2CO_3 .
25
4. A process, according to any one of claims 1 to 3, characterised in that the fluoride compound is selected from among CaF_2 , NaF and KF and is added to a concentration comprised between 0.01 M and 0.1 M.
- 30 5. A process, according to claim 4, characterised in that the fluoride compound is CaF_2 which is added to a concentration of 0.05 M.

6. Citrate-coated and fluoride-doped amorphous calcium phosphate nanoparticles obtained by a process as defined in claims 1 to 5, characterised in that they have a spherical shape and a size comprised between 30 nm and 80 nm and Na, Ca, P, citrate, carbonate, fluoride and water content comprised:

- 5 - between 3.1% and 3.5% by weight of Na
- between 27.0% and 27.4% by weight of Ca
- between 37.0% and 37.8% by weight of P
- between 3.5% and 5.0% by weight of citrate
- between 5.4% and 7.0% by weight of carbonate
- 10 - between 6% and 10% by weight of water
- between 2% and 5% by weight of fluoride.

7. Use of citrate-coated and fluoride-doped amorphous calcium phosphate nanoparticles, as defined in claim 6, as vehicles for biomolecules, drugs or both.

15

8. Use of fluoride-doped citrate-coated amorphous calcium phosphate nanoparticles, as defined in claim 6, as biomaterials in orthopaedic applications.

9. Use of fluoride-doped citrate-coated amorphous calcium phosphate

20

10. Use, according to claim 9, as a material for preparing cements for filling, sealing or both operations in root canals and dental repairs.

11. Use, according to claim 9, as a component of toothpastes, chewing gums, mouthwashes, fluoride varnishes and gels for favouring the remineralisation of enamel through the gradual release of calcium, phosphate and fluoride ions.

