



(51) International Patent Classification:

A61K 9/00 (2006.01) *A61P 35/00* (2006.01)
A61K 9/51 (2006.01) *B82Y 5/00* (2011.01)
A61K 33/24 (2006.01) *C01F 17/00* (2006.01)

(21) International Application Number:

PCT/EP2017/057570

(22) International Filing Date:

30 March 2017 (30.03.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

16163838.2 5 April 2016 (05.04.2016) EP

(71) Applicants: **HOSPITAL CLÍNIC DE BARCELONA** [ES/ES]; C. Villarroel 170, 08036 Barcelona (ES). **FUNDACIÓ INSTITUT CATALÀ DE NANOCIÈNCIA I NANOTECNOLOGIA** [ES/ES]; Campus de la UAB, Edificio ICN2, 08193 Bellaterra (ES). **INSTITUCIÓ CATALANA DE RECERCA I ESTUDIS AVANÇATS** [ES/ES]; Pg. Lluís Companys, 23, 3^a pta., 08010 Barcelona (ES). **UNIVERSITAT DE BARCELONA** [ES/ES]; Centre de Patents de la UB, Baldiri Reixac 4 - Torre D, 08028 Barcelona (ES). **CENTRO DE INVESTIGACIÓN BIOMÉDICA EN RED** [ES/ES]; Instituto de Salud Carlos III, Pabellón 11 planta baja, C. Monforte de Lemos 3-5, 28029 Madrid (ES).

(72) Inventors: **JIMÉNEZ POVEDANO, Wladimiro**; C. Magi Casanovas 22, 2^o2^a, 08870 Sitges (ES). **FRANCO PUNTES, Víctor**; C. Miracle, 14, 08028 Barcelona (ES). **FERNÁNDEZ VARO, Guillermo**; C. Roques 23, 08211 Castellar Del Vallès (ES).

(74) Agent: **ZBM PATENTS - ZEA, BARLOCCI & MARK-VARSEN**; Pl. Catalunya, 1 2nd floor, 08002 Barcelona (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, DJ, 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, KH, KN, KP, KR, KW, 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).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

Published:

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

(54) Title: CERIA NANOPARTICLES FOR USE IN THE TREATMENT OF HEPATOCELLULAR CARCINOMA

(57) Abstract: The invention relates to cerium oxide nanoparticles useful in the treatment of hepatocellular carcinoma (HCC), said nanoparticles being cerium oxide single-crystalline nanoparticles of formula (I) NP-(A)_n (I), wherein NP is a single-crystalline cerium oxide nanoparticle with a crystal diameter from 3 to 24 nm; A is a molecule selected from the group consisting of albumin, polyvinylpyrrolidone, and combinations thereof; and n is an integer from 0 to 40, and wherein if the crystal diameter of the nanoparticle is from 3 to 7 nm, then n is an integer from 1 to 12. The invention proposes also particular nanoparticles of formula (I) as conjugates, as well as pharmaceutical compositions comprising them.



Ceria nanoparticles for use in the treatment of hepatocellular carcinoma

This application claims the benefit of European Patent Application 16163838.2 filed April 5, 2016.

5

The present invention relates to the field of medicine, in particular to cancer treatment. It also relates to nanoparticles and conjugates comprising them, as well as to pharmaceutically or veterinary compositions for the treatment of cancer.

10

BACKGROUND ART

Liver cancer is a major health problem for being the second cause of cancer death worldwide. Hepatocellular carcinoma (HCC) is the most common type of liver cancer. Recommended treatments relate to surgical resection, liver transplantation and local ablation. In addition, Sorafenib, a receptor tyrosine kinase inhibitor, may be used in patients with advanced hepatocellular carcinoma. Sorafenib is a small molecule that inhibits tumor-cell proliferation and tumor angiogenesis.

20

Recently, nanomaterials have been proposed for use in the treatment of certain cancers. As a way of example, dextran-coated cerium oxide nanoparticles have been administered to an A375 xenograft model of nude mice in order to test them in a melanoma model. The data are summarized in Alili et al., "Downregulation of Tumor Growth and Invasion by Redox-Active Nanoparticles", Antioxidant & Redox Signaling (Original Research Communication) – 2013, vol. 00, pp.: 1-14 (DOI: 10.1089/ars.2012.4831). Alili et al. concluded that redox-active cerium oxide nanoparticles (CNPs) had selective pro-oxidative and antioxidative properties and could be the basis of a new paradigm in the treatment and prevention of cancer. Dextran-coated cerium oxide nanoparticles implied moreover the advantage of avoiding agglomeration when the individual nanoparticles were of the size 3-5 nm.

Cerium oxide nanoparticles (CNPs) have emerged thus in biomedical applications due to their superoxide dismutase (SOD) and catalase mimetic activity. CNPs act as catalysts with mixed valence that can exist in a reduced (+3) or oxidized (+4) state.

35

However, tests performed in human hepatoma cells have demonstrated that cerium oxide nanoparticles are toxic. As an example, Cheng et al., in "Cerium oxide nanoparticles induce cytotoxicity in human hepatoma SMMC-7721 cells via oxidative stress and the activation of MAPK signalling pathways",
5 Toxicology in vitro – 2013, vol. no. 27, pp.: 1082-1088, demonstrated that CeO₂ nanoparticles, widely used in many industrial fields, promoted a reduced viability, a dramatic morphological damage and induced apoptosis in SMMC-7721 cells. The nanoparticles used by Cheng et al. were hexahedral CeO₂
10 nanoparticles with a primary particle diameter of 20-30 nm. Sizes of particles higher than 25 nm tend to accumulate in tissue and they exert there toxic effects. In addition, it is known that high particle diameters may even not be catalytic, thus ineffective.

15 On the other side, CNPs, or any other nanoparticles with low diameters (fewer than 7 nm) have low residence time in the body, and poor solubility (See Hak Soo et al., "Renal clearance of quantum dots", Nature biotechnology – 2007, vol. no. 25(10), pp.: 1165-1170). Besides, during the preparation of CNPs toxic reagents are employed (such as hexamethylenetetramine; HMT), making
20 them hazardous for biomedical applications if they are not separated from the nanoparticles before, which sometimes is difficult or imply expensive procedures.

Thus, being still a need to find alternative therapeutic approaches for HCC, it
25 is also a need to find compounds with the beneficial effects of nanoparticles in cancer, but avoiding the problems associated with its administration, mainly due to agglomeration and low residence time in the body.

SUMMARY OF THE INVENTION

30 Inventors propose using particular cerium oxide nanoparticles in the treatment of HCC, which surprisingly allowed increasing the survival of treated animals in a remarkably way. Moreover, proliferation of cancer cells was highly reduced when the nanoparticles were administered to mammals with induced
35 HCC. Thus, the treatment avoided tumour growth.

A first aspect the invention relates to single-crystal cerium oxide nanoparticles

of formula (I) for use in the treatment of hepatocellular carcinoma, formula (I) defined by:

NP-(A)_n (I), wherein

5

NP is a cerium oxide nanoparticle with a crystal diameter measured by transmission electron microscopy (TEM) from 3 to 24 nm;

A is a molecule selected from the group consisting of albumin, polyvinylpyrrolidone (PVP), and combinations thereof; and

10

n is an integer from 0 to 40,

and wherein if the crystal diameter of the nanoparticle is from 3 to 7 nm, then n is an integer from 1 to 12.

15

When n is higher than 0, this means that the nanoparticles are conjugated with A, so that they are also called in the present invention as conjugates.

20

Although other cancers have been treated with cerium oxide nanoparticles (see Alili et al., *supra*) effectivity cannot be extrapolated to other cancer types and less in liver cancer, where in vitro data have correlated with high toxic effects (see Cheng et al., *supra*). Thus, the invention provides the unexpected effect of conjugates with CNPs or of CNPs that can be safely applied to HCC treatment. The conjugates or CNPs are effective due to a proper non-agglomeration (or non-aggregation) of the nanoparticles, and due to a size that, though relative small, can be retained in liver.

25

In a second aspect, the invention relates to particular single-crystal cerium oxide nanoparticles of formula (I),

NP-(A)_n (I), wherein

30

NP is a cerium oxide nanoparticle with a crystal diameter from 3 to 7 nm;

A is a molecule selected from the group consisting of albumin, polyvinylpyrrolidone, and combinations thereof; and

35

n is an integer from 1 to 12.

These particular single-crystal cerium oxide nanoparticles of formula (I) are

herewith also named conjugates as above indicated, since n is always an integer higher than 0, namely from 1 to 40 nm.

As exposed before, also these particular conjugates imply the advantages of being effective against liver cancer cells, since despite being small (crystal diameter size of 3-7 nm) and without agglomerating, they are retained in the liver. Once retained, they can exert its function, because they maintain its catalytic function against reactive oxygen species (ROS). In addition they are not toxic at effective doses.

Yet another aspect of the invention is a conjugated single-crystal cerium oxide nanoparticle of formula (I):

NP-(A)_n (I), wherein

NP is a cerium oxide nanoparticle with a crystal diameter from 8 to 24 nm;

A is a molecule of albumin; and

N is an integer from 5 to 40.

These later albumin conjugates with CNPs of formula (I) do not aggregate, and due to its size they are retained in the liver, thus performing their action.

Some authors have reported recently the effects of cerium oxide encapsulated albumin nanoparticles. At this respect it is mentioned the document of Bushan et al., "Antioxidant nanozyme: a facile synthesis and evaluation of the reactive oxygen species scavenging potential of nanoceria encapsulated albumin nanoparticles", Journal of Materials Chemistry B – 2015, vol. no. 3, pp.: 4843-4852. Encapsulation of CNPs (diameter of 4.35 ± 1.07 nm, measured with TEM) in albumin nanoparticles did not alter CNPs functionality in terms of protecting cells from entering oxidative stress (from gene expression studies in L-132 cell line of cervix). The cells were viable (assay performed with MTT dye); and the cellular uptake of CNPs was found to be increased with encapsulated CNPs in a time dependent manner. CNPs of Bushan et al. were synthesized by the hydrothermal method. By TEM images it was seen that the average size of CNPs was of 4.35 ± 1.07 nm. CNPs were then encapsulated inside albumin nanoparticles via a desolvation technique using ethanol as the

desolvating agent and glutaraldehyde as a cross linking agent to obtain least aggregated spherical BCNPs with uniform distribution.

The so synthesized BCNPs allowed concluding that a novel artificial antioxidant encapsulated delivery system had been developed, but the data from Bushan et al. are not conclusive for cancer treatment. In addition, these ceria encapsulated albumin nanoparticles are different from conjugates of formula (I) of present invention.

Another study showing how bovine serum albumin (BSA) is adsorbed to CNPs is derivable from Marsalek et al., "Adsorption of Bovine Serum Albumin on CeO_2 ", International Journal of Chemical, Nuclear, Materials and Metallurgical Engineering -2014, Vol. no. 8(12), pp.: 1269-1272. In this document CNPs are formed and then BSA is adsorbed by means of a vigorous stirring, sonication and further centrifugation. Authors conclude that BSA is preferentially adsorbed onto positively charged surface of the CNPs. With the methodology used by Marsalek et al., it is post-synthesis of the nanoparticles that they are coated with BSA. This procedure has nothing to do with avoiding aggregation of the nanoparticles. Besides, in many of the by Marsalek's disclosed conditions BSA is denaturalized.

The single-crystal cerium oxide nanoparticles of the invention can be administered to mammals in several forms appropriated for administration. Thus, another aspect of the invention is a pharmaceutical or veterinary composition comprising these nanoparticles of formula (I) as defined above, together with one or more pharmaceutically or veterinary acceptable excipients or carriers. These pharmaceutical or veterinary compositions comprising the new single-crystal cerium oxide nanoparticles of the invention are also for use in the treatment of hepatocellular carcinoma.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1, related with Example 2, shows in (A) optical microscope images (200X) of Ki-67-tinted hepatic cells from Wistar rats treated with cerium oxide nanoparticles (CeO_2NPs) or with vehicle, which was a suspension of TMAOH 0.8 mM in phosphate saline buffer (PBS). In FIG. 1(B) the percentage of Ki-67 nuclei hepatocytes per animal are shown for each group.

