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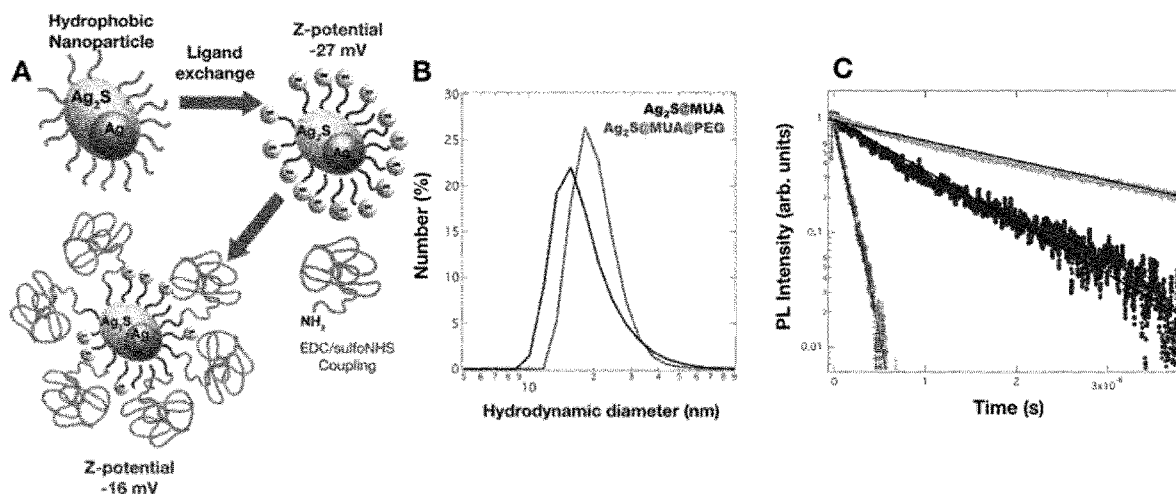
(54) Title: AG₂S NANOPARTICLES AND METHODS OF PRODUCTION THEREOF

Fig. 6

(57) Abstract: In this invention, we have developed a novel methodology that permits a significant increment in the quantum yield of Ag₂S dots. The process is partly based on the irradiation of chloroform-dispersed Ag₂S/Ag heterodimers with femtosecond laser pulses that lead to the formation of a protective AgCl shell. This reduces the surface traps while minimizing dot-to-medium transfer via non-radiative events. The Ag₂S superdots generated as a result of this process outperform all currently available NIR-II contrast agents, enabling whole body in vivo imaging at ultra-low excitation intensities.



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Ag₂S nanoparticles and methods of production thereof.

TECHNICAL FIELD OF THE INVENTION

The present invention refers to the biomedical field, in particular to nanoparticles for use
5 as imaging fluorescent agents for in vitro and in vivo applications.

BACKGROUND OF THE INVENTION

Semiconductor nanocrystals emerged almost two decades ago as a new generation of
fluorescent contrast agents capable of overcoming the intrinsic limitations of traditional
10 fluorescent biomarkers (dyes and fluorescent proteins). The initial demonstration of the
ability of these nanocrystals to generate high-contrast images at the cellular level was a
major milestone in biophotonics and promoted the research in the field for the following
years. The optimization of their chemical stability, biocompatibility and brightness was
the major focus of the subsequent investigation. That resulted in the development of
15 core/shell architectures, which constituted the second generation of semiconductor
nanocrystals. These produced unparalleled results as cellular imaging probes but failed
to enable deep-tissue *in vivo* imaging due to the poor penetration and high scattering of
visible light into tissues. This limitation was overcome by the development of a third
generation of semiconductor nanocrystals, emitting in the second near infrared window
20 (NIR-II, 1,000-1,700 nm). In this spectral range, both tissue absorption and scattering
coefficients are reduced to a minimum, allowing high-contrast, high-resolution **in vivo**
imaging at large (> 1 cm) tissue depths⁹. NIR-II fluorescent semiconductor nanocrystals
have changed the game in preclinical **fluorescence imaging**, enabling previously
unthinkable feats such as **high-resolution anatomical imaging, brain vasculature**
25 **mapping and even dynamic tracking of metabolic processes**. NIR-II emitting Ag₂S
nanocrystals are free from heavy metal ions, unlike other nanoprobe operating in this
spectral range, minimizing biocompatibility concerns and making them the most
promising system among all currently reported NIR-II fluorophores. Their properties have
been exploited for multiple applications, including subcutaneous and transcranial
30 thermometry¹⁶, in vivo heart imaging, [<https://doi.org/10.1007/s12274-019-2280-4>],
photoacoustic imaging, [<https://doi.org/10.1021/acsnano.6b07866>], theranostics
[<https://doi.org/10.1007/s12274-018-2210-x>], tumour diagnosis and dynamic imaging of
the cardiovascular system.

35 To enhance the clinical potential of Ag₂S dots, their properties need to be substantially

improved to enable deep-tissue imaging at irradiation intensities well below the established safety threshold. Clinical use of NIR-II fluorescence imaging requires the improvement in the fluorescent properties of currently available NIR-II probes in order to increase the maximum penetration depth of the obtained images. In particular, the translation of Ag₂S dots into clinics requires a substantial increase in their fluorescence brightness, which is limited by their low quantum yield (QY) and short fluorescence lifetimes. Chemically synthesized Ag₂S dots typically present absolute QYs below 1% and lifetimes below <180 ns ²⁰. These features have been attributed to the presence of surface and structural defects and to the dot-solvent interactions that favour electronic deexcitation via non-radiative pathways. Furthermore, the high redox potential of silver ions and the high temperatures required for Ag₂S dot synthesis lead to the simultaneous formation of metallic silver nanoparticles (NPs) that could also reduce the overall brightness due to plasmon coupling events. Currently available chemical synthesis routes have failed to avoid these non-radiative channels in Ag₂S dots. Therefore, the design and development of alternative and successful approaches becomes essential.

In this invention, we have developed two novel methodologies that permits a significant increment in the quantum yield of Ag₂S dots.

