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- (54) **Title:** METAL-ENCAPSULATED CARBONACEOUS DOTS

(57) **Abstract:** Nanoparticles described as metal-encapsulated carbonaceous dots or M@C-dots are disclosed. Also disclosed are specific M@C-dots with gadolinium, so called Gd@C-dots. These nanoparticles are biologically inert and preclude the release of metal in biological environments. In addition, despite a dimension exceeding the commonly recognized threshold for renal clearance, the disclosed nanoparticles can be efficiently cleared via urine after systematic injection. Methods of making and using such nanoparticles are also disclosed.



## METAL-ENCAPSULATED CARBONACEOUS DOTS

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5 awarded by the National Institutes of Health. The government has certain rights in the invention.

### FIELD

The subject matter disclosed herein generally relates to nanoparticles containing a metal encapsulated in a carbonaceous shell, and methods of making and using thereof.

### BACKGROUND

10 Main Gadolinium(III)-based contrast probes have been widely used in clinical MRI. So far, there are at least nine formulations of Gd-containing contrast agents approved for human use in the U.S., and they are assisting more than 10 million magnetic resonance imaging (MRI) scans per year. Free Gd is known to have a high toxicity profile, hence clinically used Gadolinium agents are all in the form of Gd-chelator complexes. Despite the complexation,  
15 however, these contrast agents are found to cause severe nephrogenic systemic fibrosis (NSF), especially for patients with renal diseases or poor renal functions (Perazella, *Curr. Drug Safety* 2008, 3(1):67-75; Chrysochou *et al.*, *Clin. J. Am. Soc. Neph.* 2010, 5(3): 484-489; Perazella *et al.*, *Clin. J. Am. Soc. Neph.* 2007, 2(2):200-202). For this reason, the FDA has issued warnings on the use of several Gd-based contrast agents in patients with kidney dysfunction (Thomsen *et al.*, *Clin. Rad.* 2006, 61(11):905-906; Thomsen, *Eur. Rad.* 2006, 16(12):2619-2621; Hellman, *Sem. Neph.* 2011, 31(3):310-316.5-7). This status underscores the significance of developing  
20 alternative contrast agents with more favorable safety profiles.

One approach that has been investigated is to load or imbed Gd(III) into a nanoparticle capsule/carrier that can suppress the Gd release while maintaining the  $T_1$ -shortening capacity.  
25 Examples of this approach include Gd<sub>2</sub>O<sub>3</sub> nanoparticles (Bridot *et al.*, *J. Am. Chem. Soc.* 2007, 129(16):5076-5084), Gd-loaded silica nanoparticles (Vivero-Escoto *et al.*, *Small* 2013, 9(20):3523-3531), and Gd-doped Fe<sub>3</sub>O<sub>4</sub> nanoparticles (Zhou *et al.*, *Adv. Mater.* 2012, 24(46):6223-6228). Due to their relatively large sizes, however, these nanoparticles are mostly accumulated in the reticuloendothelial (RES) organs after injection, most prominently the liver.  
30 Subsequent particle degradation may cause slow release of Gd(III) to the surroundings, and the long-term impact is largely unknown. Thus, what are needed are new Gd-based contrast agents that have improved safety profiles. The compositions and methods disclosed herein address these and other needs.

## SUMMARY

In accordance with the purposes of the described materials, compounds, compositions, articles, and methods, as embodied and broadly described herein, the subject matter described herein, in one aspect, relates to compositions and methods for preparing and using such compositions. In a further aspect, the disclosed subject matter relates to a nanoparticle comprising a metal encapsulated in an amorphous carbon shell. In particular examples, the disclosed subject matter relates to a nanoparticle comprising gadolinium encapsulated in an amorphous carbon shell. The amorphous carbon shell of the disclosed nanoparticles can comprise carboxyl groups. The average diameter of the disclosed nanoparticle can be from about 2 nm to about 34 nm.

The subject matter described herein also relates to methods of making the disclosed nanoparticles comprise calcining a metal with a chelator. Methods of functionalizing the disclosed nanoparticles are also disclosed. Methods of using the disclosed nanoparticles, *e.g.*, for imaging are also disclosed.

Additional advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

## BRIEF DESCRIPTION OF THE FIGURES

The accompanying figures, which are incorporated in and constitute a part of this specification, illustrate several aspects of the disclosure, and together with the description, serve to explain the principles of the disclosure.

Figure 1 displays characterizations of Gd@C-dots. Panel (a) is a schematic illustration of Gd@C-dots. Panel (b) is a TEM and Panel (c) is a HRTEM image of 12 nm Gd@C-dots. Panel (d) shows elemental mapping (Gd) of Gd@C-dots. Panel (e) is a STEM image of a single 12 nm Gd@C-dot. Panel (f) is an EDX line profile across the nanoparticle in Panel (e). Points “A” and “B” corresponded to those labeled respectively in Panel (e). Panel (g) shows the DLS result of Gd@C-dots. Panel (h) shows the Zeta potential of Gd@C-dots.

Figure 2 displays the optical and magnetic properties of Gd@C-dots. Panel (a) is the absorption and photoluminescence spectra of Gd@C-dots. Panel (b) shows a photostability study. Gd@C-dots were under continuous irradiation by UV light (30 W, 254 nm) and the photoluminescence (ex/em 360/425 nm) intensity was monitored over time. Panel (c) shows a comparison of photostability among FITC, CdSe/ZnS QDs, and Gd@C-dots. The three

solutions were under continuous irradiation by UV light (30 W, 254 nm) for different amounts of time. Panel (d) shows  $T_1$ -weight MR images of Gd@C-dot agarose samples of different Gd concentrations. Panel (e) shows the linear correlation between  $R_1$  ( $T_1^{-1}$ ) and Gd concentration, based on readings from Panel (d). The  $r_1$  relaxivity, which is the slope of the curve, was

5 determined to be  $5.88 \text{ mM}^{-1}\text{s}^{-1}$ .

Figure 3 displays cytotoxicity and cell targeting data. Panel (a) shows the photoluminescence intensity (ex/em 360/425 nm) change when Gd@C-dots were incubated in buffers of different pH values. Panel (b) shows Gd release from Gd@C-dots over time. The nanoparticles were incubated in solutions with pH 5 or 7.4. #: The overall Gd concentrations in

10 the solutions. Panel (c) shows cell viability, evaluated by MTT assays with U87MG cells. 2.5 mM Ca(II) was added in the incubation medium. Panel (d) displays a cell targeting study. RGD-Gd@C-dots were incubated with U87MG cells for 30 min and the cells were then imaged under a fluorescence microscope (scale bar, 10  $\mu\text{m}$ ). For controls, cells were incubated with Gd@C-dots at the same Gd concentration or with RGD-Gd@C-dots in the presence of free

15 c(RGDyK) (30 $\times$ ). Panel (e) shows  $T_1$ -weighted MR images of cell pellets, where cells had been incubated with either RGD-Gd@C-dots or Gd@C-dots.

Figure 4, panel (a), shows  $T_1$ -weighted MR images acquired at different time points after injection of Gd@C-dots or RGD-Gd@C-dots. Panel (b) shows the signal change in the bladder (bl) and liver (lv), based on region of interest (ROI) analysis on images from panel (a). Panel (c)

20 shows a photoluminescence analysis on urine samples taken 60 min after the injection of RGD-Gd@C-dots.

Figure 5, panel (a), shows  $T_1$ -weighted transverse MR images. Gd@C-dots or RGD@C-dots (3.2 mg/kg) were intravenously injected into U87MG tumor bearing mice. Images were acquired at 0, 10, 30, 45, 60 and 240 min. For both types of nanoparticles, strong signals in the

25 bladder were observed soon after the particle injection, indicating fast renal clearance. Panel (b) shows  $T_1$ -weighted coronal MR images. Significant signal enhancement was observed in tumors of animals injected with RGD@Gd-dots. Panel (c) shows the relative signal change at different time points, based imaging results from panel (b). Panel (d) shows an immunofluorescence histology study with tumor samples. Good overlap was observed between RGD-Gd@C-dots and

30 positive integrin  $\beta_3$  staining. As a comparison, Gd@C-dots showed minimal tumor uptake. Red, integrin  $\beta_3$  (Cy5); blue, fluorescence from C-dots. Scale bars, 50  $\mu\text{m}$ .

Figure 6 shows the size distribution analysis of Gd@C-dots (panel (a)), RGD-Gd@C-dots (panel (b)), and Gd@C-dots in FBS (panel (c)) for 24 h.

Figure 7 shows the Zeta potentials of Gd@C-dots and RGD-Gd@C-dots.

Figure 8 shows the FTIR spectra of Gd@C-dots and DTPA.