FIG. 2, related with Example 2, is a graphic with the survival (percentage of survival; %S) of the rats versus the time (T, weeks (W)) after reception of last dose of single-crystal cerium oxide nanoparticles of formula (I) in which A is albumin (CeO₂NPs) or of vehicle as disclosed for FIG. 1.

FIG. 3, related with Example 1 depicts the monitoring of the synthesis kinetics of CeO₂NPs (single-crystal cerium oxide nanoparticles of formula (I) in which A is either albumin or PVP). In FIG. 3 (A) the pH evolution of the synthesis solution. In FIG. 3 (B) UV-visible spectrum of initial cerium (III) precursor (black line); as-synthesized NPs at 1h of reaction, diluted 1/20 (soft grey line); as-synthesized NPs at 48h of reaction, diluted 1/20 (dark grey line). Time in minutes (min), pH_i means initial pH and pH_f means final pH; UV-vis Abs is the absorption in arbitrary units (u.a); and the wavelength is in nanometers (nm).

FIG. 4, also related with Example 1, shows the characterization of PVP-conjugated CeO₂NPs (single-crystal cerium oxide nanoparticles of formula (I) in which A is PVP) by transmission electron microscopy (TEM). In FIG. 4 a) high resolution TEM micrograph (HR-TEM) at high (400.000X) magnification, revealing the atomic planes of single-crystal nanoparticles; in FIG. 4 b) High Angle Annular Dark Field-Scanning Transmission Electron Microscopy (STEM-HAADF) image; in FIG. 4 c) HR-TEM image at low magnification (7.000 check); in FIG. 4 d) atomic resolution HR-TEM image of one single (single-crystal) CeO₂NP, showing spherical morphology and the correspondent atomic planes; in FIG. 4 e) Fast Fourier Transformation (FFT) digital diffractogram calculated from the particle shown in FIG. 4 d); in FIG. 4 f) Electron energy-loss spectroscopy (EELS, X-axis Energy in kiloelectrovolts (keV); Y-axis counts (C) in arbitrary units (a.u.)) map of the chemical composition of as synthesized CeO₂NP. Panels d), e) and F appear in Cont.FIG. 4 as a magnified image of squared single-crystal nanoparticle of panel c) in FIG. 4.

FIG. 5, related with Example 1, is an X-ray diffraction (XRD) pattern of a single-crystal CeO₂ nanoparticle of formula (I) with 5 nm of crystal size diameter.

FIG. 6, related with Example 1, depicts the Dynamic Light Scattering (DLS) of

BSA-coated CeO₂NPs of the invention (single-crystal cerium oxide nanoparticles of formula (I) in which A is albumin). In FIG. 6 a) DLS of the as-synthesized BSA-coated (1 mM) CeO₂NPs, by Intensity. In FIG. 6 b) DLS of the as-synthesized BSA-coated (1 mM) CeO₂NPs, by Number. In FIG. 6 c) DLS of the supernatant only, containing free BSA, by Intensity. In FIG. 6 d) DLS of the supernatant only, containing free BSA, by Number. In X-axis the size (hydrodynamic diameter in nm; d, nm. Y-axis shows percentage (Percent) of Intensity (I) or the percentage of Number (N).

FIG. 7 is an image of X ray contrast imaging of samples at different CeO₂NPs concentrations ranging from 0 to 100 mg/ml.

FIG. 8 is a graphic showing the CeO₂NPs antioxidant effect on fluorescently-labeled hydrogen peroxidase (EuTc-H₂O₂ complex). (a) Commercial 25-nm-sized CeO₂NPs; (b) PVP-coated 5 nm-sized CeO₂NPs (PVP-conjugated CeO₂NPs of formula (I) in which A is PVP); (c) surfactant-free mix of CeO₂NPs (mix of sizes from 6 to dozens of nm; mix, naked); (d) surfactant-free 5-nm-sized CeO₂NPs (naked). (F) is fluorescence and (t) time in minutes(min).

DETAILED DESCRIPTION OF THE INVENTION

All terms as used herein, unless otherwise stated, shall be understood in their ordinary meaning as known in the art. Other more specific definitions for certain terms as used in the present application are as set forth below and are intended to apply uniformly throughout the description and claims unless an otherwise expressly set out definition provides a broader definition.

The term “nanoconjugate” refers to a single-crystal cerium oxide nanoparticle which is attached to another compound selected from polyvinylpyrrolidone and proteins, in particular globular proteins, such as albumin. In particular, the term conjugate, also referred to as NP-A, refers to a single-crystal cerium oxide nanoparticle (NP) which is adsorbed by coordinate bonding to a molecule (A), that can be PVP and/or albumin. By “single-crystal cerium oxide nanoparticle” it is to be understood that said nanoparticle is not aggregated (by weak physical interaction) with other nanoparticles, in such a way that the nanoconjugate comprises only one nanoparticle as a core of the conjugate

and of a particular crystal size diameter (or simply crystal diameter). All these structures can be seen by X-Ray diffraction and TEM. Thus, single-crystal or monocrystal means that there is only one crystal of cerium oxide configuring the nanoparticle, with a particular crystal diameter.

5

The term "nanoparticle" as used herein, refers to a particle with at least two dimensions at the nanoscale, particularly with all three dimensions at the nanoscale, where the nanoscale is the range about 1 nm to about 100 nm, more particularly from 1 to 50 nm, and even more particularly from 5 to 20 nm.

10

Preferred crystal diameters of the nanoparticles of the invention are from 5 to 10 nm (including, 5, 6, 7, 8, 9, and 10 nm), more preferably 5 nm. As regards the shape of the nanoparticles described herein, there are included spheres and polyhedral. In a particular embodiment the nanoparticle is spherical.

15

Particularly, when the nanoparticle is substantially rod-shaped with a substantially circular cross-section, such as a nanowire or a nanotube, the "nanoparticle" refers to a particle with at least two dimensions at the nanoscale, this two dimensions being the cross-section of the nanoparticle.

As used herein, the term "size" refers to a characteristic physical dimension.

20

For example, in the case of a nanoparticle that is substantially spherical, the size of the nanoparticle corresponds to the diameter of the nanoparticle. When referring to a set of nanoparticles as being of a particular size, it is contemplated that the set of nanoparticles can have a distribution of sizes around the specified size. Thus, as used herein, a size of a set of

25

nanoparticles can refer to a mode of a distribution of sizes, such as a peak size of the distribution of sizes. In addition, when not perfectly spherical, the diameter is the equivalent diameter of the spherical body including the object.

The term "cerium oxide" or "ceria" refers to cerium (III) oxide (Ce^{3+}) and cerium (IV) oxide (Ce^{4+}) species that are both present when constituting the nanoparticles. Although many of the cerium oxide nanoparticles are usually in the (Ce^{4+}) oxidation state, small cerium oxide nanoparticles are also in the (Ce^{3+}) oxidation state. Cerium oxide nanoparticles (abbreviated also as Ceria-NP, CeO_2 -NP or simply CNP) are used in a variety of applications mainly due to its high surface area and the ability of cerium oxide to cycle between (III and IV) oxidation states. In a particular embodiment, cerium oxide nanoparticles are in the (Ce^{4+}) oxidation state

35

When in this description the diameter of the nanoparticle is mentioned, it relates to the crystal size diameter (single-crystal). Crystal size is usually measured from X-ray diffraction patterns while particle size is measured by TEM. In the case of single-crystal nanoparticles, XRD and TEM sizes coincide. Typical crystal size diameters of the nanoparticles used in the present invention range from 3 to 24 nm (including 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 and 24 nm), when measured by TEM, more particularly the crystal size diameter of the nanoparticles is about 5 nm.

The “hydrodynamic diameter” determined by dynamic light scattering (DLS) techniques based on the Stoke-Einstein equation is used herewith to refer to the single-crystal nanoparticle conjugated (also termed herein coated/encapsulated) with the compound defined by A. It can be measured by illuminating the particles with a laser and analysing the intensity fluctuations in the scattered light. Dynamic light scattering measures Brownian motion and relates it to the size of the particles for which light intensity is proportional to the square of the volume of the particle. The resulting diameter corresponds not only to the single-crystal particle diameter, but also to the molecules strongly adsorbed onto its surface. Therefore, the hydrodynamic size is always larger than the size observed by transmission electron microscopy, this later the one of the single-crystal nanoparticles. Typical hydrodynamic diameters of the single-crystal cerium oxide nanoparticles of formula (I) with n higher than 0 and used in the present invention range from 5 to 60 nm, more particularly from 5 to 30 nm. In this particular case, the hydrodynamic diameter is a function of n in formula (I).

Finally, when in the present invention it appears the expression “combinations thereof”, it relates to the combination of molecules of albumin and polyvinylpyrrolidone in the conjugated cerium oxide nanoparticles of formula (I), each of them adsorbed on the nanoparticle surface in order to make the nanoparticles compatible for the body (biocompatible). The combinations encompass one molecule of each type, or several molecules of each type depending of the surface of the nanoparticle directly correlated with its hydrodynamic diameter. The conjugates, in particularly those comprising albumin or other proteins adsorbed on the nanoparticle surface, have the

property of being captured on the cell surface, mainly due to this so-called “protein corona”. They can so penetrate into the cells, in this case into hepatocytes, and the release of the metal ions performs therein its effects, mainly by scavenging reactive oxygen species (ROS).

5

In a particular embodiment of the first aspect of the invention, single-crystal cerium oxide nanoparticles of formula (I) for use in the treatment of HCC have a NP with a crystal diameter from 3 to 15 nm. In yet another particular embodiment the crystal size diameter of the NP are from 3 to 12 nm, more particularly from 3 to 7 nm, and even more particularly from 4 to 7 nm, more in particular 5 nm. As above indicated when the crystal size diameter of NP is from 3 to 7 nm, then n is from 1 to 12.

10

In this particular case, wherein the crystal size diameter of the NP is from 3 to 7 nm, it is also said that the single-crystal cerium oxide nanoparticles of formula (I) are in form of a conjugate of formula (I) for being used in the treatment of HCC:

15

NP-(A)_n (I), wherein

20

NP is a cerium oxide nanoparticle with a crystal diameter from 3 to 7 nm;

A is a molecule selected from the group consisting of albumin, polyvinylpyrrolidone, and combinations thereof; and

25

n is an integer from 1 to 12.

On the other side, when crystal size diameters of NP in formula (I) are higher than 7 nm, these single-crystal cerium oxide nanoparticles of formula (I) can be used without being conjugated to albumin and/or PVP, thus n being an integer with value 0. The reason for that higher than 7 nm crystal size diameter, is to avoid fast renal clearance of the nanoparticles.

30

In another particular embodiment of the first aspect, optionally in combination with any embodiment above or below, NP is a cerium oxide nanoparticle with a crystal diameter from 3 to 24 nm; A is a molecule selected from the group consisting of albumin, polyvinylpyrrolidone, and combinations thereof; and n is an integer from 1 to 40. In this particular embodiment, the single-crystal

35

cerium oxide nanoparticles for use in the treatment of HCC are conjugates.

In yet another particular embodiment of the first aspect, the single-crystal cerium oxide nanoparticle is one in which NP is a cerium oxide nanoparticle
5 with a crystal diameter from 3 to 12 nm.

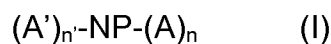
In another particular embodiment, the single-crystal cerium oxide nanoparticle for use in HCC, is one in which NP is a cerium oxide nanoparticle with a crystal diameter from 3 to 7 nm, more particularly from 4 to 5 nm, A is a
10 molecule selected from the group consisting of albumin, polyvinylpyrrolidone, and combinations thereof; and n is an integer from 1 to 12.

In another more particular embodiment of the first aspect, optionally in combination with the embodiments above or below, the single-crystal cerium
15 oxide nanoparticles are those of formula (I) in the form of conjugates, wherein A is a molecule of albumin, n is an integer from 1 to 40, and NP is a cerium oxide nanoparticle with a crystal size diameter from 3 to 24 nm.

Another particular embodiment of the first aspect relates to a single-crystal
20 cerium oxide nanoparticle for use in the treatment of HCC, in which NP is a cerium oxide nanoparticle with a crystal size diameter from 3 to 7 nm, A is a molecule of albumin; and n is an integer from 1 to 12. In yet another more particular embodiment, NP is a single-crystal cerium oxide nanoparticle with a crystal size diameter from 4 to 5 nm, and n is from 1 to 4, more particularly, n is
25 1.