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1/3

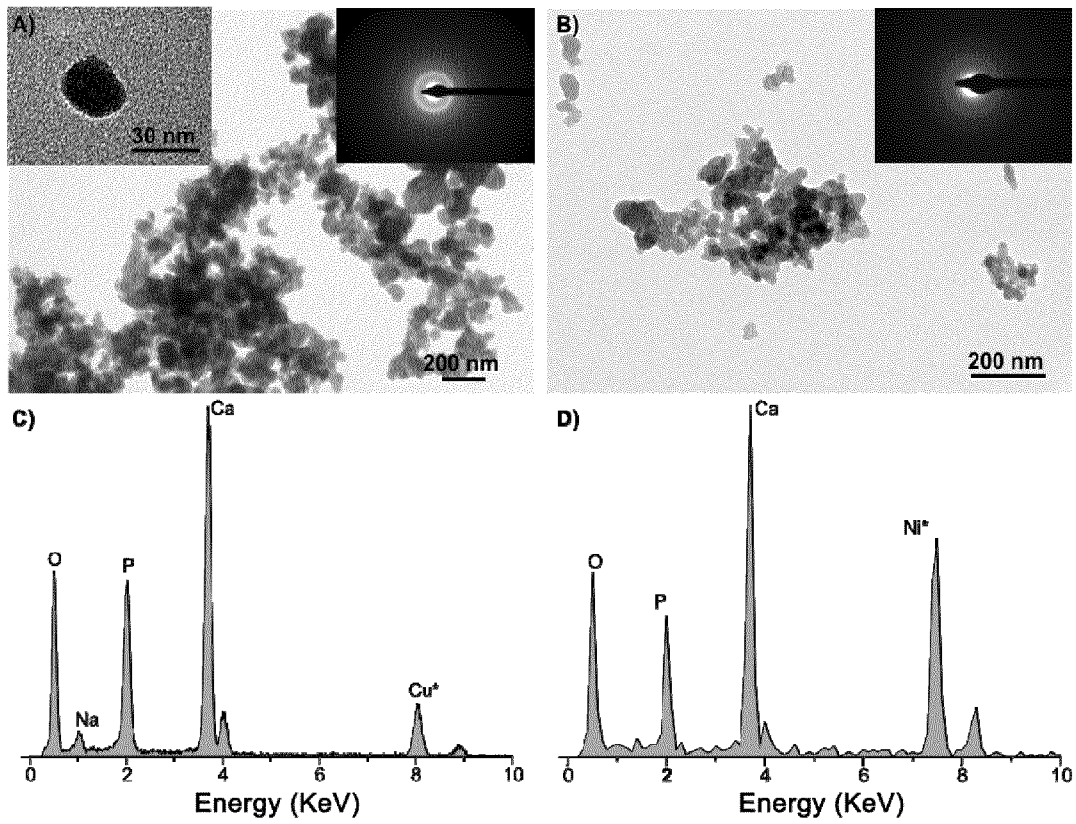
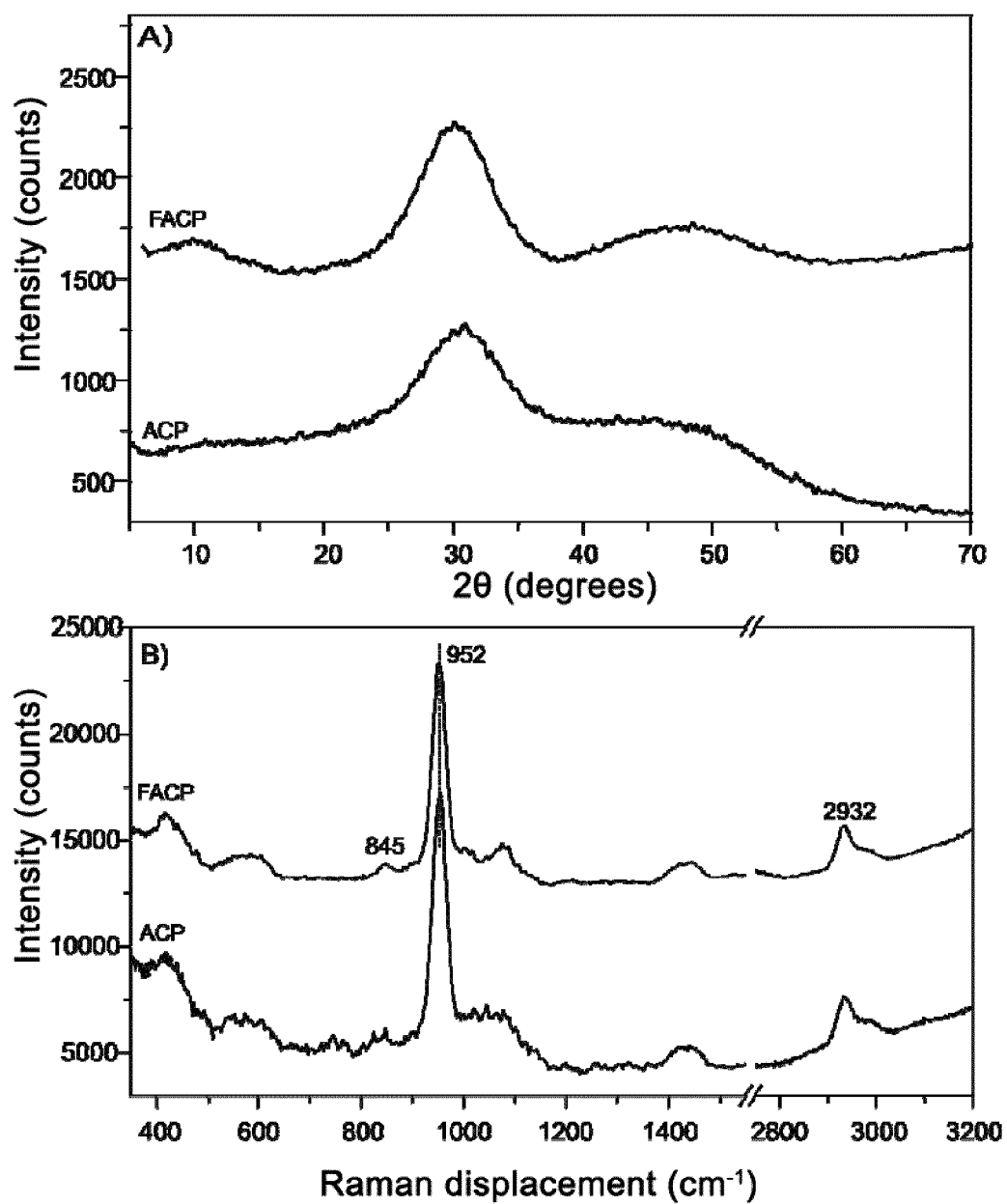