Figure 9 shows the fluorescent spectra of metal-containing C-dots.

#### DETAILED DESCRIPTION

The present disclosure can be understood more readily by reference to the following detailed description and the Examples included therein and to the Figures and their previous and following description.

As used in the specification and the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a particle” includes mixtures of two or more such particles, reference to “the compound” includes mixtures of two or more such compositions, and the like.

Ranges can be expressed herein as from “about” one particular value, and/or to “about” another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent “about,” it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. Unless stated to the contrary “about” a particular value means within 5% of the particular value, *e.g.*, within 2% or 1% of the particular value.

By “amorphous” is meant non-crystalline and without structural order over a long range, *e.g.*, a majority of the nanoparticle. An amorphous shell can contain some ordered structure over a short range atomic length scale, but the majority of the shell is not ordered and non-crystalline.

All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference and are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present disclosure is not entitled to antedate such publication by virtue of prior disclosure. Further, the dates of publication provided could be different from the actual publication dates that may need to be independently confirmed.

#### Compositions

Disclosed herein are nanoparticles where a metal is encapsulated in an amorphous carbon shell. The metal can be pure metal, metal oxide, metal complexes, or mixtures of these. These metal encapsulated carbon dots (herein referred to as M@C-dots) can have a wide variety of uses. In some specific examples, the metal is gadolinium; thus disclosed herein are Gd encapsulated carbon dots (hereafter referred to as Gd@C-dots). Also, reference to M@C-dots herein is meant to specifically include reference to Gd@C-dots.

Unlike most other nanocarriers/nanocapsules, carbon has low-toxicity and is highly biologically inert. Thus, the disclosed nanoparticle M@C-dots can remain intact even in harsh biological environments, therefore precluding the risk of metal release to the surroundings (Cao *et al.*, *Theranostics* 2012, 2(3):295-301). With specific reference to gadolinium, stemming from the inert carbon coating, the disclosed nanoparticles are immune to the issue of Gd leaking that is often observed with complex-based Gd agents. Leakage of other metals from the disclosed nanoparticles is also expected.

The disclosed nanoparticles can have an average size of about 12 nm in diameter. For example, the disclosed nanoparticles can have an average size of from about 2 nm to about 34, from about 4 nm to about 32 nm, from about 6 nm to about 30 nm, from 8 to about 28 nm, from about 10 nm to about 24 nm, from about 12 nm to about 22 nm, from about 14 nm to about 20 nm, from about 26 nm to about 18 nm, from about 8 nm to about 16 nm, from about 10 nm to about 14 nm, from about 12 nm to about 20 nm, or from about 14 nm to about 18 nm. In still other examples, the disclosed nanoparticles can have an average size of 2, 4, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 28, 30, 32, or 34 nm, where any of the stated values can form an upper or lower endpoint of a range.

Also, the disclosed nanoparticles, for example, the Gd@C-dots, can have a high  $r_1$  relaxivity compared to complex-based Gd reagents. For example, for 12 nm Gd@C-dots,  $r_1$  relaxivity is about  $5.88 \text{ mM}^{-1}\text{s}^{-1}$  (on per Gd basis). The  $r_1$  relaxivity can be tuned up to  $50 \text{ mM}^{-1}\text{s}^{-1}$  by adjusting the size and composition of Gd@C-dots. Thus, the disclosed Gd@C dots can have  $r_1$  relaxivity from about 0 to about  $50 \text{ mM}^{-1}\text{s}^{-1}$ , *e.g.*, about 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, or  $50 \text{ mM}^{-1}\text{s}^{-1}$ , where any of the stated values can form an upper or lower end point of a range. In specific examples, the disclosed Gd@C-dots can have a  $r_1$  relaxivity from about 0 to about 10, from about 5 to about 15, from about 10 to about 20, from about 15 to about 25, from about 20 to about 30, from about 25 to about 35, from about 30 to about 40, from about 35 to about 45, from about 40 to about 50, from about 0 to about 25, or from about 25 to about  $50 \text{ mM}^{-1}\text{s}^{-1}$ .

Moreover, the disclosed Gd@C-dots also possess bright photoluminescence, boasting a high quantum yield and excellent photostability that is even superior to quantum dots. The QY is found to be 19.7% for 12 nm Gd@C-dots but can be tuned up to 80% by adjusting the size and composition of Gd@C-dots. The QY of the disclosed Gd@C-dots is comparable to the highest reported QYs of C-dots (Bhunia *et al.*, *Sci. Rep.* 2013, 3, 1473). Notably, however, for most of the previously reported C-dots, a post-synthesis surface passivation step is usually needed to grant particles with strong luminescence (Sun *et al.*, *J. Am. Chem. Soc.* 2006, 128(24):7756-7757; Wang *et al.*, *Angew. Chem. Int. Ed.* 2012, 51(37):9297-9301; Liu *et al.*, *Adv. Mater.* 2012, 24(15):2037-2041). In comparison, the strong luminescence of the Gd@C-dots can be achieved

in one-step calcination with no added ligands. In specific examples, the disclosed Gd@C-dots can have a QY of from about 0 to about 80, from about 15 to about 65, from about 30 to about 80, from about 0 to about 35, from about 15 to about 75, from about 0 to about 20, or from about 20 to about 35%.

5           Uniquely, despite having dimensions that can exceed the commonly recognized threshold for renal clearance (about 5.5 nm) (Choi *et al.*, *Nat. Nanotech.* 2010, 5(1):42-47), systematically injected nanoparticle M@C-dots can be efficiently excreted *via* urine, further minimizing toxicity risks. After systematic injection, it can be desirable that an imaging probe can home efficiently to the diseased area (*e.g.*, a tumor), with the unbound probes rapidly excreted from the  
10   host. This can be challenging for nanoparticle-based imaging probes, most of which have a relatively large size, a high tendency of opsonization, and as a result, a high level of liver accumulation (Jokerst *et al.*, *Nanomedicine* 2011, 6(4):715-728; Liu *et al.*, *Biochim. Biophys. Acta.* 1992, 1104(1):95-101). Studies showed that when the overall size was below 5.5 nm, nanoparticles could be excreted by renal clearance, thereby avoiding extended durations in the  
15   host (Choi *et al.*, *Nat. Nanotech.* 2010, 5(1):42-47). This size criterion, however, is difficult to meet for many nanomaterials, including most Gd-containing nanoparticles under investigation. It is in this sense that the renal clearance of the disclosed Gd@C-dots is particularly significant. Both conjugated RGD-Gd@C-dots and Gd@C-dots have sizes well above the recognized threshold for renal clearance and yet are able to be efficiently excreted *via* urine. Though not  
20   wishing to be bound by theory, it is believed that the unique surface of the disclosed nanoparticles play a role in renal clearance. With a shell made of amorphous carbon but decorated with carboxyl groups, the surface of the disclosed nanoparticles lies between hydrophobic and hydrophilic. This affords the particles with good colloidal stability and still gives them the capacity to cross certain types of biological barriers. It is noted that recently,  
25   there was another report by Gao *et al.* showing that 11.8 nm QDs had efficient renal clearance (Gao *et al.*, *Nano Lett.* 2012, 12(1):281-286). The authors also attributed the uncommon phenomenon to the surface coating, which in that case was a dendron polymer. Aside from particle dimension, there are surface related parameters that can assist with fast particle excretion.

30           The disclosed nanoparticles are not endohedral fullerenes, which are much smaller than the disclosed M@C-dots. That is, endohedral fullerenes are usually less than about 1 nm. C<sub>60</sub>, for example, has a diameter of 0.7 nm. Also, the fullerene structure is highly ordered, where as the disclosed nanoparticles have an amorphous shell that functionalized with carboxyl groups.

## Conjugates

The surface of the disclosed nanoparticles contains carboxyl groups that can be used to functionalize the surface of the nanoparticles. The carbonyl groups are electrophiles that can be used in nucleophilic substitution reactions or carbodiimide coupling reactions with any desirable functionalizing reagent. In certain examples, the functionalizing reagent can contain a targeting moiety that can be used to direct the functionalized nanoparticles to specific locations in the patient. Thus disclosed herein are M@C-dot nanoparticles functionalized with a targeting moiety. For example, RGD-peptides and cyclic RGD-peptides, when coupled to the disclosed M@C-dots, can direct the nanoparticles to target tumors. Similarly, EGFR targeting peptides, EGFR targeting therapeutics, VEGF targeting peptides, VEGF targeting therapeutics, and the like can be coupled/attached to the disclosed nanoparticles. Different types of antibodies, such as Herceptin, Avastin, and Erbitux, etc., can be coupled to the particle surface for facilitating particle targeting to tumors. Small molecule drugs such as doxorubicin, methotrexate or paclitaxel or their derivatives and can also be coupled to the surface of the nanoparticles, and in these cases the particles are used as drug carriers. Functionalizing the surface of the nanoparticles can also be used to assist the passage of the M@C-dots across certain cell membranes. For instance, the particle surface can be coated with a layer of positively charged polymer such as polyethylenimine and the resulting conjugates can be used as carriers for gene delivery (e.g., siRNA) due to assisting gene therapeutics passing through negatively charged cell membranes.