Another particular embodiment of the first aspect of the invention relates to a single-crystal cerium oxide nanoparticle for use in the treatment of HCC, in which NP is a cerium oxide nanoparticle with a crystal diameter from 3 to 24
30 nm, A is a molecule of polyvinylpyrrolidone, and n is an integer from 1 to 40. In another more particular embodiment, in the single-crystal cerium oxide nanoparticle, NP is a cerium oxide nanoparticle with a crystal diameter from 3 to 7 nm, and n is an integer from 1 to 12. In yet a more particular embodiment, A is a molecule of polyvinylpyrrolidone; NP is a single-crystal cerium oxide
35 nanoparticle with a crystal size diameter from 4 to 5 nm, and n is from 1 to 4, more particularly, n is 1.

Another particular embodiment of the first aspect of the invention relates to single-crystal cerium oxide nanoparticles of formula (I) for use in the treatment of HCC, in which NP is a cerium oxide nanoparticle with a crystal size diameter from 3 to 7 nm, A is a combination of molecules of albumin and polyvinylpyrrolidone; and n is an integer from 2 to 12, most particularly 2 to 4. The particular conjugates, in which a combination of molecules of albumin and polyvinylpyrrolidone are adsorbed to the NP can be represented as a subgroup of conjugates of formula (I) as follows:



A' is polyvinylpyrrolidone; A is albumin; and n and n' are integers from 1 to 2, and particularly n and n' are both 1.

All these above disclosed cerium oxide nanoparticles of formula (I) are in particular for use as inhibitors of cell proliferation in hepatocellular carcinoma.

In yet another particular embodiment of the first aspect of the invention, the single-crystal cerium oxide nanoparticles of formula (I) are adapted for use as a co-treatment of an hepatocellular carcinoma therapy selected from the group consisting of radiotherapy, microwave ablation (MWA), radiofrequency ablation (RFA), transarterial radioembolization (TARE), chemotherapy, surgical resection, liver transplantation, targeted therapy, hyperthermia and combinations thereof.

More in particular, the single-crystal cerium oxide nanoparticles of formula (I) are adapted for use as a co-treatment of an hepatocellular carcinoma therapy selected from the group consisting of radiotherapy, transarterial radioembolization (TARE), chemotherapy, targeted therapy, hyperthermia and combinations thereof.

As "co-treatment" is to be understood that the nanoparticles are for simultaneous, separate or sequential use with other HCC therapies.

Thus, the single-crystal cerium oxide nanoparticles of formula (I) are for use in the treatment of hepatocellular carcinoma in combination with an

hepatocellular carcinoma therapy agent and/or drug, in particular, the agent and/or drug selected from the group consisting of a radiotherapy agent, a chemotherapeutic drug, a targeted therapy drug, and combinations thereof.

- 5 This means that the single-crystal cerium oxide nanoparticles of formula (I) for use in the treatment of HCC, are for use in combination with an hepatocellular carcinoma therapy agent and/or drug selected from the group consisting of a radiotherapy agent, a chemotherapeutic drug, a targeted therapy drug, and combinations thereof.

10

As “an hepatocellular carcinoma therapy agent and/or drug” is to be understood as any drug used in this kind of cancer, as well as any compound commonly used in hepatocellular carcinoma therapies, such as the compounds used in radiotherapy for promoting radiosensitization and radioenhancement in combination with the radiation itself. It also encompasses agents used in transcatheter arterial chemoembolization (TACE), heat sensitive compounds (i.e, liposomes) for use in radiofrequency ablation (RFA), as well as drugs (i.e sorafenib) for a targeted therapy.

15

- 20 As will be shown in the examples below, cerium oxide nanoparticles of formula (I) absorb radiation, such as ionizing radiation (including X radiation, gamma rays and higher part of ultraviolet radiation) in a highly efficient manner. The efficiency of this process (for both energy transfer and biodistribution) is mainly due to heavy atoms nanoparticles with small size and to the fact that they are not aggregated (but single-crystals). For the same reason, transfer of energy is also high with these nanoparticles. All these properties make them good as contrast agents (in particular as X-ray contrast agents), as well as good in radiotherapy (radiosensitization and radioenhancement), since effect of radiation is enhanced. Thus in a more particular embodiment, the single-crystal cerium oxide nanoparticle of formula (I) are for use in the treatment of hepatocellular carcinoma in combination with a radiotherapy agent. Thus, the single-crystal cerium oxide nanoparticle of formula (I) are for use in the treatment of hepatocellular carcinoma in combination with radiotherapy.

25

30

35

Further, the single-crystal cerium oxide nanoparticle of formula (I) are for use in combination with a targeted therapy agent (also named targeted therapy compound), which can be formulated as that they are for use as co-treatment

of a targeted therapy for HCC. Targeted therapy is defined as the specially targeted delivery vehicles to increase effective levels of chemotherapy for tumor cells while reducing effective levels for other cells. This result in an increased tumor kill and/or reduced toxicity. Particular targeted therapies of HCC include administration of sorafenib. Therefore, the nanoparticles for use according to the invention are, in a particular embodiment for use in combination with sorafenib, particularly in advanced stages of the disease, and optionally with surgical resection.

In addition, CeO₂ NPs for use in the treatment of HCC can also be adapted to be used in combination (as co-treatment) with other chemotherapeutic approaches commonly used in this type of cancer and selected from the group consisting of transcatheter arterial chemoembolization (TACE), transcatheter arterial chemotherapy (TAC), intra-arterial infusion chemotherapy (HAC), systemic chemotherapy (SCT), portal vein chemotherapy (PVC), portal vein embolization (PVE), percutaneous acetic acid injection (PAI), percutaneous ethanol injection (PEI), and combinations thereof.

The single-crystal nanoparticles for use according to the invention are used in combination with one or more of the above therapies, and/or agents for use in these therapies, depending on the stage of the disease (i.e of the HCC). Thus, for example TACE is used in initial stages of HCC and HAC in advanced stages, this later in combination with surgery resection of the tumour and/or liver transplantation. Targeted therapies including the sorafenib are used also in advanced stages of HCC and in combination with surgery resection of the tumour and/or liver transplantation.

The invention relates as a second aspect to particular single-crystal cerium oxide nanoparticles of formula (I),

NP-(A)_n (I), wherein

NP is a cerium oxide nanoparticle with a crystal diameter from 3 to 7 nm;

A is a molecule selected from the group consisting of albumin, polyvinylpyrrolidone, and combinations thereof; and
n is an integer from 1 to 12.

Particular embodiments of the second aspect of the invention, thus of the conjugated single-crystal cerium oxide nanoparticles are those in which NP is a cerium oxide nanoparticle with a crystal diameter from 4 to 5 nm.

5

In a more particular embodiment of the second aspect of the invention, optionally in combination with any embodiment above or below, in the single-crystal cerium oxide nanoparticle, A is a molecule of albumin; and n is an integer from 1 to 4, more particularly n is 1. Most particularly, A is albumin; NP is a cerium oxide nanoparticle with a crystal diameter from 4 to 5 nm; and n is an integer from 1 to 4, more particularly n is 1.

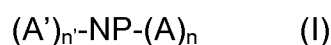
10

In another more particular embodiment of the second aspect of the invention, optionally in combination with any embodiment above or below the single-crystal cerium oxide nanoparticle is the one of formula (I), wherein A is a molecule of polyvinylpyrrolidone; and n is an integer from 1 to 4, more particularly 1. Most particularly, A is polyvinylpyrrolidone; NP is a cerium oxide nanoparticle with a crystal diameter from 4 to 5 nm; and n is an integer from 1 to 4, more particularly n is 1.

15

20

The invention relates also to single-crystal cerium oxide nanoparticles as conjugates of formula (I):



25

wherein A' is polyvinylpyrrolidone; A is albumin; and n and n' are integers from 1 to 2, and particularly n and n' are both 1.

30

In a particular embodiment of any of the first and second aspects of the invention, optionally in combination with any of the embodiments, PVP has a molecular weight from 10 kDa to 30 kDa, more in particular from 10 kDa to 20 kDa, being preferred PVP of 10 kDa.

35

In another particular embodiment of any of the aspects of the invention, optionally in combination with any of the embodiments, albumin is mammal albumin, in particular selected from human serum albumin and bovine serum albumin. Human serum albumin is the one defined in in UniProtKB database

by accession number P02768, version 255 of the entry and version 2 of the sequence of January 20, 2016. Bovine serum albumin is the one defined in in UniProtKB database by accession number P02769, version 150 of the entry and version 4 of the sequence of December 9, 2015.

5

Single-crystal cerium oxide nanoparticles of formula (I), in which n is zero and NP has a diameter higher than 7, particularly from 8 to 24 nm, are obtainable by a method comprising (a) dissolving a cerium (III) salt, particularly cerium (III) nitrate, in water or in an organic solvent, particularly ethanol; (b) adding an oxidizing and stabilizer compound, in particular selected from tetramethylammonium (TMAOH) hydroxide and hexamethylenetetramine (HMT) in a ratio of volume of solution (a) per volume of oxidizing and stabilizer compound from 95/5 (v/v) to 80/20 (v/v), more in particular of 90/10 (v/v); and (c) optionally centrifuging and resuspending the nanoparticles in aqueous solution, said aqueous solution also optionally comprising TMAOH as stabilizer. The oxidizing and stabilizer compound avoids aggregation at the moment nanoparticles are formed.

10

15

On the other side, the single-crystal cerium oxide nanoparticles of formula (I), in which n is higher than zero, thus the conjugated nanoparticles with n from 1 to 40, are obtainable by a method comprising the steps of:

20

25

30

35

- (i) Preparing a solution 1 comprising albumin, polyvinylpyrrolidone, or mixtures thereof in a buffered solvent, particularly water, with a pH from 6.0 to 8.5;
- (ii) Preparing a solution 2 of a cerium (III) salt in water wherein,
 - if in the nanoparticle of formula (I) A is albumin, and optionally PVP, then an oxidizing and stabilizer compound is added in this solution 2, in particularly selected from TMAOH and HMT, and preferably TMAOH, and
 - if in the nanoparticle of formula (I) A is only PVP next step (iii) is performed;
- (iii) Adding solution 2 to solution 1, wherein
 - if in the nanoparticle of formula (I) A is only PVP, an oxidizing and stabilizer compound is added in this step (iii), in particularly selected from TMAOH and HMT, and preferably TMAOH; and
- (iv) Let the mixture react for a period of time enough to obtain single-

crystal CeO₂NPs (CNPs), with a crystal size diameter measured by TEM from 3 to 24 nm, in particular from 3 to 15 nm and more in particular from 3 to 12, and even more in particular from 3 to 7 nm, said CNPs being conjugated with albumin, PVP or mixtures thereof, thus being single-crystal cerium oxide nanoparticles of formula (I) with n from 1 to 40, and having a hydrodynamic diameter from 10 to 32 nm, in particular from 11 to 23 nm, the hydrodynamic diameter measured by DLS.

In a particular embodiment, TMAOH is preferred in any of the steps wherein it is used, since it is non-toxic to cells. In another particular embodiment, the cerium (III) salt is cerium (III) nitrate. TMAOH acts as oxidizing agent, as surfactant and also as stabilizer because it avoids nanoparticle aggregation.

In another preferred embodiment, the buffered solvent in (i) is phosphate-buffered saline (PBS) with a pH from 6.0 to 8.5 that comprises water and salts selected from sodium hydrogenphosphate, sodium chloride and mixtures thereof, and optionally potassium chloride and potassium dihydrogenphosphate.

In another particular embodiment of the method, from which single-crystal cerium oxide nanoparticles in which n is from 1 to 40 are obtainable, solution 2 has a pH below 9, preferably from 6.0 to 8.5, more preferably from 7.0 to 8.0.

As a general rule, when the CeO₂NPs are synthesized, the pH of the solution decreases and the surface charge of the nanoparticle decreases losing the ability to remain un-aggregated thanks to electrostatic repulsion. Procedures to de-aggregate CeO₂NPs after synthesis have been proven unsuccessful (despite they work for other metal oxides as iron oxide). Therefore, the stabilizer for colloidal stability and to avoid CeO₂NPs aggregation (in this particular case of the invention, the stabilizers being PVP, albumin or a combination of both) has to be administrated before aggregation takes place, which is immediately after nucleation, this is, at the beginning of the synthesis process. Besides, other stabilizers like TMAOH can be added during synthesis.