20 DESCRIPTION OF THE INVENTION

As shown in example 1, the present invention demonstrates the crucial role of the synthetic conditions on the photoluminescence properties of Ag₂S nanoparticles. More in detail, the effect of the solvent ratio of DDT and OLA during the nanoparticle generation via thermal degradation using an Ag(DDTC) was evaluated. In total, six different solvent ratios were tested (χ_{DDT} of 0.00, 0.15, 0.30, 0.60, 0.90 and 1.00). From a structural point of view, a clear decrease in nanoparticle size with an increase of DDT was observed, assumingly correlated to a more pronounced stabilization of small nanoparticles by the thiol bearing solvent DDT. Regarding the photoluminescent properties, a maximum of luminescence lifetimes and QY were observed for nanoparticles generated with a χ_{DDT} of 0.60. Measured luminescence lifetimes reached values of 1200 ns in comparison to lifetimes of 120 and 180 ns for the nanoparticles synthesized using χ_{DDT} of 0.00 and 1.00, respectively. For the QY, a 10-fold higher value of 2.3 % was determined for the nanoparticles synthesized with χ_{DDT} of 0.60 compared to the nanoparticles derived from neat solvents. As studied by EDS-mapping of the generated nanoparticles using χ_{DDT} of 0.00, 0.60 and 1.00, we were able to clearly correlate the improved photoluminescent

properties to a more precise ratio of Ag and S in the final nanoparticle matrix. In addition, the surface modification of the Ag₂S nanoparticles allows for a transfer into aqueous solution. In this media our fabricated Ag₂S nanoparticles conserve the superb optical properties, exhibiting higher luminescence lifetimes and QY compared to commercially available PEGylated Ag₂S in aqueous buffer. This permits their usage as contrast
 5 imaging agents, where the nanoparticles present an excellent biodistribution.

Therefore, a first aspect of the invention refers to a method for manufacturing, preferably hydrophobic, Ag₂S nanoparticles comprising the following steps:

- 10 a. Mixing a given amount of silver such as for example in the form of Ag(DDTC) (silver diethyldithiocarbamate), preferably in an amount of about 25 mg (0.1 mmol), preferably at room temperature, with a solvent comprising a mixture based on an i) organic long-chain molecule, comprising between 4 and 20 carbon atoms in length and comprising an amine group, and ii) DDT (1-dodecanethiol)
 15 or any other thiol based molecule with a boiling point of less than 350°C, preferably less than 200°C, preferably between 140 and 200°C, such as 1-decanethiol, 1-undecanethiol, 1-pentadecanethiol, wherein the solvent mole fraction (χ_{DDT}) calculated as $\chi_{\text{DDT}} = (\text{nDDT}) / (\text{nDDT} + \text{organic long-chain molecule (such as nOLA)})$ is between 0.30 and 0.90;
- 20 b. submitting the reaction mixture of a) to a temperature between 80°C and 350°C in, preferably a closed atmosphere; and
- c. collecting the nanoparticles.

In a preferred embodiment, the solvent mole fraction (χ_{DDT}) is between 0.45 and 0.75.
 25 More preferably, the solvent mole fraction (χ_{DDT}) is between 0.55 and 0.65. More preferably, the solvent mole fraction (χ_{DDT}) is between 0.58 and 0.62. Still more preferably, the solvent mole fraction (χ_{DDT}) is between 0.59 and 0.61. Still more preferably, the solvent mole fraction (χ_{DDT}) is between 0.595 and 0.605. Still more preferably, the solvent mole fraction (χ_{DDT}) is between 0.598 and 0.602. Still more
 30 preferably, the solvent mole fraction (χ_{DDT}) is between 0.599 and 0.601- Still more preferably, the solvent mole fraction (χ_{DDT}) is about 0.60. Still more preferably, the solvent mole fraction (χ_{DDT}) is 0.60.

In another preferred embodiment of the first aspect of the invention, silver can be provided in any form such as those selected from the list consisting of: Ag(DDTC), silver

nitrate, silver dihydrocarbyl thiophosphate, silver dioctyl sulfosuccinate, silver thiobenzoate, silver acetate, silver dodecanoate, silver tetradecanoate and silver octadecenoate.

5 In another preferred embodiment of the first aspect of the invention, the organic long-chain molecules are selected from the list consisting of octylamine, trioctylamine, dodecylamine, octadecylamine, and OLA (oleylamine). Preferably, such molecule is and OLA (oleylamine).

10 In another preferred embodiment of the first aspect of the invention or of any of its preferred embodiments, the closed atmosphere is created by using a vacuum, preferably for about 10 min, to remove air and then filled with N₂, other inert gases like Argon, or He can be also used.

15 In another preferred embodiment of the first aspect of the invention or of any of its preferred embodiments, in step b) the mixture is heated to a temperature between 120°C and 350°C, preferably between 150°C and 230°C, more preferably between 170°C and 210°C, still more preferably to a temperature about 190°C, under magnetic stirring, preferably with a heating rate of 20°C/min and under slow magnetic stirring.

In another preferred embodiment of the first aspect of the invention or of any of its preferred embodiments, prior to step c) the reaction is cooled down by any suitable means, preferably by naturally cooling the reaction.

20 In another preferred embodiment of the first aspect of the invention or of any of its preferred embodiments, in step c) the nanoparticles are preferably collected by the addition of ethanol, which reduces the colloidal stability of the nanoparticles, preferably by adding about 10 mL of ethanol to the raw product and centrifuging at about 10,000 rpm for 10 min, and preferably repeating this process twice.

25 In another preferred embodiment of the first aspect of the invention or of any of its preferred embodiments, the resultant product of step c) is dispersed in chloroform and stored.

A second aspect of the invention refers to a method for manufacturing pegylated hydrophobic Ag₂S nanoparticles comprising the following steps:

30 a. adding a hydrophilic agent such as 11-mercaptoundecanoic acid (MUA), dihydrolipoic acid, 2,3 dimercaptosuccinic acid, mercaptopropionic acid, or cysteine, to a dispersion containing Ag₂S/Ag nanoparticles as obtained from the method of the first aspect of the invention, preferably in chloroform at room temperature;

- b. Precipitating and collecting the product of step d);
- c. Covering the precipitates of step e) with PEG, preferably PEG-NH₂, more preferably via EDC/NHS coupling; and
- d. Collecting the nanoparticles.

5

In a preferred embodiment of the second aspect of the invention, the precipitating step e) is performed by sonicating the mixture in an ultrasonic bath, preferably for 10 minutes, until the Ag₂S/Ag nanoparticles lose their colloidal stability and precipitate.