FIG. 1

**FIG. 2**

3/3

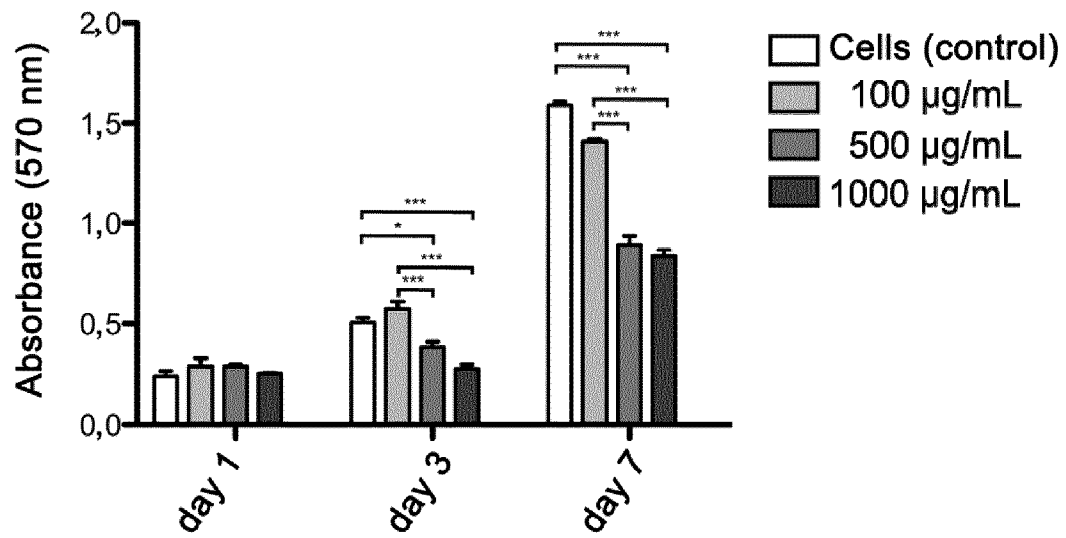


FIG. 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/066651

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K9/19 A61K6/00 A61K9/51 A61L24/00 ADD.											
According to International Patent Classification (IPC) or to both national classification and IPC											
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K A61L Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, EMBASE, WPI Data, BIOSIS											
C. DOCUMENTS CONSIDERED TO BE RELEVANT <table border="1"> <thead> <tr> <th>Category*</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td>Y</td> <td>CN 101 428 779 A (UNIV JIANGSU [CN]) 13 May 2009 (2009-05-13) the whole document</td> <td>1-11</td> </tr> <tr> <td>Y</td> <td>US 2006/110306 A1 (CHOW LAURENCE C [US] ET AL) 25 May 2006 (2006-05-25) claims 1, 8 paragraphs [0073] - [0074], [0007] ----- -/--</td> <td>1-11</td> </tr> </tbody> </table>			Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	Y	CN 101 428 779 A (UNIV JIANGSU [CN]) 13 May 2009 (2009-05-13) the whole document	1-11	Y	US 2006/110306 A1 (CHOW LAURENCE C [US] ET AL) 25 May 2006 (2006-05-25) claims 1, 8 paragraphs [0073] - [0074], [0007] ----- -/--	1-11
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Y	CN 101 428 779 A (UNIV JIANGSU [CN]) 13 May 2009 (2009-05-13) the whole document	1-11									
Y	US 2006/110306 A1 (CHOW LAURENCE C [US] ET AL) 25 May 2006 (2006-05-25) claims 1, 8 paragraphs [0073] - [0074], [0007] ----- -/--	1-11									
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.											
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family											
Date of the actual completion of the international search		Date of mailing of the international search report									
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Schwald, Claudia									

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/066651

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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