In another particular embodiment, the cerium (III) salt is particularly cerium (III)

nitrate, and in yet another more particular embodiment it is in a concentration at solution 2 to give an initial cerium (III) concentration from 9.0 mM to 10.0 mM.

- 5 In a particular embodiment of the single-crystal cerium oxide nanoparticles obtainable by this method, the final concentration of albumin in solution 1 is from 0.70 to 0.80 mM, more particularly 0.75 mM.

10 In all these embodiments, during steps (iii) and (iv) exchange of adsorbed TMAOH by albumin, PVP or the combinations of both on the surface of cerium oxide nanoparticles takes place. Residual traces of TMAOH can be present in the final single-crystal cerium oxide nanoparticles of formula (I). When added, TMAOH is preferably added dropwise.

15 Thus, in a particular embodiment, the single-crystal cerium oxide nanoparticles of formula (I) of the invention, wherein NP is a cerium oxide nanoparticle with a crystal diameter from 3 to 7 nm; A is a molecule selected from the group consisting of albumin, polyvinylpyrrolidone, and combinations thereof; and n is an integer from 1 to 12, are obtainable by a method
20 comprising the steps of:

- (v) Preparing a solution 1 comprising albumin, polyvinylpyrrolidone, or mixtures thereof in a buffered solvent, particularly water, with a pH from 6.0 to 8.5;
- 25 (vi) Preparing a solution 2 of a cerium (III) salt in water wherein,
 - if in the nanoparticle of formula (I) A is albumin, and optionally PVP, then an oxidizing and stabilizer compound is added in this solution 2, in particularly selected from TMAOH and HTM, and preferably TMAOH, and
 - 30 - if in the nanoparticle of formula (I) A is only PVP next step (iii) is performed;
- (vii) Adding solution 2 to solution 1, wherein
 - if in the nanoparticle of formula (I) A is only PVP, an oxidizing and stabilizer compound is added in this step (iii), in particularly
35 selected from TMAOH and HTM, and preferably TMAOH; and
- (viii) Let the mixture react for a period of time enough to obtain single-crystal CeO₂NPs (CNPs), with a crystal size diameter measured by

TEM from 3 to 7 nm, said CNPs being conjugated with albumin, PVP or mixtures thereof, thus being single-crystal cerium oxide nanoparticles of formula (I) with n from 1 to 12, and having a hydrodynamic diameter from 11 to 23 nm, the hydrodynamic diameter measured by DLS.

With these methods, and as above reasoned, aggregation of the nanoparticles does not take place, thus the nanoconjugates are constituted by single-crystal nanoparticles, duly conjugated with albumin, PVP or both. In addition, these single-crystal cerium oxide nanoparticles of formula (I), in which n is higher than zero, thus the conjugated nanoparticles with n from 1 to 40, and obtainable as indicated in any of the above embodiments, are catalytically active single-crystal cerium oxide nanoparticles. The nanoparticles being catalytically active means that they retain its superoxide dismutase (SOD) and catalase mimetic activity. This catalytically active feature can be measured by many technologies, such as for example, by means of the CeO₂NPs antioxidant effect on fluorescently-labeled hydrogen peroxidase (EuTc-H₂O₂ complex), Amplex Red or 2',7'-dichlorofluorescein diacetate (DCFDA).

Particular pharmaceutical or veterinary compositions comprising the nanoparticles as defined above, are those further comprising physiologic saline buffer, such as Phosphate-buffered saline (PBS). By physiologic saline buffer is to be understood a water-based salt solution with osmolarity, pH (6-7.5) and ion concentrations of mammal bodies, in particular of humans. Particular salts and ions are sodium hydrogenphosphate, sodium chloride and optionally potassium chloride and potassium dihydrogenphosphate. In another particular embodiment, the pharmaceutical or veterinary compositions comprise the serum of the individual mammal to which the composition is to be administered.

Throughout the description and claims the word "comprise" and variations of the word, are not intended to exclude other technical features, additives, components, or steps. Furthermore, the word "comprise" encompasses the case of "consisting of". Additional objects, advantages and features of the invention will become apparent to those skilled in the art upon examination of the description or may be learned by practice of the invention. The following

examples are provided by way of illustration, and they are not intended to be limiting of the present invention. Furthermore, the present invention covers all possible combinations of particular and preferred embodiments described herein.

5

EXAMPLES

Characterization techniques

10 1) Transmission microscopy:

Morphology of the engineered NPs was analyzed by High-Resolution Transmission Electron Microscopy (HR-TEM, FEI Tecnai G2 F20 200 kV). The ultrafin 200-mesh copper TEM grids (Ted-Pella, Inc.) were placed on a filter paper. Afterwards, fifty microliters of the NPs colloidal suspension were deposited drop by drop over the grid and left to dry in air. The obtained HR-TEM images were analyzed by Image J program: for each sample, at least 150 NPs were measured and the average size and size-distribution were obtained.

15 2) UV-Vis Spectroscopy:

UV-visible spectra was acquired with Shimadzu UV-2400 spectrophotometer. Regarding the detection limits of the apparatus and a high concentration of the synthesis, an aliquot of the synthesis solution was diluted in water (1/15) and then measured in a wavelength range between 260 and 800 nm.

3) ZP, DLS:

25 A 1 ml aliquot of the synthesis solution was placed in a sizing cuvette and analyzed by a Zetasizer Nano-ZS (Malvern Instruments). Thus, the surface charge (Zeta Potential, ZP), the Dynamic Light Scattering (DLS) and the Isoelectric Point (pI) of the nanoparticles were measured.

4) X-ray diffraction (XRD):

A 10 ml aliquot of the synthesis solution was centrifuged twice (45 min at 12.000g), in order to ensure a maximal precipitation of the nanoparticles. A supernatant was discarded and the precipitates were dried at room temperature for 2 days, to avoid further NP transformations.

- 5 The data were collected on a PANalytical X-Pert PRO MPD diffractometer using a Cu K α 1 radiation source ($\lambda = 1.541 \text{ \AA}$). The 2θ diffraction (Bragg) angles were measured by scanning the goniometer from 20° to 100° .

The peak positions and their width at half maximum (FWHM) were determined by the X-Pert High Score program after the baseline correction.

10

Example 1. Synthesis of cerium oxide nanoparticles.

To obtain lipopolysaccharide (LPS)-free CeO₂NPs, the synthesis was performed in fume hoods, replacing distilled water with pure milliQ water (18.2 M Ω conductivity and < 5 ppb TOC). Before the synthesis started, all the glass material and stirring bars were heat treated in an oven at 260°C for 1h; naturally cooled down to room temperature and rinsed with milliQ water (to remove calcined traces). The consistent use of gloves and sterile pipette tips was also strict.

20

1.1. Synthesis of PVP-coated 5nm CeO₂ NPs:

An amount of 100ml of milli-Q water was placed under vigorous stirring (500 rpm) in a glass bottle at room temperature. Then, 5g of Polyvinylpyrrolidone (PVP of 10kDa, Sigma Aldrich) was added, to give a 5mM concentration of PVP. The solution was left at room temperature under vigorous stirring (500 rpm) for 30 min, until all solid was homogeneously dissolved. Besides, a solution of cerium (III) nitrate hexahydrated was prepared in water with 0.434 g of cerium (III) nitrate hexahydrated (Ce(NO₃)₃·6H₂O, 99%, Sigma). This solution was added to that of PVP, to give a 10 mM concentration of initial cerium (III) precursor. The solution was vigorously mixed during 15 minutes. Just after that, 2.2 ml of an as-received TMAOH (1M solution) was gently added dropwise during half of a minute (20-30 seconds), approximately. The solution suffered several changes in color, from pale yellow to whitish, during

the first two hours of reaction. 24h later, the PVP-coated 5nm nanoparticles were ready at 1 mg/ml concentration and stored at 4°C.

1.2 Synthesis of BSA-coated 5 nm CeO₂ NPs:

5

Solution 1. An amount of 25 ml of PBS (Dulbecco's phosphate buffered saline 10x, Sigma) was placed under vigorous stirring (500 rpm) in a glass bottle at room temperature. Then, 0.625 g of bovine serum albumin (BSA, Sigma) was added under vigorous stirring, to give a homogeneous solution at 0.752 mM concentration.

10

Solution 2. In parallel, in another glass bottle, an amount of 25 ml of milli-Q water was placed under vigorous stirring (500 rpm). Then, 0.217 g of cerium (III) nitrate hexahydrate (Ce(NO₃)₃·6H₂O, 99%, Sigma) were added, to give a 10 mM concentration of initial cerium (III) precursor. Following, a volume of 1.1 ml of an as-received TMAOH (Sigma, 1M) was gently added dropwise, during half of a minute approximately (dropwise for a period from 20 to 30 seconds). The reaction was left under vigorous stirring during five minutes.

15

After this time, the solution 2 was gently added to the solution 1 under vigorous stirring during one minute approximately. The resultant solution was left at room temperature and under vigorous stirring for 24 h. 24 h hours later, the BSA-coated 5nm CeO₂NPs were ready and stored at 4°C.

20

1.3. Monitoring of CeO₂NPs formation with UV-vis spectroscopy:

25

The optical study with ultraviolet-visible spectroscopy (UV-vis) was carried out to corroborate the NPs formation (any of 1.1; PVP-conjugated, or 1.2; BSA-conjugated) and to monitor the reaction kinetics. Despite the unspecificity of UV light absorption at short spectra wavelengths, it resulted in a good technique to monitor the progress and evolution of the reaction. FIG. 3 (A) shows pH evolution of the synthesis solution, meanwhile FIG. 3 (B) corresponds to the non-oxidized initial Cerium (III) precursor (black line); of the sample taken at 1 h of reaction (soft grey line) and of the sample of as-synthesized NPs at 48 h of reaction. A strong characteristic UV absorption peak of Ce⁴⁺ at 298 nm, confirms particles formation (H.L.Greenhaus et al.,

30

35

Ultraviolet Spectrophotometric Determination of Cerium(III), Anal Chem -1957, vol. no. 29, pp.: 1531).

1.4. Nanoceria characterization by Electron Microscopy:

5 To minutely examine the quality of produced CeO₂NPs, in terms of morphology, size, aggregation state and chemical composition (purity), the samples were analyzed by high resolution electron microscopy (HR-TEM, STEM-HAADF, EELS). A few representative images of as-synthesized PVP-coated samples are displayed in FIG. 4. Corresponding (similar) images were
10 recorded with BSA-coated NPs. FIG. 4 (a) shows as-synthesized monodispersed PVP-coated NPs at high resolution (400.000X), displaying a particle size of 5.2 ± 1.9 nm (Image J analysis) and the atomic planes of single-crystal nanoparticles. FIG. 4 (b) shows the dark-field STEM-HAADF image of the NPs, while FIG. 4 (c) inspects the homogeneity and
15 monodispersity degree of nanoceria at lower magnification (70.000X). FIG. 4 (d) shows a typical single-crystalline CeO₂ NP and its correspondent Fourier Transformation, FFT (FIG. 4 (e)). The profile of the chemical composition, represented in FIG. 4 (f) displays absolute purity of the sample.

20 1.5. Nanoceria characterization by X-ray diffraction:

The structural and compositional characterization, phase identification and size of CeO₂NPs were analyzed by X-ray Diffraction (XRD). The XRD pattern of as-synthesized CeO₂NPs of 4 nm is displayed in FIG. 5. The XRD pattern
25 was scanned from 20 to 80 degrees and the XRD profile confirmed the monocrystalline nature of CeO₂NPs (single-crystal cerium oxide nanoparticles). The characteristic diffraction peaks were observed at $2\theta = 28.51^\circ, 33.12^\circ, 47.44^\circ, 56.30^\circ, 58.88^\circ, 69.36^\circ, 76.75^\circ, 79.02^\circ, 88.35^\circ$ and 95.34° correlated to (111), (200), (220), (311), (222), (400), (331), (402),
30 (422), (333) crystal planes, respectively. All of the reflection peaks are in agreement with the database standard (JCPDS 34-0394) of the face-centered cubic CeO₂ crystal with the fluorite structure.