10 In another preferred embodiment of the second aspect of the invention or of any of its preferred embodiments, the treatment with PEG via EDC/NHS coupling, is performed by dissolving EDC and sulfo-NHS in a saline solution such as PBS comprising the nanoparticles obtained from step e) and PEG.

15 A third aspect of the invention refers to, the preferably hydrophobic, Ag₂S nanoparticles obtained or obtainable by the method of the first aspect of the invention or of any of its preferred embodiments.

A fourth aspect of the invention refers to pegylated Ag₂S nanoparticles obtained or obtainable by the method of the second aspect of the invention or of any of its preferred embodiments.

20 A fifth aspect of the invention refers to a composition comprising the hydrophobic Ag₂S nanoparticles of the third aspect of the invention.

A sixth aspect of the invention refers to a composition comprising the pegylated hydrophobic Ag₂S nanoparticles of the fourth aspect of the invention.

25 A seventh aspect of the invention refers to the composition of any of the fifth or sixth aspects of the invention or to the nanoparticles of the third or fourth aspects of the invention for use as an in vivo imaging agent, or for in vivo **fluorescence imaging**, enabling features such as **high-resolution anatomical imaging, brain vasculature mapping and even dynamic tracking of metabolic processes**.

30 A further application of the seventh aspect of the invention refers to the composition of any of the fifth or sixth aspects of the invention or to the nanoparticles of the third or fourth aspects of the invention for use in vivo diagnosis.

A further application of the seventh aspect of the invention refers to the composition of any of the fifth or sixth aspects of the invention or to the nanoparticles of the third or

fourth aspects of the invention for use in in vitro diagnosis as a imaging agent, in particular as a **fluorescence imaging agent**.

A second part of the present invention, is shown in example 2, which demonstrates the potential of femtosecond laser pulses to radically improve the properties of luminescent nanostructures operating in the second biological window. The capacity of ultrafast laser irradiation to tune the structural and optical properties of infrared luminescent Ag₂S NPs shown here can undoubtedly expand their range of applications, especially in the field of nanomedicine. The advantages offered by these nanoparticles, as demonstrated here, including their photochemical stability, optimum spectral operation range and the 80-fold improvement in their quantum yield, constitute a large step towards their translation into the clinics. Our discovery, the ability of ultrafast laser pulses to improve the luminescent properties of nanoparticles, also stimulates new synthesis procedures that could benefit from the synergy between traditional chemical procedures and light-matter interaction processes.

Therefore, a second part of the present invention relates to hydrophobic Ag₂S nanoparticles capable of emitting in the near infrared with a quantum efficiency of 10% (representing a 50-fold increase with respect to the efficacy of the nanoparticles obtained so far) as well as to a method of synthesis of the same. Such nanoparticles can be superficially functionalized, making them especially suitable as imaging contrast agents in biological applications.

Therefore, an eight aspect of the invention refers to a method of manufacturing, preferably, hydrophobic Ag₂S nanoparticles comprising the following steps:

- a. Mixing a given amount of silver with a long chain organic molecule, preferably having between 4 and 20 carbons in length, comprising an amine group;
- b. submitting the reaction mixed of a) to a temperature between 80°C and 350°C in, preferably a closed atmosphere; and
- c. collecting the nanoparticles.

In a preferred embodiment of the eight aspect of the invention, preferably prior to collecting the nanoparticles, after step b) and preferably after a passive cooling process, a polar organic solvent is added to the mixture, centrifuged and the obtained nanoparticles are washed giving rise to the hydrophobic nanoparticles that are collected in step c).

In a preferred embodiment of the eight aspect of the invention, silver is provided by using one or more molecules selected from the list consisting of: silver nitrate, Ag(DDTC) (silver diethyldithiocarbamate), silver dihydrocarbyl thiophosphate, silver dioctyl sulfosuccinate, silver thiobenzoate, silver acetate, silver dodecanoate, silver tetradecanoate and silver octadecanoate.

In another preferred embodiment of the eight aspect of the invention, the organic long-chain molecules are selected from the list consisting of octylamine, trioctylamine, dodecylamine, octadecylamine, and OLA (oleylamine).

In a ninth aspect of the invention, the nanoparticles of the eight aspect of the invention, or the nanoparticles of the first or second aspects of the invention are treated with an ultra-fast laser treatment, wherein such treatment is performed by dispersing the Ag₂S nanoparticles in chloroform, dichloromethane, dichloroethane, trichloroethane or in any organic solvent comprising chlorine; and wherein such nanoparticles may be optionally further subjected to a ligand exchange reaction to generate hydrophilic Ag₂S nanoparticles. Preferably, the ion exchange reaction is carried out by mixing hydrophobic Ag₂S nanoparticles with an equivalent amount or an excess amount of a hydrophilic molecule comprising a thiol group so that the surface of the Ag₂S nanoparticles are functionalized by the hydrophilic group thus obtaining or resulting in hydrophilic particles. Preferably, the hydrophilic molecule is selected from the group consisting of: mercaptoacetic acid, mercaptopropionic acid, cysteine, cysteinamine, thiocetic acid, ammonium mercaptoacetate, or any combination thereof. Preferably, the laser treatment is performed by dispersing the Ag₂S nanoparticles in chloroform.

In the context of the present invention, ultra-fast laser is understood as any laser capable of producing laser pulses with a temporal duration below 500 femtoseconds.

It is noted that the ion exchange ligand reaction of the ninth aspect of the invention can be carried out by mixing hydrophobic Ag₂S nanoparticles with an equivalent amount or an excess amount of a hydrophilic molecule that contains a thiol group, in an organic solvent such as ethanol, methanol, acetone, chloroform, dichloromethane, toluene, 1-methyl-2-pyrrolidone or any combination thereof, at a temperature between 0 and 80°C for a period of about 1 hour or more. The reaction time can be modified according to the reactivity and solubility of the stabilizing molecule. The pH of the aqueous solutions used during this reaction should be between 7 and 14. After the reaction has been carried-out, the nanoparticles are preferably washed with an aqueous solution to remove any residues.