Suitable reagents for initiating a carbodiimide-mediate coupling to the carboxyl of the disclosed nanoparticles are commercially available. Specific examples of such reagents include, but are not limited to, water soluble carbodiimides such as 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-p-toluene sulfonate, alcohol and water soluble *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, and organic soluble *N,N'*-dicyclohexylcarbodiimide.

## Methods of Making

The disclosed M@C-dots can be prepared by calcining a metal with a chelator. An exemplary chelator that can be calcined with the metal is diethylenetriaminepentaacetate (DTPA). DTPA can form complexes with a wide range of transition metals, especially Gd. By calcining the corresponding metal-DTPA complexes, metal-encapsulated M@C-dots can be produced. This method was tested with complexes formed between DTPA and  $Mn^{2+}$ ,  $Ni^{2+}$ ,  $Co^{2+}$ ,  $Zn^{2+}$ ,  $Fe^{3+}$ ,  $Nd^{3+}$ ,  $Y^{3+}$ , and  $Eu^{3+}$ . In all the scenarios, metal-containing C-dots (Figure 9) were obtained, indicating the generality of the method.

Other chelator can be calcined with the metal to produce the disclosed nanoparticles. Examples of such chelators include, but are not limited to, 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), 1,4,8,11-tetraazacyclododecane-1,4,8,11-tetraacetic acid (TETA), 2,2'-(1,4,8,11-tetraazabicyclo[6.6.2]hexadecane-4,11-diyl)diacetic acid (CB-TE2A), 3,6,9,15-Tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-3,6,9-triacetic acid (PCTA), penetide (GYK-DTPA), cyclohexyldiethylenetriaminepentaacetic acid (CHX-DTPA), 2-(4,7-biscarboxymethyl[1,4,7]triazacyclononane-1-yl-ethyl)carbonyl-methylamino]acetic acid (NETA), diethylene triamine pentaacetic acid (DTPA), desferrioxamine, nitrilotriacetate (NTA), DO3A, ethylenediamine, acetylacetonate, phenanthroline, oxalate, citric acid, bipyridine, cyanide, nitrite, acetonitrile, ethylenediamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), poly-L-lysine, polyethylenimine, and polyvinylpyrrolidone (PVP). Salts, derivatives, and functionalized versions of these chelators can also be used.

### Formulations

While it can be possible for disclosed nanoparticles to be administered neat, it is also possible to present them as a pharmaceutical formulation. Accordingly, provided herein are pharmaceutical formulations which comprise one or more of the disclosed nanoparticles together with one or more pharmaceutically acceptable carriers thereof and optionally one or more other therapeutic ingredients. The carrier(s) must be “acceptable” in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof. Proper formulation is dependent upon the route of administration chosen. Any of the well-known techniques, carriers, and excipients can be used as suitable and as understood in the art; *e.g.*, in *Remington: The Science and Practice of Pharmacy*, 21<sup>st</sup> Ed., Gennaro, Ed., Lippencott Williams & Wilkins (2003). The compositions and formulations disclosed herein can be manufactured in any manner known in the art, *e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or compression processes.

A nanoparticle as disclosed herein can be incorporated into a variety of formulations for therapeutic administration, including solid, semi-solid, or liquid forms. The formulations include those suitable for oral, parenteral (including subcutaneous, intradermal, intramuscular, intravenous, intraarticular, and intramedullary), intraperitoneal, transmucosal, transdermal, rectal and topical (including dermal, buccal, sublingual and intraocular) administration although the most suitable route can depend upon for example the condition and disorder of the recipient. The formulations can conveniently be presented in unit dosage form and can be prepared by any of the methods well known in the art of pharmacy. Typically, these methods include the step of bringing into association a compound or a pharmaceutically acceptable salt thereof (“active

ingredient”) with the carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both and then, if necessary, shaping the product into the desired formulation.

5           The disclosed nanoparticles can be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions can take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing  
10   agents. The formulations can be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and can be stored in powder form or in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline or sterile pyrogen-free water, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules and tablets of the kind previously  
15   described.

          Formulations for parenteral administration include aqueous and non-aqueous (oily) sterile injection solutions of the active compounds which can contain antioxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which can include suspending agents  
20   and thickening agents. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions can contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension can also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for  
25   the preparation of highly concentrated solutions.

### Methods of Using

          The disclosed nanoparticles have properties that can afford them uses as optical, MRI, fluorescence, photoacoustic imaging probes. The disclosed nanoparticles can also be used in therapy (drug delivery, gene delivery, photodynamic therapy), catalysis, energy, and electronics  
30   applications.

          In a specific example, the disclosed nanoparticles can be used a MRI/fluorescence dual modal imaging probes. For example, the disclosed nanoparticles, or formulations containing them, can be used as imaging agents to visualize cancerous tissues, *e.g.*, tumors. In one aspect, disclosed herein is a method of detecting cancer *in vivo* comprising administering a nanoparticle  
35   (*e.g.*, Gd@C-dot) as disclosed herein to an individual and detecting a fluorescent signal and/or

magnetic resonance signal. Also, a region of interest in the individual can be imaged using a fluorescence reflectance imaging system (such as the F-Pro from Bruker), which is fitted with multiple band pass filters for excitation and emission.

In recent years, many new fluorescence imaging systems, such as endoscopes (Hsiung *et al.*, *Nat Med* 14:454 (2008); Funovics *et al.*, *Mol Imaging* 2:350 (2003)), wide-field video cameras (Knapp *et al.*, *European urology* 52:1700 (2007); van Dam *et al.*, *Nat Med* 17:1315 (2011)), and goggles (Liu *et al.*, *Surgery* 149:689 (2011); Wang *et al.*, *J Biomed Opt* 15:020509 (2010)), have been developed. Any of these systems can be used to detect the fluorescent signal, or lack thereof, in an individual to whom the disclosed nanoparticles have been administered. Further, the development of the fluorescence can be followed using a near infrared video camera (e.g., Fluoptics).

The disclosed nanoparticles can also be used as MR imaging probes. The nanoparticles disclosed herein can be used to detect/image a variety of other cancers. Examples of cancer types detectable by the compounds and compositions disclosed herein include bladder cancer, brain cancer, breast cancer, colorectal cancer, cervical cancer, gastrointestinal cancer, genitourinary cancer, head and neck cancer, lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, renal cancer, skin cancer, and testicular cancer. Further examples include cancer and/or tumors of the anus, bile duct, bone, bone marrow, bowel (including colon and rectum), eye, gall bladder, kidney, mouth, larynx, esophagus, stomach, testis, cervix, mesothelioma, neuroendocrine, penis, skin, spinal cord, thyroid, vagina, vulva, uterus, liver, muscle, blood cells (including lymphocytes and other immune system cells). Specific cancers contemplated for imaging include carcinomas, Kaposi's sarcoma, melanoma, mesothelioma, soft tissue sarcoma, pancreatic cancer, lung cancer, leukemia (acute lymphoblastic, acute myeloid, chronic lymphocytic, chronic myeloid, and other), and lymphoma (Hodgkin's and non-Hodgkin's), and multiple myeloma.

## EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary of the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

Optical measurements were performed at room temperature under ambient air conditions. UV-Vis absorption spectra were recorded on a Shimadzu 2450 UV-Vis spectrometer.

Photoluminescence (PL) measurements were performed on a Hitachi F-7000 fluorometer.

Fourier transform infrared (FT-IR) spectra were recorded on a Nicolet iS10 FT-IR Spectrometer.

- 5 The PL quantum yield (QY) was estimated using quinine sulfate in 0.1 M H<sub>2</sub>SO<sub>4</sub> (literature quantum yield: 58% at 354 nm excitation) as a reference standard, which was freshly prepared to reduce the measurement error. The formula used for QY measurements is as follows:

$$(QY)_{sm} = (QY)_{st} \times \left[ \left( PL_{area} / OD \right)_{sm} / \left( PL_{area} / OD \right)_{st} \right] \times \eta_{sm}^2 / \eta_{st}^2$$

- where Sm indicates the sample, St indicates the standard,  $\eta$  is the refractive index of the solvent, and PL area and OD are the fluorescence area and absorbance value, respectively. TEM and HR-TEM samples were prepared by dispersing the sample onto carbon-coated copper grids with the excess solvent evaporated. The TEM/HR-TEM overview images were recorded using a FEI Tecnai20 transmission electron microscope operating at 200 kV. Energy Dispersive Spectroscopy (EDS) and element mapping was characterized using Hitachi HD2000 Dedicated Scanning Transmission Electron Microscope (STEM). Dynamic light scattering (DLS) analysis was performed on a Zetasizer Nano S90 size analyzer (Malvern Corp, U.K.). Fluorescence images were acquired on an Olympus X71 fluorescence microscope (ex/em: 360/420 nm).