1.6. Crystal size diameter estimation (Crystal size estimation)

Three techniques were used to estimate the average crystal size diameter of as-synthesized in 1.1 or 1.2 CeO₂NPs: Image J analysis software for HR-TEM; size estimation using the Scherrer equation of XRD pattern and the Dynamic Light Scattering (DLS) of colloidal suspension of the NPs (measuring hydrodynamic diameter). Table 1 shows a comparison between three sizing techniques for as-synthesized CeO₂NPs: (a) Dynamic Light Scattering (DLS) and the polydispersity index (PDI) of this DLS, (b) Image J analysis of HR-TEM images, and (c) Scherrer equation calculations from XRD profile. All the data suggests good agreement for all methods regarding the NPs size. PDI is a parameter in DLS related with size distribution of molecules or particles in suspension. For a perfectly uniform sample, the PDI would be 0.0. PDI from 0.0 to 0.1 reflects a narrow non-uniform distribution type sample. From 0.1 to 0.4 the PDI indicates a moderate non-uniform distribution type sample. PDI higher than 0.4 is usually understood as a broad distribution type sample.

Table 1

(a)	(b)	(c)
DLS (PDI)	HR-TEM (St.Dev)	XRD (Scherrer equation)
8-12 nm (0.391)	5.0 (+/- 0.9) nm for PVP coated (5mM) of example 1.1	4 (+/-) nm

The size (diameter in nm) and aggregation state of the CeO₂NPs conjugated with albumin disclosed in 1.2 were again corroborated by DLS technique. In both measurements, by intensity and by number, it was observed one single peak at around 12 nm (hydrodynamic diameter). The absence of peaks at larger size axis corroborates the absence of aggregates in the designed synthesis method. Moreover, as an internal control, the BSA-coated NPs were purified by centrifugation and measured by DLS the supernatant only (FIG. 6 (c) and (d)), that contains free BSA in excess. The size distribution coincides with previously obtained data in FIG. 6 (a) and (b), wherein the DLS of the as-synthesized BSA-coated CeO₂NPs is depicted.

All these data allow concluding that single-crystal cerium oxide nanoparticles of formula (I) (NP-(A)_n) were conjugates in which NP had a crystal diameter from 3 to 7 nm, around 4-5 nm, when measured with TEM and other techniques, such as XRD. In addition albumin or PVP was disposed
5 conjugated (as a coating) with n from 1 to 4, particularly n was 1. When n is greater, the single peak appears in a position with higher nm in a graphic as that of FIG. 6.

Example 2. In vivo assay

10

An *in vivo* assay was performed with 44 Wistar rats to which HCC was induced by means of diethylnitrosamine (DEN). DEN is an hepatic carcinogenic used commonly to induce HCC in animals. All animals received weekly an injection intraperitoneally (50 mg of DEN/Kg) for 16 weeks. Two
15 protocols were carried out:

In first protocol the effect of CeO₂ NPs of Example 1.2 (BSA-conjugated CNPs) on hepatic morphology, conventional serology and cell proliferation were studied. In weeks 16 (last of DEN injection) and 17, rats were randomly
20 divided into two groups of 10 animals per group, each group receiving, respectively, two doses of CeO₂ NPs (0.1 mg/Kg) administered intravenously weekly for two weeks (Test group). The other group (Control) received the vehicle (TMAOH, 0.8 mM). At week 18, rats were sacrificed and blood and liver samples were obtained to study standard hepatic functional parameters
25 and hepatocyte proliferation.

In the second protocol (24 rats), the effect of BSA-conjugated CNPs of Example 1.2 on survival of animals with induced HCC was studied. The same procedure as in the first protocol was followed with the difference that animals
30 were sacrificed only when a badly damaged state was evident and always following criteria of Ethical and Research Committee of Hospital Clinic (Barcelona).

Liver sections of non-treated with CeO₂ NPs rats had a more dysmorphic appearance also with more superficial deformities than liver of CeO₂ NPs
35 treated rats. In the same way, the liver weight percentage in relation to total

body weight was slightly lower in animals receiving CeO₂ NPs in comparison with non-treated animals. Non meaningful differences were observed in serologic standard hepatic functional parameters and in renal parameters between bot animal groups.

5

The most important result of the first protocol was that hepatic cell proliferation measured as the percentage of hepatocyte nuclei positively stained (tinted) with Ki-67 was markedly lower in hepatic tissue of CeO₂ NPs treated rats than in the non-treated rats (21 ± 2 vs 52 ± 5 , $p < 0.001$). Data are depicted in FIG. 1 (A and B), wherein the microscope image (FIG. 1(A)) of the liver of non-treated cells (VEHICLE) shows a higher percentage of Ki-67 stained hepatocyte nuclei than in the image of CeO₂ NPs treated liver (CeO₂NPs). Same results can be concluded from FIG. 1(B), wherein for all animals of the protocol the percentage of stained Ki-67 nuclei are depicted. Lower percentages are observed in treated animals, thus demonstrating that the treatment with the CeO₂ NPs reduced cell proliferation.

10

15

In the second protocol 24 rats were randomly treated with CeO₂ NPs (n=12) or with vehicle (n=12). This assay showed that survival of HCC rats treated with CeO₂ NPs was higher than in non-treated rats. At week 21, 4 weeks after last dose of CeO₂ NPs or of vehicle, percentage of survival of non-treated rats was lower than in treated rats (10 %). This value increased up to 40 % in treated rats. Data are depicted in FIG. 2.

20

25

All these data demonstrated that CeO₂ NPs have a powerful effect on cell proliferation and on rat survival with HCC, thus being useful in the treatment of HCC patients.

Example 3. CeO₂ NPs for use in combination with radiotherapy.

30

FIG. 7 is an image of X ray contrast imaging of samples at different CeO₂NPs concentrations ranging from 0 to 100 mg/ml. The samples where imaged at 90 kV and 160 microA. The bright white signal, indicative of X-ray absorption increases with CeO₂ concentration as expected. This X-ray absorption is at the basis of X-ray imaging and X-ray therapy.

35

As shown in this FIG. 7, CeO₂ NPs, absorbed X radiation, in a highly efficient

manner. Transfer of energy was also high with these nanoparticles. Therefore, they are good contrast agents (in particular as X-ray contrast agents), as well as good to be used in combination with radiotherapy, providing to the subject the intrinsically effect of the ceria nanoparticles (due to their superoxide
5 dismutase (SOD) and catalase mimetic activity), and enhancing the effect of the administered radiotherapy.

Example 4. Catalytic activity of PVP-conjugated CeO₂NPs (single-crystal cerium oxide nanoparticles of formula (I) in which A is PVP)

Catalytic activity of PVP-conjugated CeO₂NPs was studied by detection of the the CeO₂NPs antioxidant effect on fluorescently-labeled hydrogen peroxidase (EuTc-H₂O₂ complex).

There were used:

- (a) Commercial 25-nm-sized CeO₂NPs (aggregates)
- (b) PVP-coated 5 nm-sized CeO₂NPs of the invention (synthesized as in Example 1);
- (c) surfactant-free mix of CeO₂NPs (mix of sizes from 6 to dozens of nm),
20 which where thus naked (not conjugated) CeO₂NPs;
- (d) surfactant-free 5-nm-sized CeO₂NPs, which where thus naked (not conjugated) CeO₂NPs.

Results are depicted in FIG. 8, a graphic displaying for each assayed
25 nanoparticle type the decay of fluorescence (F) versus time (t, in min).

In cell-free environment, as bigger the diameter of CeO₂NPs, lower their antioxidant capacity, which is displayed by an inefficient decay of fluorescence (F) versus time (t).

The PVP-coating slightly decreases CeO₂NPs reactivity, but as deducible from FIG. 8, PVP-coated 5nm-sized CeO₂NPs of the invention maintained the catalytic activity, and not so did commercial.

35 REFERENCES CITED IN THE APPLICATION

- Alili et al., "Downregulation of Tumor Growth and Invasion by Redox-Active Nanoparticles", Antioxidant & Redox Signaling (Original

Research Communication) – 2013, vol. 00, pp.: 1-14 (DOI: 10.1089/ars.2012.4831).

- Chen et al., in “Cerium oxide nanoparticles induce cytotoxicity in human hepatoma SMMC-7721 cells via oxidative stress and the activation of MAPK signalling pathways”, Toxicology in vitro – 2013, vol. no. 27, pp.: 1082-1088.
- Hak Soo et al., “Renal clearance of quantum dots”, Nature biotechnology – 2007, vol. no. 25(10), pp.: 1165-1170.
- Bushan et al., “Antioxidant nanozyme: a facile synthesis and evaluation of the reactive oxygen species scavenging potential of nanoceria encapsulated albumin nanoparticles”, Journal of Materials Chemistry B – 2015, vol. no. 3, pp.: 4843-4852.
- Marsalek et al., “Adsorption of Bovine Serum Albumin on CeO₂”, International Journal of Chemical, Nuclear, Materials and Metallurgical Engineering -2014, Vol. no. 8(12), pp.: 1269-1272.
- H.L.Greenhaus et al., Ultraviolet Spectrophotometric Determination of Cerium(III), Anal Chem -1957, vol. no. 29, pp.: 1531.

CLAIMS

1.- Single-crystal cerium oxide nanoparticle of formula (I)

5 NP-(A)_n (I), wherein

NP is a cerium oxide nanoparticle with a crystal diameter from 3 to 24 nm;

10 A is a molecule selected from the group consisting of albumin, polyvinylpyrrolidone, and combinations thereof; and n is an integer from 0 to 40,

15 for use in the treatment of hepatocellular carcinoma, and wherein if the crystal diameter of the cerium oxide nanoparticle is from 3 to 7 nm, then n is an integer from 1 to 12.

20 2.- The single-crystal cerium oxide nanoparticle for use according to claim 1, wherein NP is a cerium oxide nanoparticle with a crystal diameter from 3 to 24 nm; A is a molecule selected from the group consisting of albumin, polyvinylpyrrolidone, and combinations thereof; and n is an integer from 1 to 40.

25 3.- The single-crystal cerium oxide nanoparticle for use according to any one of claims 1-2, in which NP is a cerium oxide nanoparticle with a crystal diameter from 3 to 12 nm.

30 4.- The single-crystal cerium oxide nanoparticle for use according to any one of claims 1-3, in which NP is a cerium oxide nanoparticle with a crystal diameter from 3 to 7 nm, A is a molecule selected from the group consisting of albumin, polyvinylpyrrolidone, and combinations thereof; and n is an integer from 1 to 12.

35 5.- The single-crystal cerium oxide nanoparticle for use according to claim 4, wherein NP is a cerium oxide nanoparticle with a crystal diameter from 4 to 5 nm.

6.- The single-crystal cerium oxide nanoparticle for use according to any one

of claims 1-4, in which NP is a cerium oxide nanoparticle with a diameter from 3 to 7 nm, A is a molecule of albumin; and n is an integer from 1 to 12.

7.- The single-crystal cerium oxide nanoparticle for use according to claim 6,
5 wherein NP is a cerium oxide nanoparticle with a crystal diameter from 4 to 5 nm, and n is from 1 to 4.

8.- The single-crystal cerium oxide nanoparticle for use according to any one
10 of claims 1-4, in which NP is a cerium oxide nanoparticle with a crystal diameter from 3 to 7 nm, A is a molecule of polyvinylpyrrolidone; and n is an integer from 1 to 12.

9.- The single-crystal cerium oxide nanoparticle of formula (I) for use
15 according to any one of claims 1-8, which is for use as inhibitor of cell proliferation in hepatocellular carcinoma.

10.- The single-crystal cerium oxide nanoparticle of formula (I) for use
according to any one of claims 1-9, which is for use as a co-treatment of an
20 hepatocellular carcinoma therapy selected from the group consisting of radiotherapy, microwave ablation (MWA), radiofrequency ablation (RFA), transarterial radioembolization (TARE), chemotherapy, surgical resection, liver transplantation, targeted therapy, hyperthermia and combinations thereof.

11.- A single-crystal cerium oxide nanoparticle of formula (I)
25

$$\text{NP-(A)}_n \quad (\text{I}), \text{ wherein}$$

NP is a cerium oxide nanoparticle with a crystal diameter from 3 to 7
nm;

30 A is a molecule selected from the group consisting of albumin, polyvinylpyrrolidone, and combinations thereof; and n is an integer from 1 to 12.