It is further noted that the ultra-fast laser shall be applied for a period of time that will depend on the specific characteristics of the laser, among which are: wavelength, pulse length, frequency, applied power density and beam diameter size. It is still further noted that the ultrafast laser treatment of the ninth aspect of the invention, induces a structural change that eliminates defects and increases the quantum efficiency of the nanoparticles. This process is preferably performed by dispersing the Ag₂S nanoparticles in chloroform, dichloromethane, dichloroethane, trichloroethane or any organic solvent containing chlorine.

In a preferred embodiment of the ninth aspect of the invention, the nanoparticles are irradiated using an ultra-fast laser at a wavelength, preferably between 200 and 1000 nm, more preferably between 300 and 900 nm, more preferably between 400 and 800 nm, still more preferably about 800 nm; with a power density of less than 40 W/cm², preferably between 3 and 30 W/cm², more preferably between 6 and 10 W/cm², still more preferably about 9 W/cm²; with pulses of less than 500 femto-seconds, preferably with pulses of about 50 femto-seconds; and at preferably a frequency of 1 kHz for preferably approximately 10 minutes.

As a result of the methodology shown in the ninth aspect of the invention, we have developed a novel methodology that permits a significant increment in the quantum yield of Ag₂S dots. The process is partly based on the irradiation of chloroform-dispersed Ag₂S/Ag heterodimers with femtosecond laser pulses that lead to the formation of a protective AgCl shell. This reduces the surface traps while minimizing dot-to-medium transfer via non-radiative events. The Ag₂S superdots generated as a result of this process outperform all currently available NIR-II contrast agents, enabling whole body in vivo imaging at ultra-low excitation intensities. The nanoparticles as obtained by the ninth aspect of the invention obtained have a quantum efficiency close to 10% and a high fluorescence, stability and excellent biocompatibility, so they can be used as contrast agents both in cellular image and in vivo imaging.

Therefore, a tenth aspect of the invention refers to Ag₂S nanoparticles obtained or obtainable by the method of the ninth aspect of the invention or of any of its preferred embodiments.

An eleventh aspect of the invention refers to a composition comprising the Ag₂S nanoparticles of the tenth aspect of the invention.

A twelfth aspect of the invention refers to the composition of the eleventh aspect of the invention or to the nanoparticles of the tenth aspect of the invention for use as an in vivo

imaging agent, or for in vivo **fluorescence imaging**, enabling features such as **high-resolution anatomical imaging, brain vasculature mapping and even dynamic tracking of metabolic processes**.

5 A further application of the twelfth aspect of the invention refers to the composition of the eleventh aspect of the invention or to the nanoparticles of the tenth aspect of the invention for use in vivo diagnosis.

A further application of the twelfth aspect of the invention refers to the composition of the eleventh aspect of the invention or to the nanoparticles of the tenth aspect of the invention for use in in vitro diagnosis as a imaging agent, in particular as a
10 **fluorescence imaging agent**.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. HAADF-STEM micrograph of nanoparticles obtained under different synthetic conditions (from A1 to F1). The insets from A2 to F2 represent the EDS elemental mapping of silver while the insets from A3 to F3 depict the EDS elemental mapping of sulfur. From A4 to F4, merged figures resulting from the HAADF-STEM and EDS elemental analysis are showed. The insets from A5 to F5 show detailed magnifications of the merged EDS and HAADF-STEM micrographs.
15

Figure 2. XRD patterns of Ag₂S/Ag nanoparticles synthesized using different ratios between DDT/OLA, Ag₂S-1.00 black, Ag₂S-0.60 blue, Ag₂S-0.15 pink, Ag₂S-0.90 orange, Ag₂S-0.30 green, Ag₂S-0.00 cyan.
20

Figure 3. Mean size of the Ag₂S/Ag nanoparticles (green) and the Ag cores (red) as a function of the DDT/OLA ratio.

Figure 4. A) Normalized UV-Vis spectra of the nanoparticles synthesized under different solvents ratios. Inset A1 shows the absorbance change as a function of the wavelength. B) PL emission spectra of the samples. Insets B1 and B2 show the variation of the maximum of the emission wavelength and the maximum of the PL intensity as a function of the solvent ratio. C) PL decay curves of the different nanoparticles. D) Lifetimes and absolute quantum yields as a function of the solvent ratio. The color legend is as follows:
25 Ag₂S-0.00 cyan, Ag₂S-0.15 red, Ag₂S-0.30 green, Ag₂S-0.60 blue, Ag₂S-0.90 orange, Ag₂S-1.00 black, with numeric values indicating the χ_{DDT} .
30

Figure 5. EDS-mapping profile of the Ag₂S/Ag nanoparticles (A, D and G. Net X-ray profile extracted from the yellow arrow marked in the STEM images (B, E, and H). Merged STEM and nanoparticles model images represented as a function of Ag/S ratio

versus the probe position (B, E and H). Frequency distribution of the Ag/S ratio in the nanoparticles excluding the silver core (C, F and I). The nanoparticles used for EDS-mapping were derived using χ_{DDT} of 0.00 (A-B), 0.60 (D-F) and 1.00 (G-I) during synthesis.

5 **Figure 6.** A) Schematic representation for the surface modification of the nanoparticles by ligand exchange reaction with MUA and its subsequent PEGylation through EDC/sulfo-NHS coupling reaction. Graphic B) Evolution of the nanoparticle hydrodynamic diameter during the surface functionalization process. C) PL decay times of Ag₂S/Ag nanoparticles in CHCl₃ (green), Ag₂S/Ag-PEG nanoparticles in PBS (blue)
10 and Ag₂S-PEG nanoparticles synthesized by Zhang's method in PBS (red).

Figure 7. A) In vivo imaging of an anesthetized mouse 5 min after intravenous injection of the here optimized Ag₂S nanoparticles. The animal was optically excited with an 800 nm laser diode and luminescence (1000-1400 nm) was recorded with an infrared camera. B) Magnification of the lower extremities where the femoral vessels can be
15 observed. C) A cross-sectional intensity profile measured along the black dashed line in (B). D) ex vivo NIR-II image of dissected mouse of the skeleton E) lung spleen and liver and F) detailed image of femur, tibia, ilium, and sacrum of mice.

Figure 8. (a) TEM image of the as synthesized Ag₂S/Ag nanoparticles. (b) HR-TEM of a single electrodeposited nanoparticle, showing its polycrystalline structure with lattice fringe
20 0.20 and 0.23 nm. (c) HAADF-STEM image of the as synthesized Ag₂S/Ag nanoparticles. (d) EDS elemental analysis obtained from a high Z-contrast nanoparticle labelled with the red crosshair.