#### Example 1: Gd@C-Dot Synthesis and Characterization

- Gadolinium-DTPA (gadopentetic acid which is a complex of gadolinium with diethylenetriaminepentaacetate) was dried on a crucible and then calcined at 300 °C for 2 h in the air. This yielded black foam-like powder. The raw products were dispersed in water and subjected to centrifugation using centrifugal filter units (Millipore filter units: MWCO 100K, 3K), which removed aggregations of nanoparticles and unreacted precursors, respectively. The soluble portion through the filter was collected. The yielded Gd@C-dots were spherical, with an average size of about 12 nm and relatively narrow size distribution (Figure 1, panels (a) and (b)). High-resolution TEM (Figure 1, panel (c)) found low diffraction contrast and no obvious lattice fringes with the particles, indicating that the carbon was amorphous. This correlates with previous observations that calcination at low pressure typically yields amorphous structures (Bourlinos *et al.*, *Small* 2008, 4(4):455-458; Chen *et al.*, *Theranostics* 2013, 3(9):650-657). Elemental mapping revealed that Gd was distributed evenly within the carbon particles (Figure 1, panel (d)) (EDX pattern of Gd@C-dots as calculated by EDX software: C K 50.20%; O K 20.09%, N K 10.9%, Cu K 12.82%, Gd L 6.71% = 100%). The distribution pattern was also observed by scanning transmission electron microscopy (STEM) on a single C-dot particle

(Figure 1, panel (e)), showing that Gd was well encased within the carbon shell (Figure 1, panel (f)).

The as-synthesized Gd@C-dots were highly dispersible in aqueous solutions, maintaining colloidal stability for months without visible precipitation. Dynamic light scattering (DLS) analysis showed a single narrow peak at about 12 nm, which is well correlated with the TEM result (Figure 1, panel (g), Figure 6). The surface of Gd@C-dots was slightly negatively charged ( $-16.4 \pm 0.6$  mV, Figure 1, panel (h), Figure 7), attributed to carboxyl groups either inherited from the DTPA precursors and/or generated during the calcination. This is supported by FT-IR analysis, finding peaks at 3300 and 1600  $\text{cm}^{-1}$  that are characteristic absorptions of OH and C=O, respectively (Figure 8) (Li *et al.*, *Angew. Chem., Int. Ed.* 2013, 52(31):8151-8255; Bhunia *et al.*, *Sci. Rep.* 2013, 3, 1473). Despite the charged surface, however, there was little size increase when the nanoparticles were incubated in the bovine serum (Figure 6, panel (b)), indicating a minimal level of opsonization.

Gd@C-dots showed a broad absorption band between 200 to 500 nm, with a shoulder appearing at about 280 nm (Figure 2, panel (a)). The spectrum resembles those published previously of pure C-dots (Chen *et al.*, *Theranostics* 2013, 3(9):650-657; Bhunia *et al.*, *Sci. Rep.* 2013, 3, 1473; Sun *et al.*, *J. Am. Chem. Soc.* 2006, 128(24):7756-7757; Ding *et al.*, *Acc. Chem. Res.* 2014, 47, 20-30). The Gd@C-dots are also highly fluorescent, and can be excited by light of a wide range of wavelengths to emit strong photoluminescence (Figure 2, panel (a)). Such wavelength-dependent fluorescence is also similar to conventional C-dots (Bourlinos *et al.*, *Small* 2008, 4(4):455-458; Li *et al.*, *Angew. Chem., Int. Ed.* 2013, 52(31):8151-8255). Impressively, there was no drop of photoluminescence intensity of Gd@C-dots even after 24 hours of continuous UV illumination (Figure 2, panel (b)). This photostability is vastly superior to organic dye molecules, and even better than CdSe/ZnS quantum dots (Michalet *et al.*, *Science* 2005, 307(5709):538-544; Song *et al.*, *Chem. Soc. Rev.* 2010, 39(11):4234-4243), both of which were completely bleached within hours of UV exposure (Figure 2, panel (c)). The  $T_1$  contrast ability was investigated on a 7T magnet with agarose samples of Gd@C-dots. Gd@C-dots showed an  $r_1$  of 5.88  $\text{mM}^{-1}\text{s}^{-1}$  on a Gd basis (Figure 2, panels (d) and (e)), which is significantly higher than Gd-DTPA (3.10  $\text{mM}^{-1}\text{s}^{-1}$ ) (Kim *et al.*, *J. Am. Chem. Soc.* 2011, 133(9):2955-2961; Kalavagunta *et al.*, *Contrast Media & Mol. Imaging* 2014, 9(2):169-176). The enhanced  $r_1$  was mainly attributed to the increase in the rotational correlation time ( $\tau_R$ ) as a result of binding Gd to a nanoparticle (Huang *et al.*, *Curr. Top. Med. Chem.* 2013, 13(4):411-421).

**Example 2: *In vitro* testing**

Gd@C-dots were incubated in phosphate buffered saline of pH 5 or 7.4 at 37°C for 72 h. At both pH values, no drop of luminescence intensity and negligible Gd leakage from the nanoparticles was observed over time (Figure 3, panels (a) and (b)).

Gd@C-dots were also subjected to a cytotoxicity study with U87MG cells using standard MTT assays. The cells were first seeded in 96-well plates ( $1 \times 10^4$  cells per well). After 24 h, Gd@C-dots at different concentrations were added. Incubation was carried out for 24 h with or without 2.5 mM  $\text{CaCl}_2$ . To assess the particles' stability against transmetallation, which is the major cause of toxicity for conventional Gd contrast agents (Corot *et al.*, *J. Magn. Reson. Imaging* 1998, 8(3):695-702), 2.5 mM  $\text{Ca(II)}$  was added into the incubation medium. No significant drop of cell viability was observed even at high nanoparticle concentrations (0–100  $\mu\text{g Gd/mL}$ , Figure 3, panel (c)). As a comparison, Gd-DTPA induced dramatic cell death under the same conditions with an  $\text{IC}_{50}$  of 33.1  $\mu\text{g/mL}$  (Figure 3, panel (c)) (Wu *et al.*, *Pharm. Res.* 2010, 27(7):1390-1397; Aime *et al.*, *J. Magn. Reson. Imaging* 2009, 30(6):1259-1267).

**Example 3: Conjugation of Gd@C-dots**

Carboxyl groups on the Gd@C-dots surface offer a facile means to tether functional bio-species. As an example, c(RGDyK), a tumor targeting peptide, was conjugated onto the Gd@C-dots. A cyclic RGD derivative, c(RGDyK) holds strong binding affinity toward integrin  $\alpha_v\beta_3$ , a biomarker that is seen overexpressed on neoplastic blood vessels and many types of cancer cells (Ye *et al.*, *Theranostics* 2011, 1:102-126). Specifically, Gd@C-dots were dispersed in a borate buffer (pH 8.3). Into the solution, carbodiimide (EDC) and N-hydroxysuccinimide (NHS) ( $10\times$ ) in DMSO was added, and the mixture was magnetically stirred for 30 min. The intermediate was purified by centrifugation, and redispersed in PBS (pH 7.4). Into the solution, c(RGDyK) in DMSO ( $20\times$ ) was added and the mixture was incubated for 2 h with gentle agitation. The product was collected using a centrifugal filtration unit (Millipore filter unit: MWCO 3K) and redispersed in PBS (pH = 7.4). After the coupling, the nanoparticles' zeta potential increased slightly to  $-12.0 \pm 0.4$  mV, and the size to about 16.0 nm (Figure 6, panel (c)).

The targeting specificity of the c(RGDyK) conjugated Gd@C-dots (hereafter referred to as RGD-Gd@C-dots) was investigated with U87MG cells, which are integrin  $\alpha_v\beta_3$  positive.

U87MG cells were grown in a petri dish of a sterile glass bottom at 37 °C in 5%  $\text{CO}_2$ . Cells were incubated in 1 mL media containing Gd@C-dots (50  $\mu\text{g}$ ) with and without the presence of c(RGDyK) (1 mg) for 1 hour. Cells were washed three times with PBS (pH 7.4), and then imaged under an Olympus X71 fluorescence microscope. For MRI studies,  $10^5$  cells treated under similar conditions were collected in 300  $\mu\text{L}$  PCR tubes and subjected to  $T_1$ -weighted MRI.