12.- The single-crystal cerium oxide nanoparticle according to claim 11,
35 wherein NP is a cerium oxide nanoparticle with a crystal diameter from 4 to 5 nm.

13.- The single-crystal cerium oxide nanoparticle according to any one of the claims 11-12, wherein A is a molecule of albumin; an n is an integer from 1 to 4.

5 14.- A single-crystal cerium oxide nanoparticle of formula (I):

NP-(A)_n (I), wherein

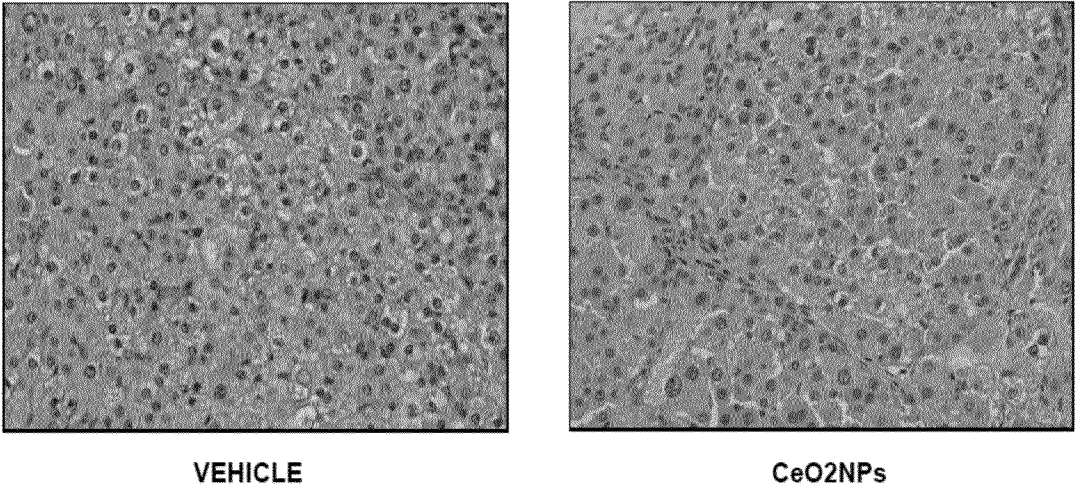
10 NP is a cerium oxide nanoparticle with a crystal diameter from 8 to 24 nm;

A is a molecule of albumin; and
n is an integer from 5 to 40.

15 15.- A pharmaceutical or veterinary composition comprising single-crystal cerium oxide nanoparticle as defined in any of claims 11-14, together with one or more pharmaceutically or veterinary acceptable excipients or carriers.

20 16.- The pharmaceutical or veterinary composition according to claim 15 comprising physiologic saline buffer.

(A)



(B)

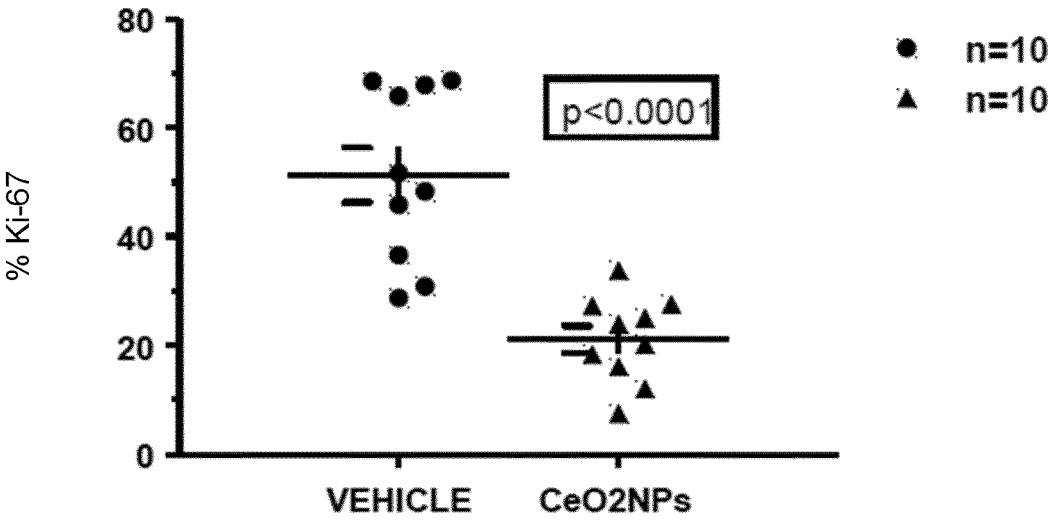
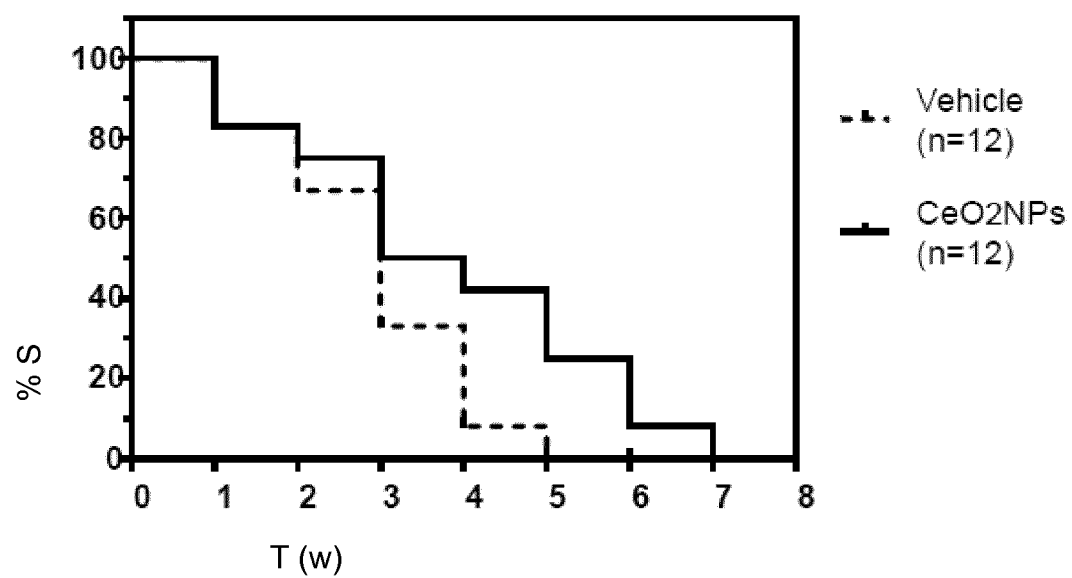
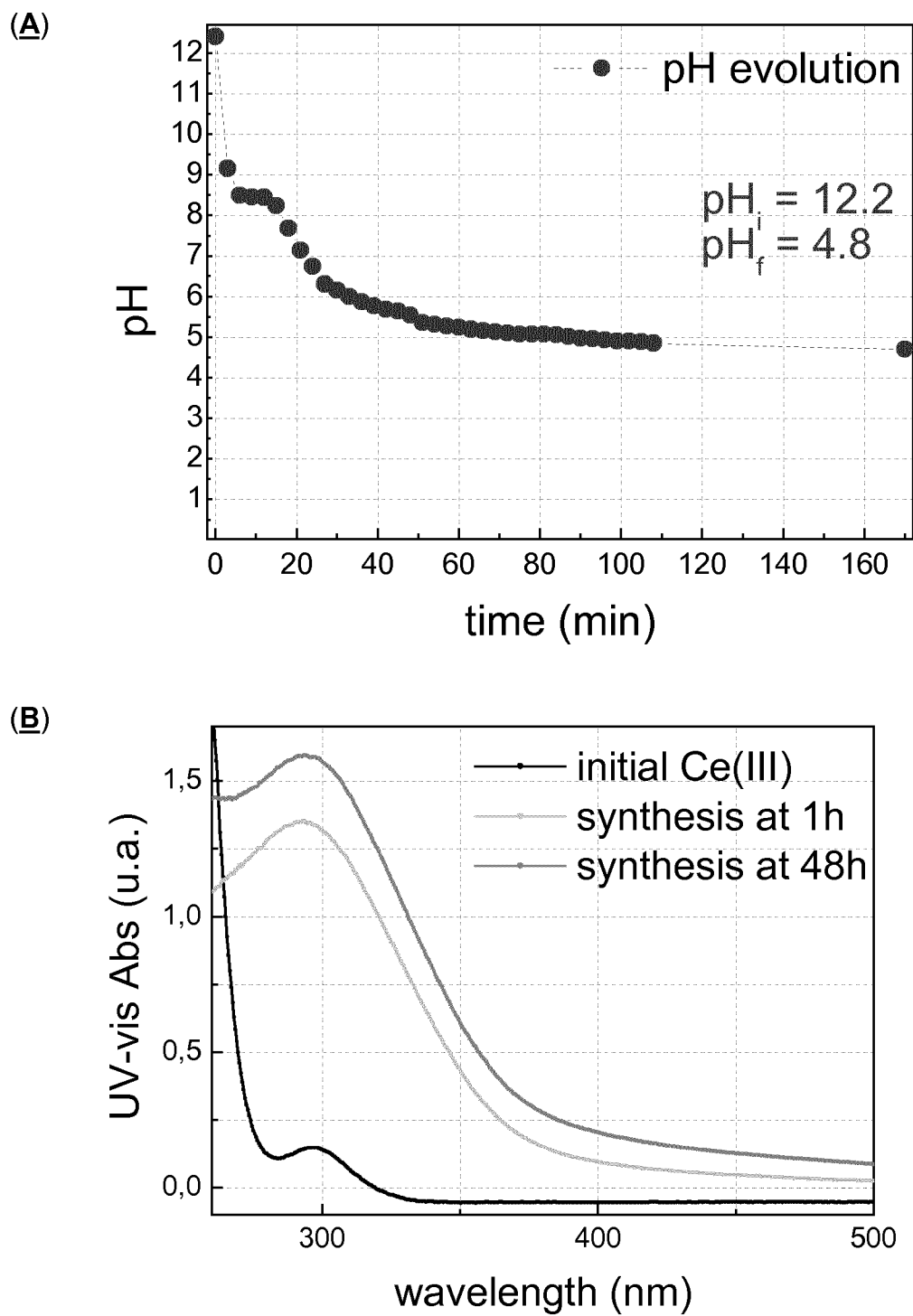
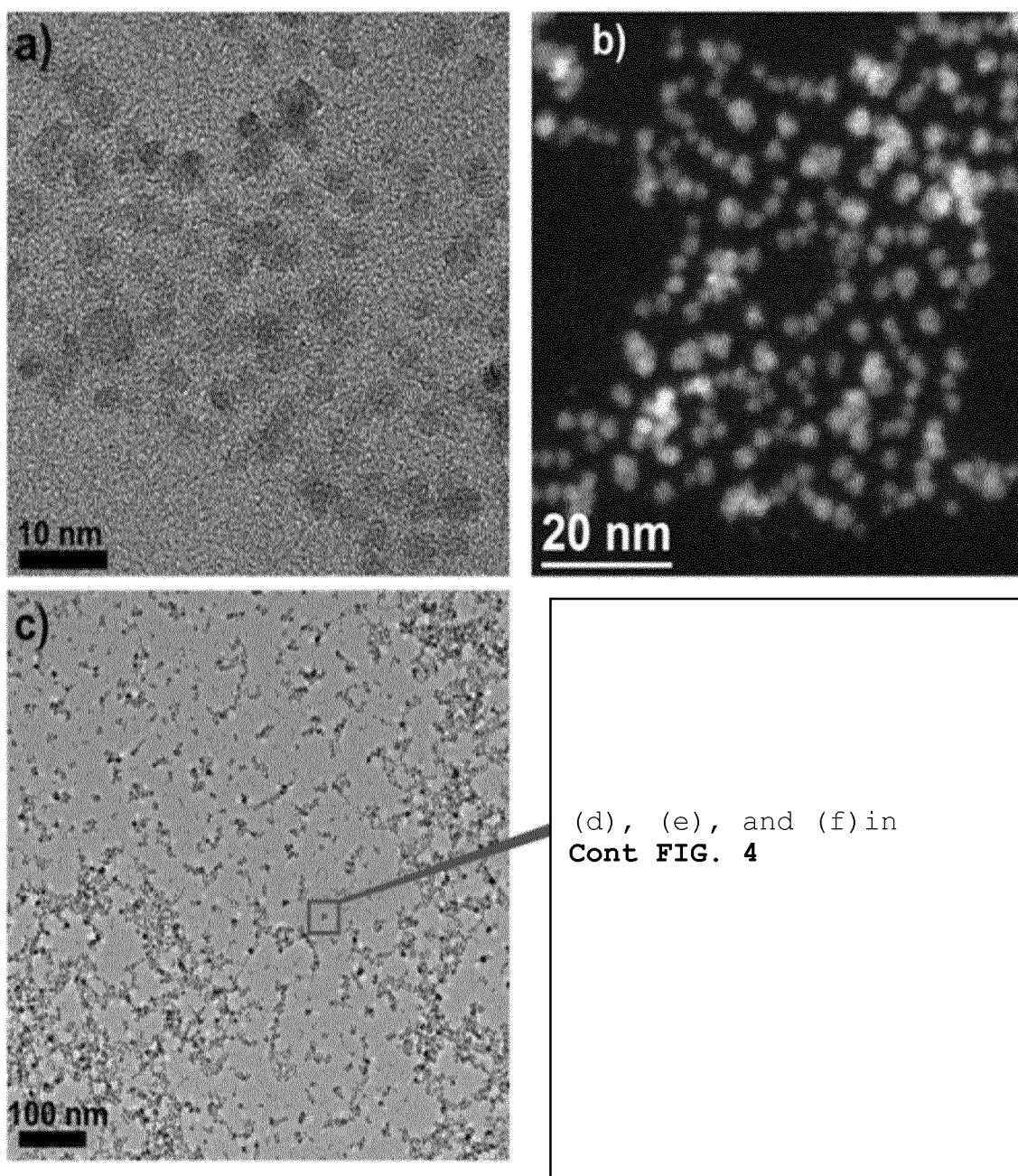