Figure 9. (a) STEM micrograph of AgCl NPs obtained by ultra-fast laser irradiation of the sample. (b) and (c) 2D EDS mapping of the spatial distribution of Cl (b) and Ag (c) reveals
25 that these NPs are basically constituted by AgCl. This result is further confirmed by EDS analysis in (d). (e) XRD analysis of the sample shows the typical reflection of cubic AgCl crystals.

Figure 10. (a) and (b) are HAADF-STEM image of Ag₂S dots and superdots, respectively. (c) and (d) show the size distributions of Ag₂S dots and superdots, respectively. Orange and red bars correspond to size distribution of Ag core and whole NP, respectively. (e) Schematic representation of the structure of Ag₂S dots. The average sizes of whole nanoparticle and Ag core are indicated. (f) Schematic representation of the structure of Ag₂S superdots, including the sizes. The average sizes of whole nanoparticle and Ag core are indicated.
30

Figure 11. Tuning structural and chemical properties through ultrafast laser irradiation. (a) HAADF-STEM micrograph of the as-synthesized Ag_2S dots. (b) High-resolution STEM of as-synthesized dots showing lattice fringes d111 and d-104 of cubic Ag and monoclinic Ag_2S respectively. 2D EDS mapping of the spatial distribution of (c) Ag, (d) S and (e) Ag+S. (f) Magnified STEM micrograph of two Ag_2S dots. (g) Net X-ray intensity profiles extracted from the green arrow marked in (f), note how the Ag/S ratio increases at the edges of the dots, which coincides with the electrodeposited area. The inset shows a model nanoparticle. (h) HAADF-STEM micrograph of Ag_2S dots after ultrafast laser irradiation with 50 fs laser pulses for 90 min and with a power density of 9 W/cm². (i) High-resolution STEM of an ultrafast laser irradiated Ag_2S dot showing lattice fringes d111 and d110 of cubic Ag and monoclinic Ag_2S respectively. Note the presence of a shell marked with a blue bracket around the nanoparticle. 2D EDS mapping of the spatial distribution of (j) Ag, (k) S, (l) Cl and (m) Ag+S+Cl of an ultrafast laser-irradiated Ag_2S dot. (n) Magnified STEM micrograph of an ultrafast laser-irradiated Ag_2S dot. (o) Net X-ray intensity profiles extracted from the green arrow marked in image (n), where we can observe the presence of Ag, S and Cl. The inset on the left shows a schematic representation of a Ag_2S heterodimer after ultrafast laser irradiation.

Figure 12. (a) XRD pattern of the as synthesized Ag_2S dots sample. The red lines represent typical reflections of monoclinic Ag_2S phase, whilst green lines represent the reflection positions of cubic Ag. (b) XANES spectra of Ag_2S dots (red) and Ag_2S used as reference (blue) and Ag foil used as reference (black). (c) EDS spectrum of Ag_2S dots. (d) EDS elemental mapping of Ag_2S , silver (yellow), sulphur (red), the inset in the bottom left is the resulting image after merging S+Ag and the inset in the bottom right is the HAADF-STEM image from which the EDS map was collected. The scale bar is 10 nm.

Figure 13. (a) XRD pattern of Ag_2S superdots. (b) XANES spectra of Ag_2S superdots (orange) and Ag_2S used as reference (blue), AgCl used as reference (red) and Ag foil used as reference (black). (c) EDS spectrum of Ag_2S superdots. (d) EDS elemental mapping of Ag_2S superdots, silver (yellow), sulphur (red) and chlorine (blue). The inset in the bottom right is the resulting image after merging the S, Ag and Cl HAADF-STEM images. The scale bar is 10 nm.

Figure 14. Optical transformation of Ag_2S dots into superdots. (a) Optical images, (b) VIS-UV extinction spectra, and (c) NIR-II fluorescence images of a colloidal dispersion of Ag_2S dots in CHCl_3 before and after ultrafast laser irradiation (50 fs, 90 min, 9 W/cm²). For the acquisition of NIR-II fluorescence images, the dispersions were optically excited with an 808 nm continuous wave laser diode (100 mW/cm²). (d) NIR-II emission spectra

generated by a colloidal suspension of dots in CHCl_3 during a 90-minute-long ultrafast laser irradiation. The inset shows the normalized emission spectra of the sample before and after the laser irradiation. (e) Fluorescence quantum yield and (f) fluorescence lifetime of Ag_2S dots in CHCl_3 after being subjected to ultrafast laser irradiations processes of different durations. Error bars correspond to the standard deviations as obtained by measuring and analysing up to 10 decay curves for each sample. In (e) and (f), squares and circular points refer to the experimental data and the red lines are guides for the eyes.

Figure 15. (a) VIS-NIR extinction spectra of colloidal dispersions of Ag_2S dots in CHCl_3 after ultrafast laser irradiation for different times. (b) Extinction coefficient at 400 nm as a function of the ultrafast laser irradiation time, obtained from the spectra shown in (a). (c) Extinction coefficient at 808 nm as a function of the ultrafast laser irradiation time, as obtained from (a). (d) Fluorescence decay curves of colloidal dispersions of Ag_2S dots in CHCl_3 after ultrafast laser irradiation for different times. In all cases, the dispersions were treated with an ultrafast laser with the same pulse width, power density (50 fs, 9 W/cm^2).

Figure 16. Illustrative examples of the spectra used for the determination of the QY. Data correspond to dispersions of Ag_2S dots and superdots in CHCl_3 and in PBS. The spectra of the excitation light (808 nm) is measured with and without sample. From the difference between these spectra, the number of absorbed photons is calculated in each case. The number of emitted photons generated by each sample is calculated from the emission spectra recorded in presence of the sample. Then, the QY is calculated by dividing the number of emitted photons by the number of absorbed photons. All the emission spectra are corrected by the system response.