After 30 min incubation, there was a high level of nanoparticle uptake (ex/em: 360/460 nm), with many signals concentrated in the cell endosomes/lysosomes (Figure 3, panel (d)). The cell uptake was dramatically suppressed when Gd@C-dots were co-incubated with free c(RGDyK) (30×), indicating that the uptake was mostly mediated by RGD-integrin interaction.

Such difference in cell uptake was also discerned by MRI. Figure 3, panel (e), shows a  $T_1$ -weighted MR image of 105 U87MG cells that had been incubated with either RGD-Gd@C-dot or Gd@C-dots. Compared to the control, significantly enhanced signals were observed in cells incubated with RGD@C-dots (Figure 3, panel (e)).

#### Example 4: *In vivo* testing

Animal studies were performed according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of University of Georgia. Before *in vivo* experiments, the Gd@C-dots and RGD-Gd@C-dots were filtered through sterilized membrane filters (pore size 0.22  $\mu$ m) and stored in sterilized vials. For *in vivo* MRI studies, whole body transverse images of normal athymic nude mice were first acquired. The mice were then intravenously (i.v.) injected with 100  $\mu$ L Gd@C-dots and RGD-Gd@C-dots (0.8 mg Gd/kg). Transverse and coronal  $T_1$ -weighted MR images were acquired at 10, 30, 45 min, 60 min and 4 h post the nanoparticle injection (p.i.). The images were acquired using the following parameters: TR/TE= 500/12 ms, field-of-view (FOV) = 70  $\times$  70 mm<sup>2</sup>, matrix size = 256  $\times$  256, slice = 4, and thickness = 1 mm.

There was an initial increase of signals, followed by a signal decay starting from 60 min. After 4 h, all the signals had subsided to the pre-injection levels, indicating efficient clearance of Gd@C-dots from the body (Figure 4, panel (a)). Unexpectedly, the signal change in the liver was found to be small throughout the course of the experiment (Figure 4, panels (a) and (b)). Instead, there was a dramatic increase of signals in the bladder, indicating that most of the nanoparticles were excreted through renal clearance. Similar pharmacokinetics was observed with animals injected with RGD-Gd@C-dots (Figure 4, panel (a) and (b)).

To validate, urine was collected from the animals about 60 min after the particle injection, and by centrifuging, nanoparticles were separated from the rest of the sample. Subsequent analysis found a large amount of Gd (by inductively coupled plasma mass spectroscopy, or ICP- MS) in the nanoparticle fragment, along with photoluminescence that was characteristic of C-dots (Figure 4, panel (c)). On the other hand, no Gd was detected in the rest of the sample. This result confirmed the renal clearance of Gd@C-dots, and that the Gd was still encapsulated within the carbon shell at the time of excretion.

RGD-Gd@C-dots were also tested as tumor imaging probes in U87MG tumor-bearing mice. Tumor models were developed in 5-6 week athymic nude mice (Harlan) by subcutaneous

implantation of  $10^6$  human glioblastoma U87MG cells suspended in 100  $\mu$ L of serum-free DMEM to the right lower flank of a mouse. Imaging studies were conducted 3-4 weeks later. Specifically, the tumor-bearing mice were intravenously injected with Gd@C-dots and RGD-Gd@C-dots (3.2 mg Gd/kg). Transverse and coronal  $T_1$ -weighted images were acquired at 15, 30, 60, 120, and 240 min post injection using the following parameters: TR/TE= 500/12 ms, field-of-view (FOV) =  $70 \times 70$  mm<sup>2</sup>, matrix size =  $256 \times 256$ , slice = 4, thickness = 1 mm. To quantify the signal change, the signal-to-background ratio (SBR) was calculated by finely analyzing regions of interest (ROIs) of the MR images and calculating the values of SBR/SBR<sub>0</sub> to represent the signal changes. Signal intensity (SI) of normal liver, kidney, brain, and muscle were measured before and after injection of Gd@C nanoparticles. The mean SI measurements of 3 mice per group were used for statistical analysis. Because of slight changes in the position of the mice at different imaging stages, pre and post ROIs were determined manually on each image as reproducible as possible. For each animal, 3-5 ROIs were selected to measure the SI of the liver, kidney, brain and muscle. The SBR values were calculated according to  $SBR = SI_{organ}/SI_{muscle}$  for coronal plane, and  $SBR/SBR_0 = SI/SI_0$  for transverse plane. In a control group, Gd@C-dots at the same Gd dose were injected.

Similar to the above experiment, there was fast renal clearance of RGD-Gd@C-dots, evidenced by strongly enhanced intensities in the bladder (Figure 5, panel (a)). After 4 h, signals in the normal tissues had receded to the normal level for both RGD-Gd@C-dots and Gd@C-dots injected animals. However, there was a signal enhancement in tumors of  $42.6 \pm 0.08\%$  in animals injected with RGD-Gd@C-dots compared to those injected with Gd@C-dots (Figure 5, panels (b) and (c)). This retention of particles in tumors was attributed to RGD-integrin interaction, which was confirmed by immunofluorescent studies that found good overlap between RGD-Gd@C-dots (ex/em: 360/460 nm) and positive integrin  $\beta_3$  staining (Figure 5, panel (d)).

It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

## CLAIMS

What is claimed is:

1. A nanoparticle, comprising: gadolinium encapsulated in an amorphous carbon shell, wherein the shell comprises carboxyl groups and wherein the average diameter of the nanoparticle is from about 2 nm to about 34 nm.
2. The nanoparticle of claim 1, wherein the gadolinium is Gd(III).
3. The nanoparticle of any one of the preceding claims, wherein the average diameter is from about 10 nm to about 14 nm.
4. The nanoparticle of any one of the preceding claims, wherein the average diameter is about 12 nm.
5. The nanoparticle of any one of the preceding claims, wherein the nanoparticle has a  $r_1$  relaxivity of from about 0 to about 50 mM<sup>-1</sup>s<sup>-1</sup>.
6. The nanoparticle of any one of the preceding claims, wherein the nanoparticle has a  $r_1$  relaxivity of from about 5 to about 25 mM<sup>-1</sup>s<sup>-1</sup>.
7. The nanoparticle of any one of the preceding claims, wherein the nanoparticle has a quantum yield of from about 0 to about 80%.
8. The nanoparticle of any one of the preceding claims, wherein the nanoparticle has a quantum yield of from about 15 to about 50%.
9. The nanoparticle of any one of the preceding claims, wherein the nanoparticle is conjugated to a targeting moiety.
10. The nanoparticle of any one of the preceding claims, wherein the nanoparticle is conjugated to a cyclic RDG peptide.
11. The nanoparticle of any one of the preceding claims, wherein the nanoparticle is conjugated to Herceptin, Avastin, Erbitux, doxorubicin, methotrexate or paclitaxel, or polyethylenimine.

12. A method of imaging cancer in a subject, comprising: administering to the subject a nanoparticle of any one of the preceding claims; and detecting a fluorescent and/or magnetic resonance signal from the cancer.
13. A method of making a nanoparticle of any one of claims 1-11, comprising: calcining gadolinium with a chelator.
14. The method of claim 13, wherein the chelator is diethylenetriaminepentaacetate.
15. The method of claim 13, wherein the chelator is 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA), 1,4,7,10-tetraazacyclododecane-1, 4, 7,10-tetraacetic acid (DOTA), 1,4,8,11-tetraazacyclododecane-1,4,8,11-tetraacetic acid (TETA), 2,2'-(1,4,8,11-tetraazabicyclo[6.6.2]hexadecane-4,11-diyl)diacetic acid (CB-TE2A), 3,6,9,15-Tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-3,6,9-triacetic acid (PCTA), pendetide (GYK-DTPA), cyclohexyldiethylenetriaminepentaacetic acid (CHX-DTPA), 2-(4,7-biscarboxymethyl[1,4,7]triazacyclonona-1-yl-ethyl)carbonyl-methylamino]acetic acid (NETA), diethylene triamine pentaacetic acid (DTPA), desferrioxamine, nitrilotriacetate (NTA), DO3A, ethylenediammine, acetylacetonate, phenanthroline, oxalate, citric acid, bipyridine, cyanide, nitrite, acetonitrile, ethylenediamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), poly-L-lysine, polyethylenimine, or polyvinylpyrrolidone (PVP), or any salt, derivative, functionalized analog, or mixture of these.
16. A nanoparticle, comprising: a metal encapsulated in an amorphous carbon shell, wherein the shell comprises carboxyl groups and wherein the average diameter of the nanoparticle is from about 2 nm to about 34 nm.
17. The nanoparticle of claim 16, wherein the metal is Fe, Mn, Ti, Co, Ni, Cu, Zn, Ga, Ge, Y, Au, Ag, Pt, Cr, Cd, Pb, Ce, Eu, Dy, Tb, Ho, Er, Tm, or Yb.
18. A method of making a nanoparticle of claims 16 or 17, comprising: calcining a metal with a chelator.
19. The method of claim 18, wherein the metal is Fe, Mn, Ti, Co, Ni, Cu, Zn, Ga, Ge, Y, Au, Ag, Pt, Cr, Cd, Pb, Ce, Eu, Dy, Tb, Ho, Er, Tm, or Yb.