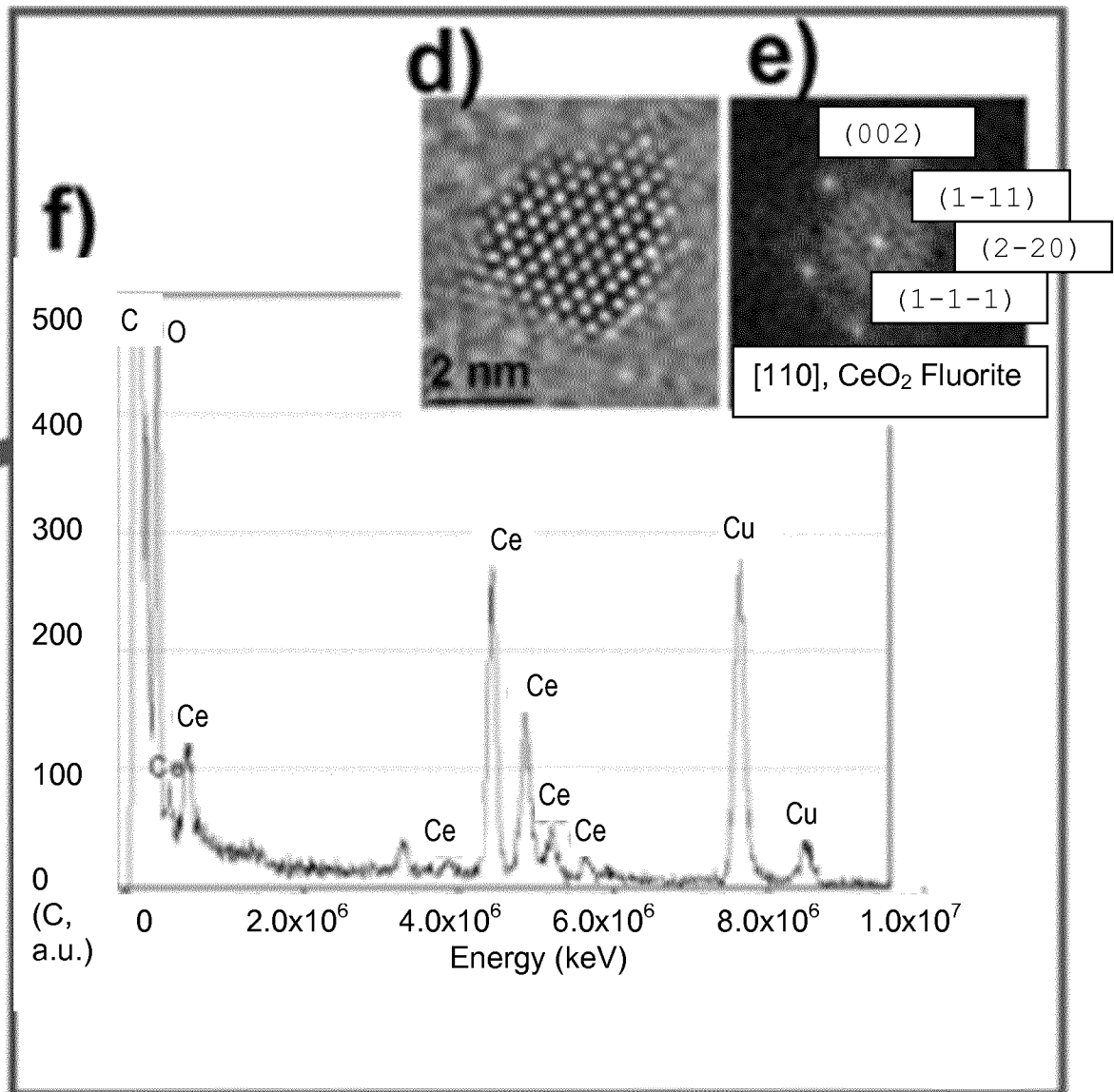
FIG. 1

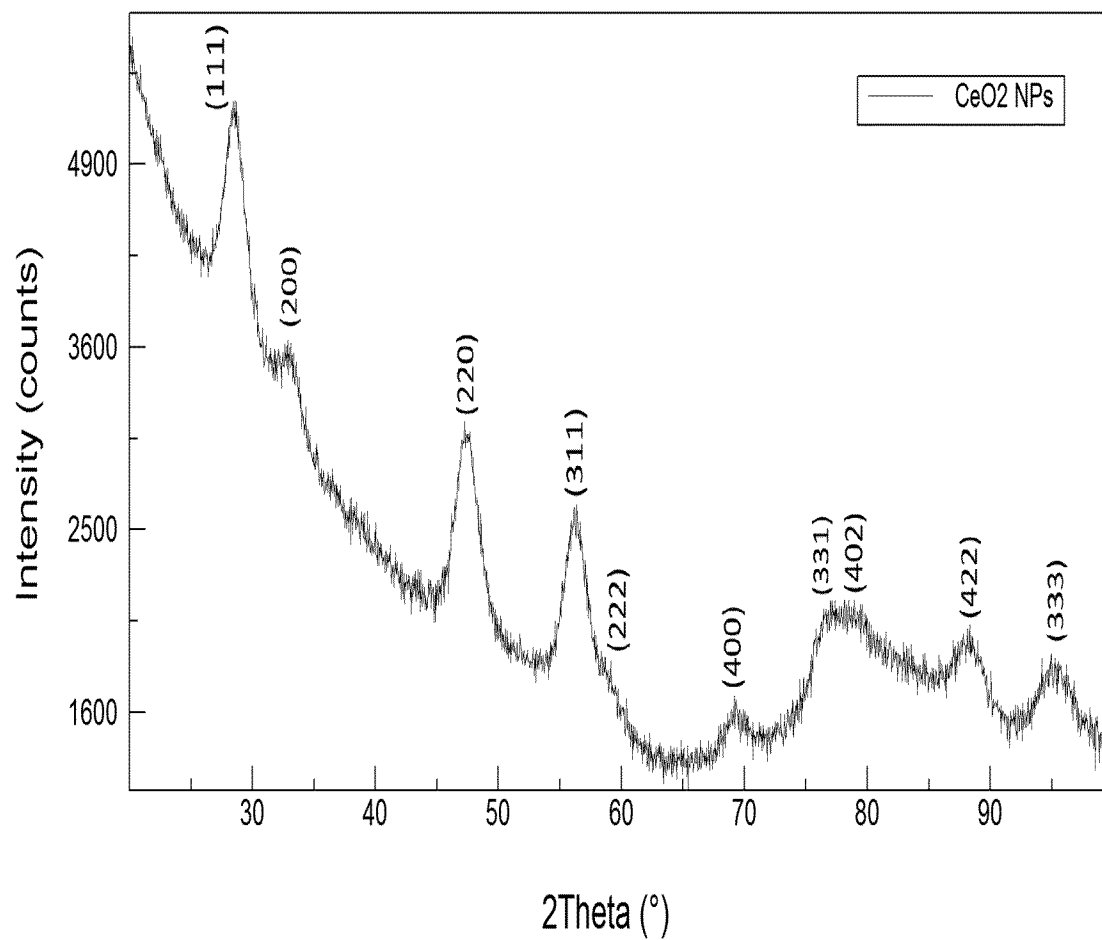
2 / 10

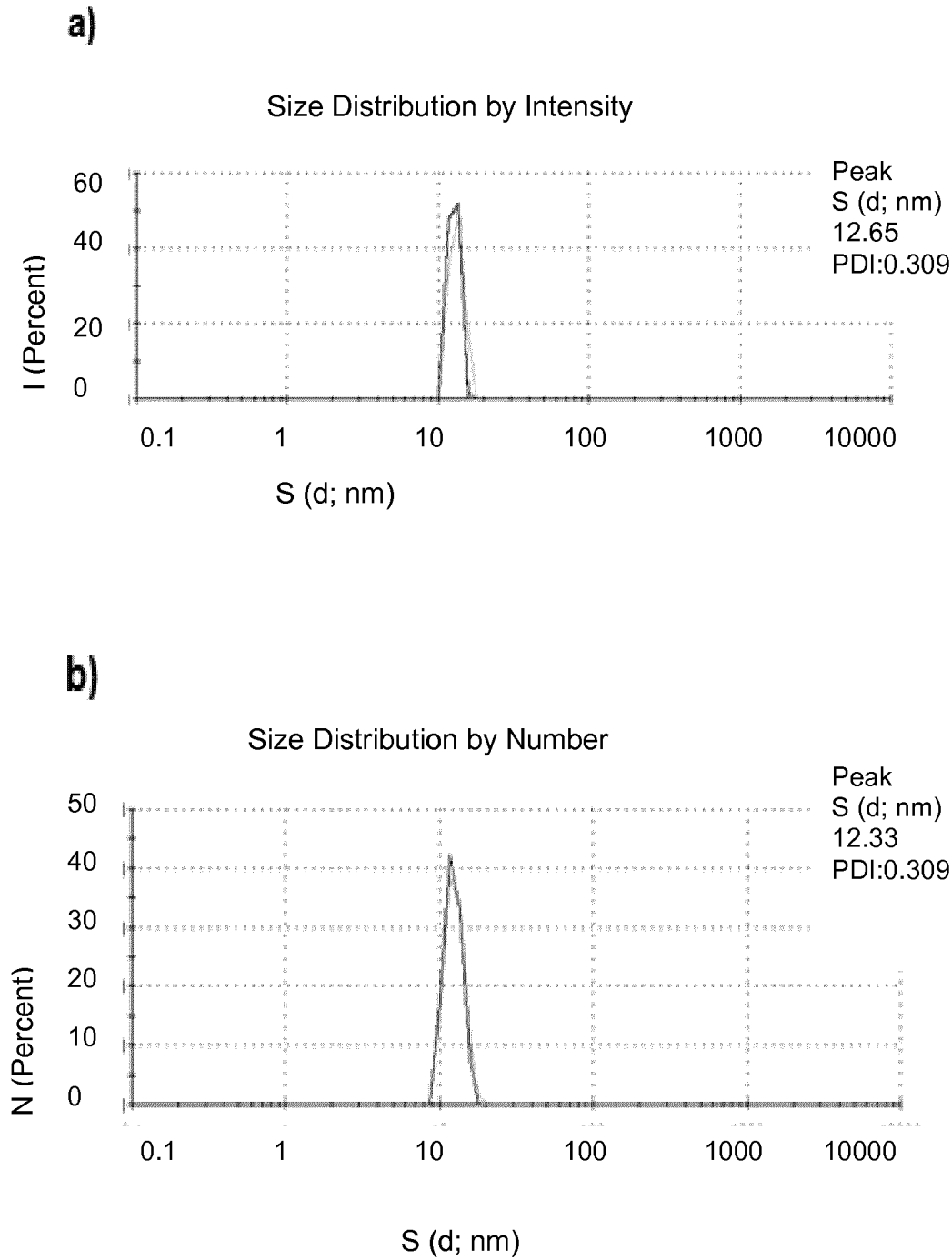
**FIG. 2**

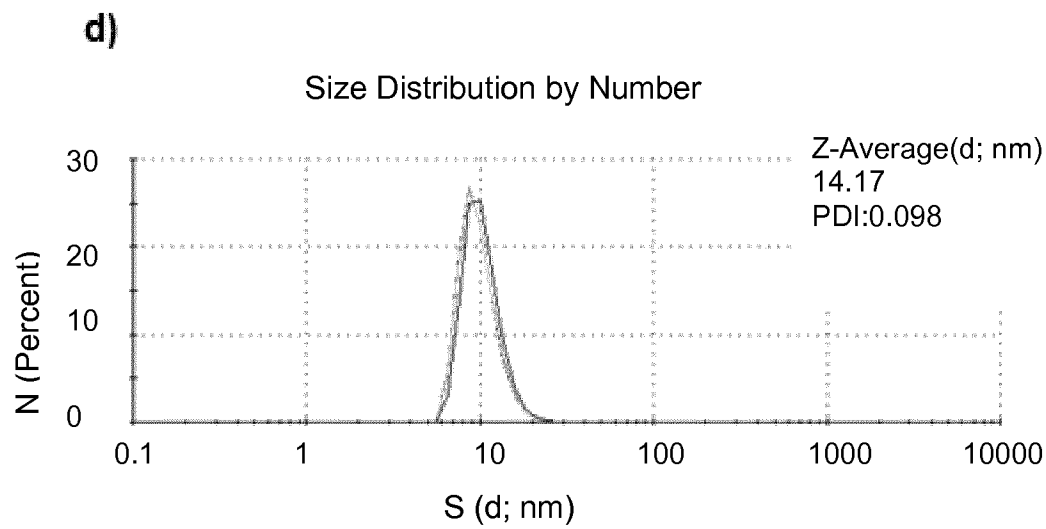
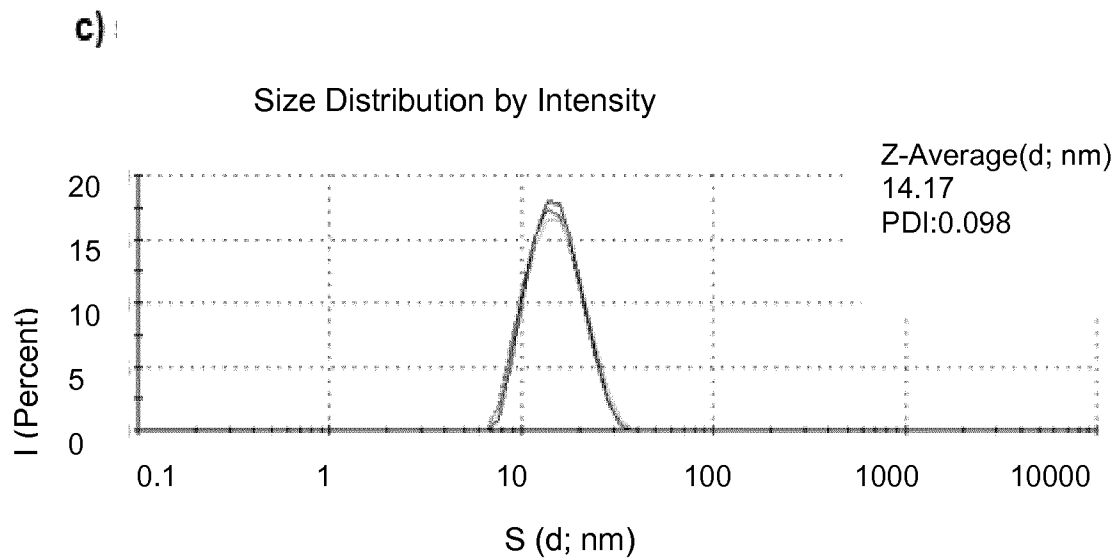
**FIG. 3**

**FIG. 4**

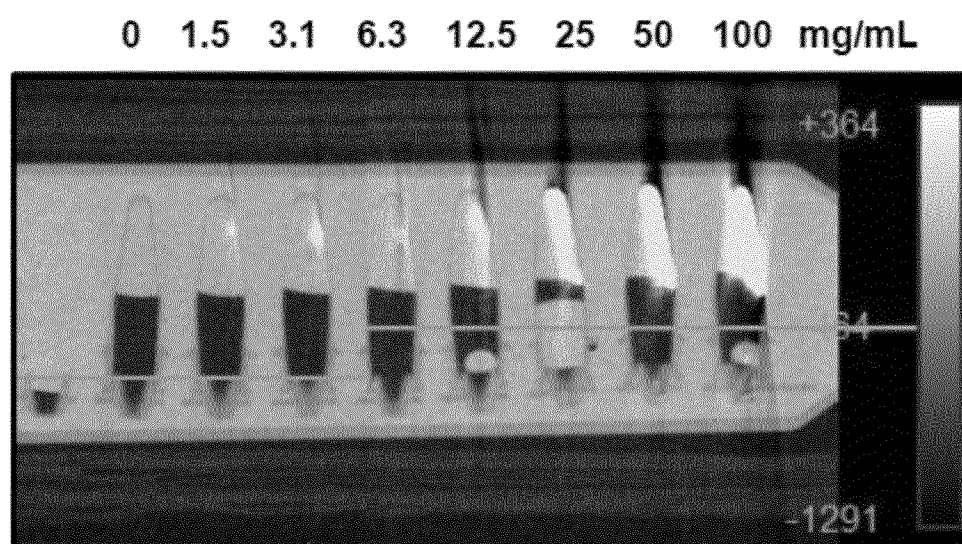
**Cont. FIG. 4**

**FIG. 5**





Cont. FIG. 6

**FIG. 7**

F
(EuTc-
H₂O₂)

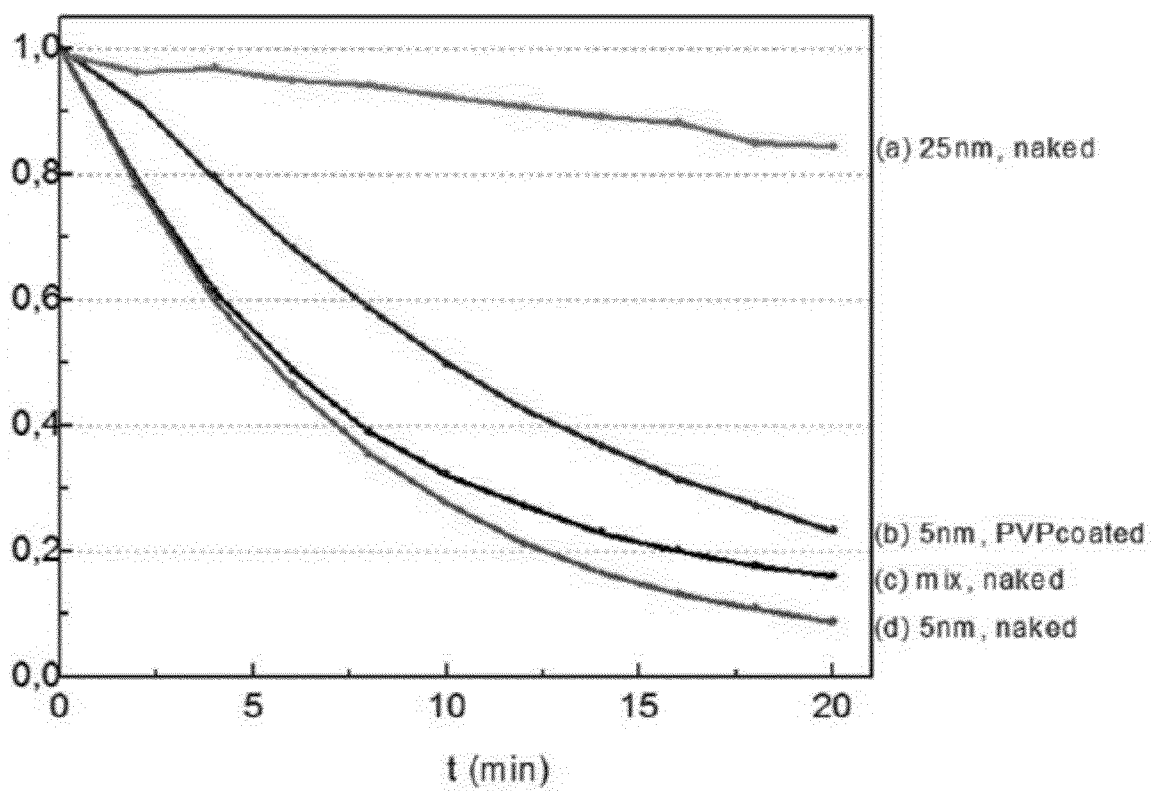


FIG. 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/057570

A. CLASSIFICATION OF SUBJECT MATTER		
INV.	A61K9/00 C01F17/00	A61K9/51 A61K33/24 A61P35/00 B82Y5/00
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 B82Y C01F		
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, BIOSIS, CHEM ABS Data, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHANG LI ET AL: "Selective cytotoxicity effect of cerium oxide nanoparticles under UV irradiation.", JOURNAL OF BIOMEDICAL NANOTECHNOLOGY FEB 2014, vol. 10, no. 2, February 2014 (2014-02), pages 278-286, XP009191656, ISSN: 1550-7033 abstract figure 1 page 284 - page 285 -----	1-5, 7-12,15, 16
X	WO 2016/011328 A1 (BAKER CHERYL [US]) 21 January 2016 (2016-01-21) example 1; table 2 example 6 claim 21 ----- -/-	1,3,5,9, 10,15,16
☒ Further documents are listed in the continuation of Box C. ☒ 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 15 May 2017		Date of mailing of the international search report 30/05/2017
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 Kollmannsberger, M