Figure 17. Required conditions and mechanisms of laser induced dot-to-superdot transformation. (a) Time evolution of the NIR-II fluorescence generated by colloidal dispersions of Ag_2S dots in CHCl_3 under irradiation at different power densities. In all cases, the pulse width and repetition rate were set to 50 fs and 1 kHz, respectively. (b) Time evolution of the NIR-II fluorescence generated by Ag_2S dots dispersed in different solvents under laser irradiation with 50 fs, 808 nm laser pulses. (c) Time evolution of the NIR-II fluorescence generated by Ag_2S dots under laser irradiation with 50 fs, 808 nm laser pulses in presence and absence of Ag NPs. (d) Time evolution of the NIR-II fluorescence intensity generated by Ag_2S dots in CHCl_3 as obtained for different pulse durations. For all cases in (b), (c), and (d), the ultrafast laser power density was set to 9 W/cm^2 . (e) Schematic representation of the physicochemical mechanisms underlying the

ultrafast laser-induced dot-to-superdot transformation. Upon illumination with ultrafast infrared laser pulses, multi-photon excitation of Ag NPs leads to their coulomb explosion (step 1). The Ag atoms generated in this process react with CHCl_3 molecules forming AgCl (step 2), which in turn reacts with the surface of the Ag_2S dots forming a protective layer (steps 3 and 4).

Figure 18. (a) Optical images and (b) NIR-II emission intensity for a dispersion of Ag_2S in CHCl_3 after a 100-minute-long ultrafast laser irradiation at different power densities.

Figure 19. In vivo brightness of Ag_2S superdots: a comparison with their competitors.

(a) NIR-II fluorescence decay curves of PEG-coated Ag_2S dots (provided by Sinano Corp., China) and Ag_2S superdots, both dispersed in PBS. (b) NIR-II fluorescence images of a group of mice subcutaneously injected with colloidal aqueous dispersions containing Ag_2S superdots, commercial Ag_2S dots, SWNTs and Nd-doped NPs. The same NP concentration was injected in all cases. The different images for same optical probe correspond to different 808 nm illumination power densities. (c) The signal-to-noise ratio (SNR) as a function of power density quantified from the analysis of images in (b) as obtained for the four NIR-II nanoprobe evaluated. Note that Ag_2S superdots enable the use of illumination power densities almost two orders of magnitude below those required for imaging with other NIR-II probes. (d) Fluorescence images of Ag_2S superdots accumulated in the liver obtained at three different excitation power densities with an 808 nm laser (200, 140, and 10 mW/cm^2 from left to right). (e) Power density dependence of SNR calculated from the in vivo NIR-II fluorescence at different excitation power densities (10-200 mW/cm^2). (f) and (g) NIR-II fluorescence images of the left leg of CD1 mice obtained 15 seconds after injection of 100 μL of Ag_2S superdots and Ag_2S dots, respectively. Concentration of the solution injected was 0.15 mg/mL . (h) Cross-section intensity profiles of Ag_2S superdots and Ag_2S dots obtained along the dashed line depicted in (f) and (g) that corresponds to a secondary vessel. Dots are experimental data obtained from fluorescence images and solid line is the best fit to a Gaussian function from which the spatial resolution of the fluorescence image is estimated. Note that when using Ag_2S dots their lower brightness do not allow the visualization of this secondary vessel.

Figure 20. In vivo time-resolved imaging with Ag_2S superdots. (a) NIR-II fluorescence images as obtained at different times after intravenous injection of 100 μL of a dispersion of Ag_2S superdots in PBS at a concentration of 0.15 mg/mL . (b) In vivo biodistribution of Ag_2S superdots: time course of the fluorescence emission intensity at the liver, spleen, heart and femoral artery. (c) NIR-II ex vivo fluorescence and optical images of the liver,

spleen, heart and lungs. The mouse was euthanized 100 min after intravenous injection of Ag₂S superdots. (d) Time evolution of daily food intake (e) weight and (f) body temperature of CD1 mice after intravenous injection of 300 µL of a dispersion of Ag₂S superdots in PBS (0.5 mg/mL, leading to a total dose of 150 µg of Ag₂S superdots). The results obtained from a control group, which was intravenously injected with 300 µL of PBS, are included for comparison. (g)-(i) Serum concentration of hepatic enzymes for mice intravenously injected with Ag₂S superdots and for control mice as obtained 1 and 28 days after injection. (j)-(k) Serum concentration of creatinine and bilirubin corresponding to mice subjected to an intravenous injection of Ag₂S superdots and for control mice as obtained 1 and 28 days after injection. (n= 3 for each group). Error bars corresponding to standard error of the mean ± SEM.

Examples

15 Example 1.

Experimental Section

1.1. Chemicals

Silver nitrate (99%), sodium diethyldithiocarbamate (NaDDTC) (ACS reagent grade), oleylamine (70%) (OLA), 1-dodecanethiol (≤98%) (DDT), 11-mercaptoundecanoic acid (95%) (MUA), CHCl₃ (99,6%), ethanol (99.9%), *N*-(3-(dimethylamino)propyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) (99%), *N*-hydroxysulfosuccinimide sodium salt (Sulfo-NHS) (98%), *O*-(2-aminoethyl)-*O'*-methyl- polyethylene glycol (PEG-NH₂, M_p = 5.000 g/mol), and PBS tablets were purchased from Sigma-Aldrich and used as received. Commercial Ag₂S-PEG nanoparticles were bought from NIR Optics technology.

1.2. Characterization

Transmission electron microscopy (TEM) studies were carried out using a TEM Talos F200X operated at 80 kV. Zeta-potential experiments were carried out using a Malvern Nano-ZS. The FTIR spectra were obtained using a Nicolet IR200 FTIR spectrometer. X-ray diffraction patterns were recorded on a Philips X'pert diffractometer (Cu-K α radiation, 45 kV, 40 mA). Data were collected from 2 θ = 20–90° with a step size of 0.02° and a normalized count time of 1 s/step. The emission spectra upon illuminating the samples with an 800 nm CW laser were collected with an Andor iDus InGaAs 491 cooled to -90°C. The absolute photoluminescence QY was measured with a calibrated

spectrofluorometer (Edinburgh Instruments, FLS920) equipped with an integrating sphere (Jobin-Yvon). A Xe lamp has been used as excitation source, filtered with a long-pass filter (610 nm) and a monochromator (wavelength: 800 nm, bandwidth: 20 nm). Luminescence was detected by a liquid nitrogen cooled NIR photo-multiplier tube (Hamamatsu, R5509-72). The QY has been calculated by dividing the total number of emitted photons in the 900-1700 nm range by the total number of absorbed photons at 800 nm. Luminescence decay curves were obtained by exciting the colloidal suspensions of nanoparticles by an OPO oscillator (Lotis) tuned to 800 nm, which provides 8 ns pulses at a repetition rate of 10 Hz. Fluorescence intensity was detected with a Peltier cooled photomultiplier tube with enhanced sensitivity in the NIR-II (Hamamatsu R5509-73). The contribution of scattered laser radiation was removed by using two band-pass filters (FEL850 from Thorlabs) as well as a high brightness monochromator (Shamrock 320 from Andor). The time evolution of the fluorescence signal was finally recorded and averaged by a digital oscilloscope (LeCroyWaverunner 6000).