20. The method of claims 18 or 19, wherein the chelator is diethylenetriaminepentaacetate, 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), 1,4,8,11-tetraazacyclododecane-1,4,8,11-tetraacetic acid (TETA), 2,2'-(1,4,8,11-tetraazabicyclo[6.6.2]hexadecane-4,11-diyl)diacetic acid (CB-TE2A), 3,6,9,15-Tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-3,6,9-triacetic acid (PCTA), pendentide (GYK-DTPA), cyclohexyldiethylenetriaminepentaacetic acid (CHX-DTPA), 2-(4,7-biscarboxymethyl[1,4,7]triazacyclononane-1-yl-ethyl)carbonyl-methylamino]acetic acid (NETA), diethylene triamine pentaacetic acid (DTPA), desferrioxamine, nitrilotriacetate (NTA), DO3A, ethylenediamine, acetylacetonate, phenanthroline, oxalate, citric acid, bipyridine, cyanide, nitrite, acetonitrile, ethylenediamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), poly-L-lysine, polyethylenimine, or polyvinylpyrrolidone (PVP) or any salt, derivative, functionalized analog, or mixture of these.

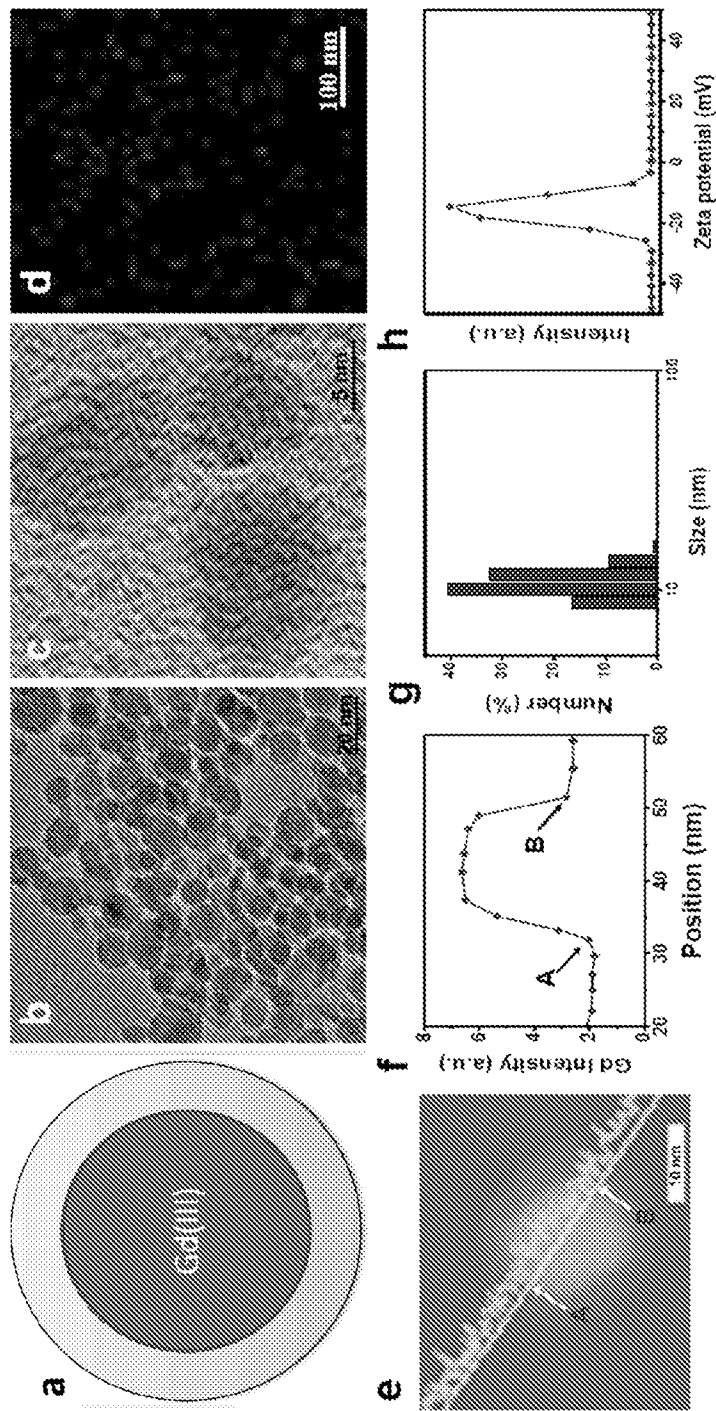
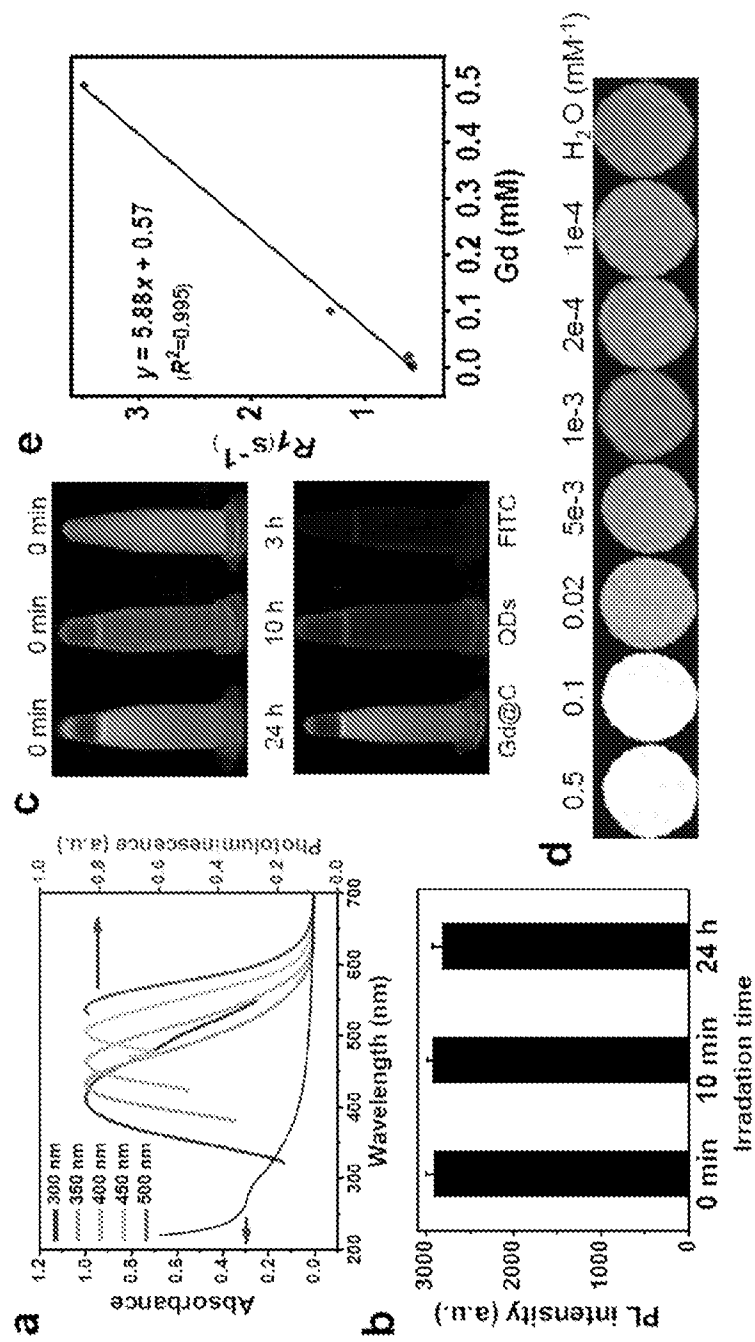