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RUTH C. MERRIFIELD ET AL: "Synthesis and Characterization of Polyvinylpyrrolidone Coated Cerium Oxide Nanoparticles", ENVIRONMENTAL SCIENCE & TECHNOLOGY, vol. 47, no. 21, 5 November 2013 (2013-11-05), pages 12426-12433, XP055302490, US ISSN: 0013-936X, DOI: 10.1021/es402541z page 12429, column 2 figure 1 table 1 -----	11,12,15
X	PATIL ET AL: "Protein adsorption and cellular uptake of cerium oxide nanoparticles as a function of zeta potential", BIOMATERIALS, ELSEVIER SCIENCE PUBLISHERS BV., BARKING, GB, vol. 28, no. 31, 18 August 2007 (2007-08-18), pages 4600-4607, XP022207000, ISSN: 0142-9612, DOI: 10.1016/J.BIOMATERIALS.2007.07.029 figures 2,4 -----	11-15
X	BHARAT BHUSHAN ET AL: "Antioxidant nanozyme: a facile synthesis and evaluation of the reactive oxygen species scavenging potential of nanoceria encapsulated albumin nanoparticles", JOURNAL OF MATERIALS CHEMISTRY B, vol. 3, no. 24, 1 January 2015 (2015-01-01), pages 4843-4852, XP055302510, GB ISSN: 2050-750X, DOI: 10.1039/C5TB00572H page 4844 -----	11-13,15
A	LONG WANG ET AL: "Nano-cerium-element-doped titanium dioxide induces apoptosis of Bel 7402 human hepatoma cells in the presence of visible light", WORLD JOURNAL OF GASTROENTEROLOGY, vol. 13, no. 29, 1 January 2007 (2007-01-01), pages 4011-4014, XP055302404, CN ISSN: 1007-9327 the whole document -----	1-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2017/057570

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2016011328 A1	21-01-2016	AU 2015289504 A1	23-02-2017
		CA 2955384 A1	21-01-2016
		CN 106604719 A	26-04-2017
		EP 3169312 A1	24-05-2017
		WO 2016011328 A1	21-01-2016