High-angle annular dark field (HAADF) scanning TEM and EDX mappings were conducted by using a FEI Talos F200X (FEI, USA) coupled to a EDX detector. Samples for TEM were prepared by adding 10 μ L of each dispersion on a Cu grid with a carbon support membrane, followed by drying.

1.3. *in vivo* imaging

NIR-II *in vivo* images were obtained in a homemade NIR-II system. A fiber-coupled diode laser operating at 800 nm was used as excitation source (LIMO30-F200-DL808). The illumination intensity was controlled via adjustment of diode current. An anesthetized mouse was placed on a homemade temperature-controlled plate operating at 36 °C. The NIR-II fluorescence image was acquired with a Peltier cooled InGaAs camera (Xeva 320 from Xenics). The InGaAs detector was cooled down to -40 °C. Two long-pass filters (FEL850 from Thorlabs) were used to remove the background signal generated by the scattered laser radiation.

in vivo experiments were approved by the regional authority for animal experimentation of Comunidad de Madrid and were conducted in agreement with the Universidad Autónoma de Madrid (UAM) Ethics Committee, in compliance with the European Union directives 63/2010UE and Spanish regulation RD 53/2013. For this study, a total of 15 CD1 female mice (8-14 weeks old, weighing 25-39 g) bred at the animal facility at UAM were used. Mice were anesthetized prior to the imaging experiments in an induction chamber with a continuous flow of 4% isoflurane (Forane, AbbVie Spain, S.L.U) in 100%

oxygen until loss of righting reflex was confirmed and breathing rhythm was significantly slowed. Anesthesia was maintained throughout the experiments by means of facemask inhalation of 1.5% isoflurane and core body temperature was kept at 36 ± 1 °C, as measured with a rectal probe, using a heating pad.

5

1.4. Synthesis of the Ag₂S/Ag-PEG nanoparticles

The synthesis of the Ag₂S/Ag-PEG nanoparticles was carried out in different steps as explained below.

10 1.4.1. Synthesis of Ag₂S/Ag nanoparticles

The production of the Ag₂S/Ag nanoparticles was carried out as follows: a given amount of Ag(DDTC) typically 25 mg (0.1 mmol) was added into a two-necked round bottom flask at room temperature, which contained 5 mL of a solvent mixture based on OLA and DDT. Table 1 shows the solvent mole fraction (χ_{DDT}) used in each synthesis, where $\chi_{\text{DDT}} =$
 15 $(n_{\text{DDT}}/(n_{\text{DDT}} + n_{\text{OLA}}))$.

Table 1. Solvent mixtures used in each synthesis. In all the experiments the amount of precursor Ag(DDTC) was kept constant at 25 mg (0.1 mmol).

Synthesis name	Solvent mole fraction (χ_{DDT})	mmol DDT	mmol OLA
A	0	0.0	15.2
B	0.15	2.4	13.7
C	0.30	5.2	11.4
D	0.60	10.4	7.6
E	0.9	18.8	1.5
F	1	20.9	0

20

Once all the reagents were introduced within the flask, the mixture was submitted to vacuum for 10 min to remove air and then filled with N₂. After that, the mixture was heated to 190°C under magnetic stirring with a heating rate of 20°C/min and under slow

magnetic stirring. The reaction was kept for 1 hour and subsequently cooled down naturally. The synthesized nanoparticles were collected by addition of ethanol, which reduces the colloidal stability of the nanoparticles. To do that, 10 mL of ethanol were added to the raw product and centrifuged at 10,000 rpm for 10 min, this process was repeated twice. Finally, the as-prepared product was dispersed in 10 mL of chloroform and stored for further steps.

1.4.2. PEGylation of Ag₂S/Ag heterodimers

With the aim of providing hydrophilicity to the synthesized nanoparticles, they were treated with MUA. This molecule can displace the hydrophobic ligands on the nanoparticles' surface and introduce carboxylic groups on the surface of the nanoparticles, thereby providing good colloidal stability in water. With that purpose, 20 mg (0.1 mmol) of MUA was added to a 1 mL dispersion containing 1 mg/mL of Ag₂S/Ag nanoparticles in chloroform at room temperature. After that, the mixture was sonicated in an ultrasonic bath for 10 minutes until the Ag₂S/Ag nanoparticles lost their colloidal stability and precipitated at the bottom of the flask. Then, the precipitate was collected and dispersed in 1 mL of PBS at pH 7.4. Subsequently, these nanoparticles were covered with PEG-NH₂ (M_p = 5000 g/mol) via EDC/NHS coupling. To do that, 0.5 mg of EDC and 0.7 mg of sulfo-NHS were dissolved in 1 mL of PBS containing 1 mg of the previously prepared Ag₂S/Ag presenting MUA ligands and 1 mg of PEG-NH₂. The mixture was gently stirred for 2 hours and after this time the nanoparticles were collected by centrifugation at 12.000 rpm for 2 hours. This process was repeated three times and the resulting nanoparticles were dispersed in 1 mL of PBS and stored at 4°C.

2. Results and discussion

Figure 1 depicts HAADF-STEM images of the Ag₂S nanoparticles synthesized using different solvent ratios. As observed, all the nanoparticles exhibit two well-differentiated regions, a white area with higher Z-contrast, corresponding to the electrodense parts of the nanoparticles, probably due to the presence of metallic Ag into the nanoparticle. In addition, the figure shows the existence of less electrodense areas that appear as grey regions, corresponding to Ag₂S.