FIGURE 1



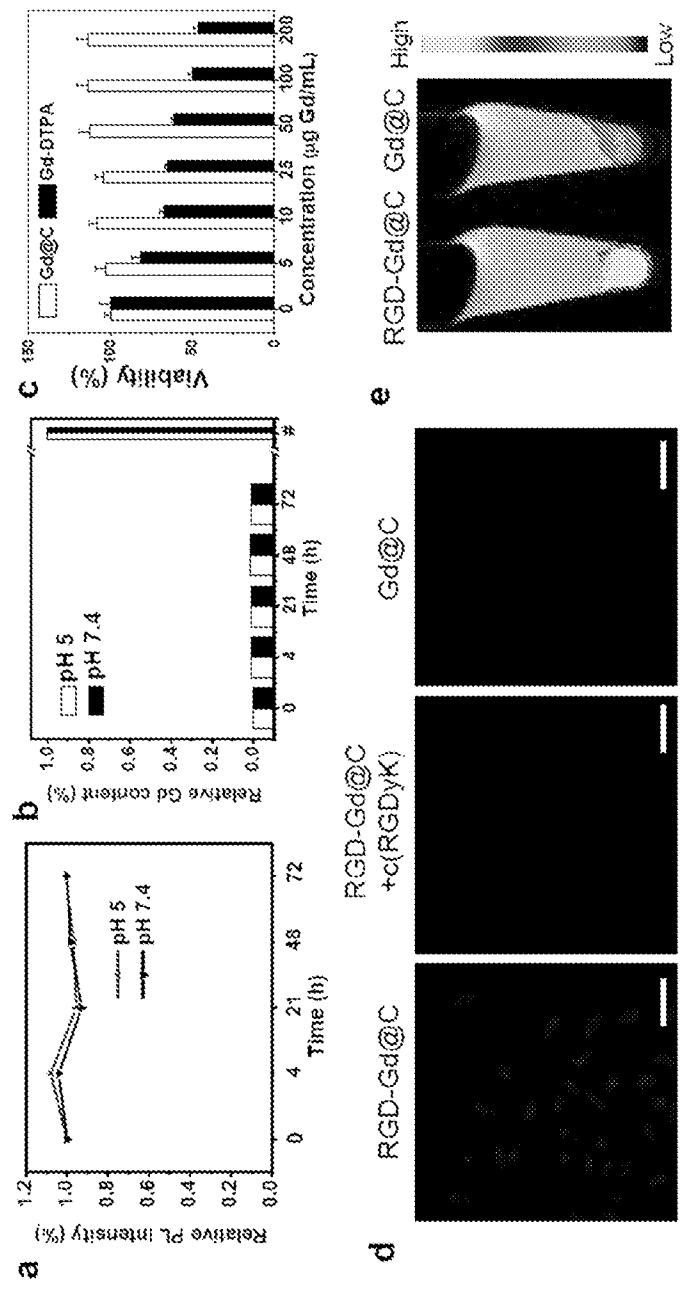


FIGURE 3

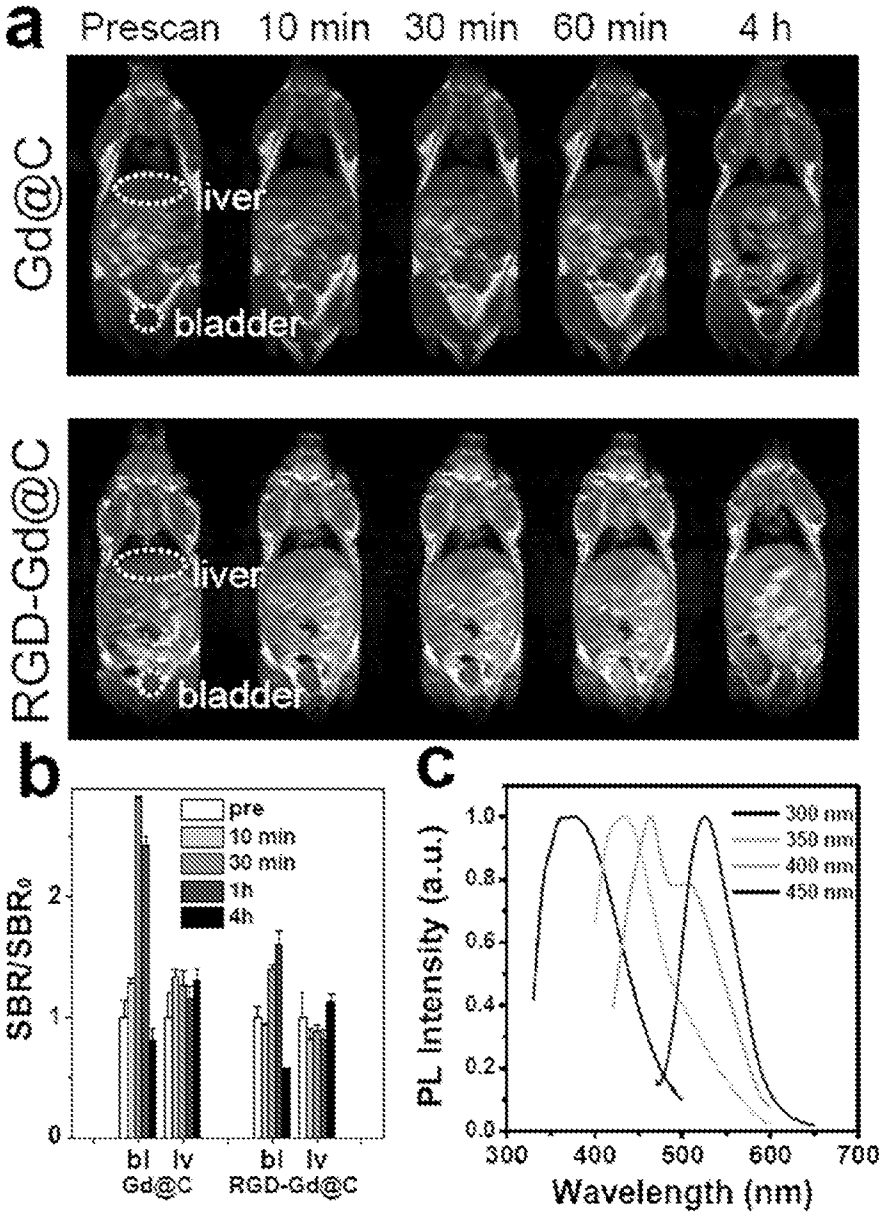


FIGURE 4

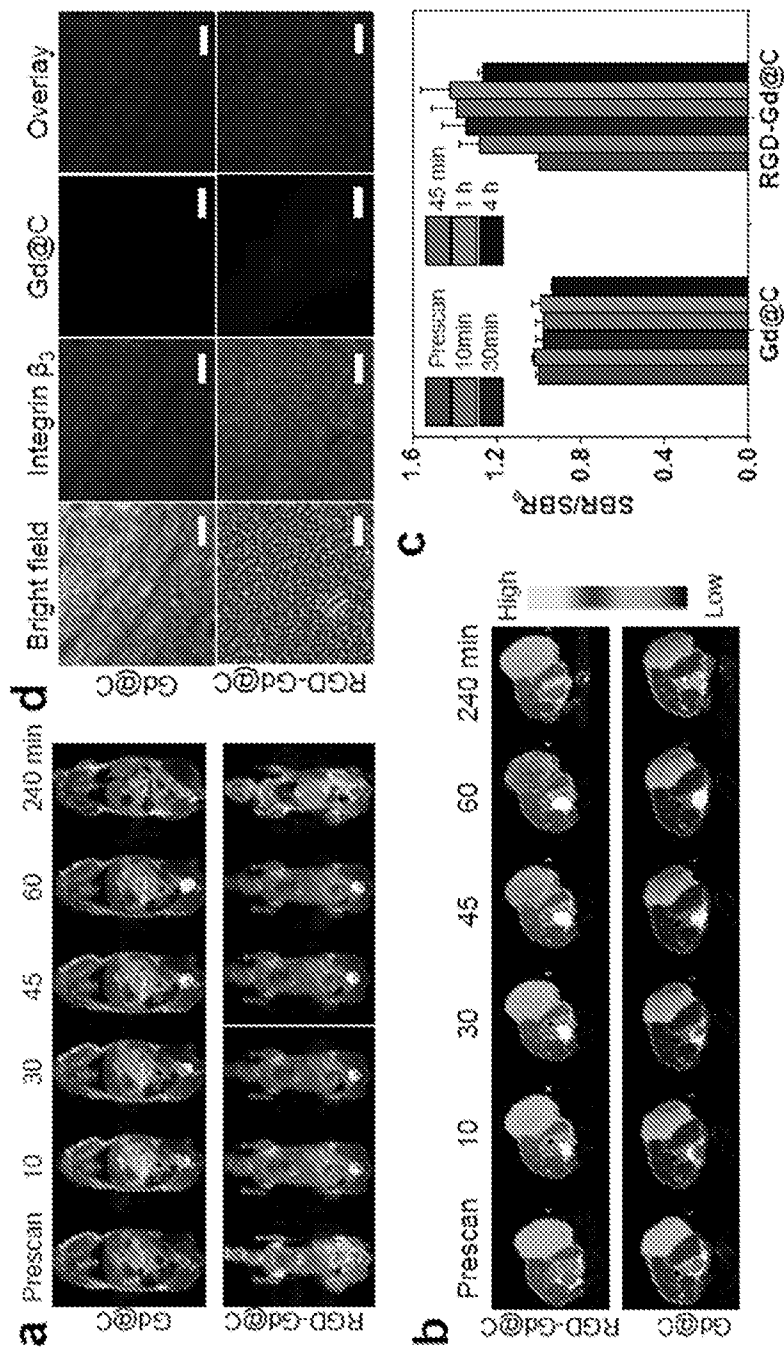


FIGURE 5

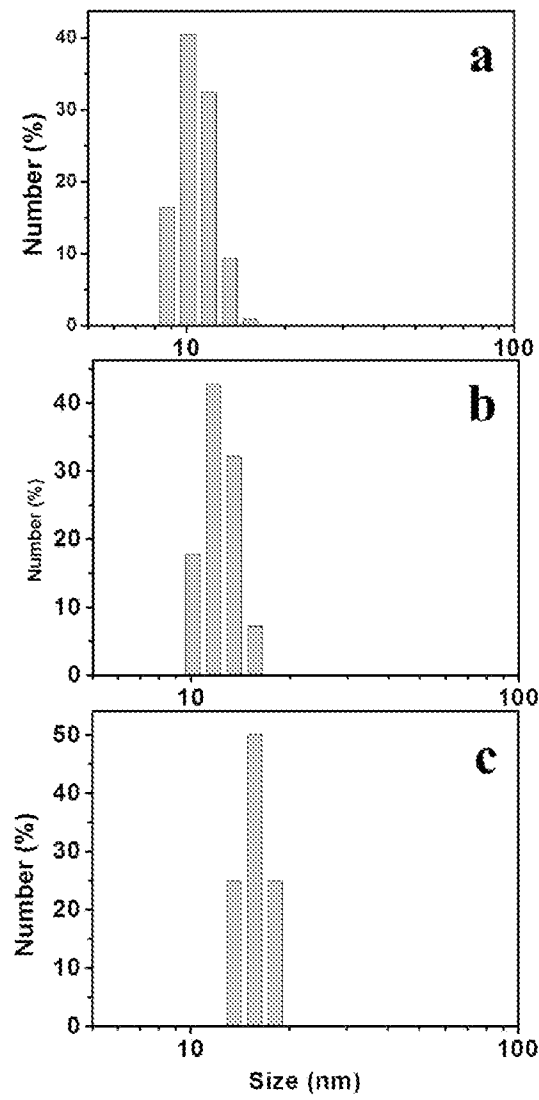


FIGURE 6

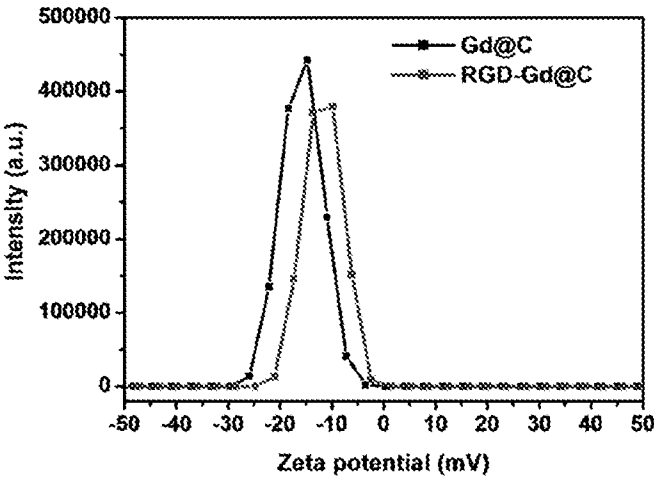


FIGURE 7

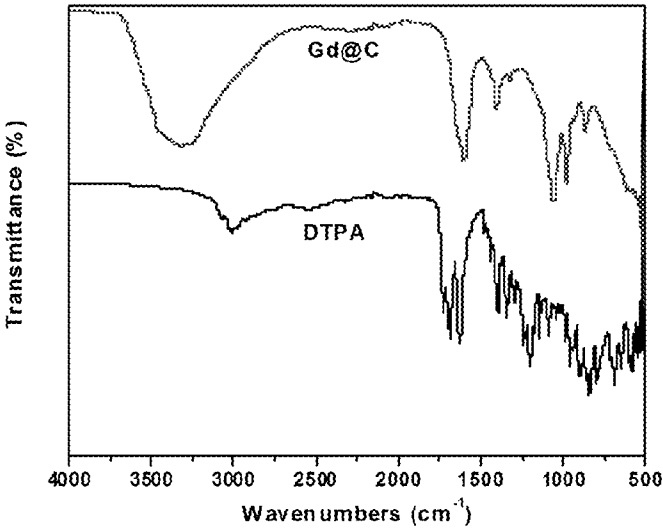


FIGURE 8

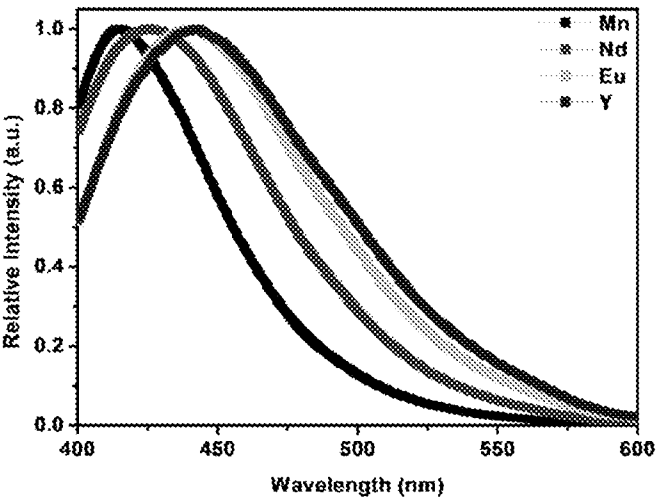


FIGURE 9

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US15/42787

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 49/08, 49/06; A61B 5/055 (2015.01)

CPC - B82Y 30/00; A61K 49/06; A61B 6/481

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)

IPC(8): A61K 49/08, 49/06; A61B 5/055 (2015.01)

CPC: B82Y 30/00; A61K 49/06; A61B 6/481, 5/055

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)

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); Google; Google Scholar; PubMed; Dialog ProQuest; nanoparticle, 'Carbon dot,' gadolinium, 'Carbon shell,' metal, calcining

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                    | Relevant to claim No.            |
|-----------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------|
| Y         | REN, X et al. Facile Preparation of Gadolinium (III) Chelates Functionalized Carbon Quantum Dots-based Contrast Agent for Magnetic Resonance/Fluorescence Multimodal Imaging. Journal of Materials Chemistry B. 24 June 2014, Vol. 2, pp 5541-5549; abstract; page 5, first column, third paragraph; page 5, second column fourth paragraph. DOI: 10.1039/C4TB00709C. | 1-3, 16-19                       |
| Y         | WO 2007/095454 A2 (BOARD OF SUPERVISORS OF LOUISIANA STATE UNIVERSITY AND AGRICULTURAL AND MECHANICAL COLLEGE) August 23, 2007; paragraph [0087]                                                                                                                                                                                                                      | 1-3, 16-19                       |
| Y         | BHUNIA, SK et al. Carbon Nanoparticle-Based Fluorescent Bioimaging Probes. Scientific Reports. 18 March 2013, Vol. 3, No. 1473; pages 1-7; abstract; DOI: 10.1038/srep01473.                                                                                                                                                                                          | 31/, 3/2                         |
| Y         | THIRUMAVALAVAN, M et al. Preparation And Morphology Studies Of Nano Zinc Oxide Obtained Using Native And Modified Chitosans. Materials. 18 September 2013, Vol. 6, pp 4198-4212; abstract; page 4199, second paragraph; page 4201, second paragraph. DOI: 10.3390/ma6094198.                                                                                          | 18/16, 18/17, 19/18/16, 19/18/17 |

☐ 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

"&amp;" document member of the same patent family

Date of the actual completion of the international search

05 October 2015 (05.10.2015)

Date of mailing of the international search report

29 OCT 2015

Name and mailing address of the ISA/

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Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300  
PCT OSP: 571-272-7774

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US15/42787

## Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-15, 20  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.