The EDS elemental mappings showed in Figure 1 reveal the anisotropic spatial distribution of Ag (red) and S (green) within the nanoparticles. These micrographs unveil the presence of Ag enriched regions that overlap with those that exhibited higher Z-contrast (white regions in the HAADF-STEM). Interestingly, this silver core appears more eccentrically located when lower χ_{DDT} is used during the synthesis. XRD patterns of these nanoparticles reveal the presence of two crystalline phases, which could be attributed to

monoclinic Ag₂S (JCPDS card No. 14-0072; lattice constants: $a = 4.229 \text{ \AA}$, $b = 6.931 \text{ \AA}$, $c = 7.862 \text{ \AA}$) and cubic Ag (JCPDS card No. 04-0783; lattice constants: $a = 4.0862 \text{ \AA}$), as seen in **Figure 2**. Interestingly, the synthetic conditions induce variations in the structure and properties of the synthesized nanoparticles. In fact, a decrease in overall nanoparticle size can be clearly correlated to an increase of χ_{DDT} during the reaction.

Figure 3 shows the average size of each synthesis as well as the size of the electrodeposited region that corresponds to the silver core. This result can be explained in terms of the higher capacity of the DDT molecules to interact with the nanoparticles through the thiol group, which exhibits the highest binding energy towards noble metals (200 kJ/mol).³⁴ Therefore, a high χ_{DDT} (high amount of DDT) leads to an increased colloidal stability of small nanoparticles formed during the course of the reaction and thus preventing the particles from aggregation resulting in a smaller overall nanoparticle size.

Figure 4 summarizes the impact of the synthetic conditions on the spectroscopic properties of the nanoparticles (absorption, photoluminescence, lifetime and QY). **Figure 4A** shows the normalized absorption spectra for all samples in the range between 300 to 900 nm. In all cases, a strong absorption in the visible region with weak shoulder at 465 nm and a long tail until the NIR region can be observed. The shoulder at ~465 nm may be attributed to the plasmonic contribution of the metallic Ag present in the nanoparticle. **It is important to note that this band only appears in those syntheses in which OLA is used.** This indicates the role of the amino groups in the formation of metallic silver during the synthesis of the nanoparticles at high temperatures. In fact, it is well known that the synthesis of Ag₂S nanoparticles through the thermal decomposition of a silver precursor, like Ag-monothiobenzoate or Ag-diethyldithiocarbamate, metallic Ag nanocrystals are formed as side products. This is because the dissociation energy of the Ag-S bond (206.45 kJ/mol) is lower compared to that of the S-C bond (265.95 kJ/mol) and, under a heating-up process, Ag ions are released in the absence of reactive sulfur. The addition of alkylamines in the reaction may lower the dissociation energy of the S-C bonds of the silver precursor via nucleophilic addition of an amine to the carbonyl group, thereby potentially providing a higher amount of reactive sulfur in the reaction resulting in an increased Ag₂S formation. Such an effect can be observed in **Figure 2**, here one can observe the reduction of the size of the Ag₂S matrix when increasing χ_{DDT} , whilst the size of the Ag core remains unchanged.

Figure 4B shows the emission band of the different samples as obtained under 800 nm optical excitation. The emission band is clearly affected by the synthesis conditions. Firstly, we observe that the peak wavelength is red-shifted when increasing χ_{DDT} . In fact,

the nanoparticles synthesized only in the presence of OLA exhibit its maximum emission at 1160 nm, whilst those synthesized using only DDT as solvent showed its emission peak at 1220 nm. These variations indicate an increment in the bandgap of the nanoparticles when increasing the χ_{DDT} , which cannot be attributed to quantum confinement effects, since the size of all these nanoparticles are well above the Bohr radius of the Ag_2S . Another effect observed is a prominent variation of the luminescence emission intensity as a χ_{DDT} , which could be related to different emission efficiencies. For a better understanding, luminescence decay times of the nanoparticles were measured, see **Figure 4C**. From these results, we can observe an enhancement of the nanoparticle lifetimes **from 120 ns to 1200 ns when increasing χ_{DDT} up to 0.60**, see **Figure 4D**. Such an increment of the lifetimes is concomitant with the enhancement of the QY that increases from 0.2% to 2.3%. Surprisingly, a further increase of χ_{DDT} provoked a subsequent reduction of the lifetimes from 1200 ns to 180 ns as well as the QY values, from 2.4% down to 0.2%. For the lowest and the highest χ_{DDT} values, decreased PL lifetimes and QYs could be attributed to the increment of non-radiative pathways in the nanoparticles. All these results allowed us to identify the optimal synthetic conditions for the production of highly efficient Ag_2S nanoparticles.

In order to understand how synthetic conditions could affect the emission of the nanoparticles, we have analyzed the composition of the generated nanoparticles by **EDS-mapping profile** as seen in **Figure 5**. It is evidenced how the synthetic conditions affect tremendously the size, the shape, the position of the Ag enriched core and also the atomic ratio of Ag/S within the nanoparticles. In fact, the analysis of the Net X-ray profile of those nanoparticles synthesized with neat OLA (χ_{DDT} of 0.00, **Figure 5A**) allows to infer the Ag/S atomic ratio profile of such nanoparticles as seen in **Figure 5B** that is characterized by the presence of peaks that match with the position of the silver enriched region, while the rest of the regions present Ag/S atomic ratios around 2. When we represent the frequency distribution of the Ag/S ratio obtained in these nanoparticles, we can observe the presence of two Gaussian populations, one cantered at 1.8 and other at 2.2, which differs from a theoretical ratio of 2, which should correspond to a perfect Ag_2S matrix (**Figure 5C**). Such a result would indicate the presence of compositional inhomogeneities in the Ag_2S matrix that could render in structural defects. This fact would explain the low QY observed in this sample. Interestingly, those nanoparticles synthesized using a χ_{DDT} of 0.60 depict and Net X-ray profile different than the previous nanoparticles, see **Figure 5D**. That informs us of Ag/S ratios very close to 2 for those areas distinct to the silver enriched regions, as seen in **Figure 5G**. When we represent

the frequency distribution of the Ag/S ratio, we obtain a mean value centred at 1.9, which is closer to the hypothetical ratio of 2 the Ag₂S matrix and would indicate the reduction of possible defects as shown in **Figure 5F**. That would be the reason why these nanoparticles exhibit an increment of the static emission as well as the PL lifetime and QY. Finally, in the case of those nanoparticles synthesized in the presence of neat DDT (χ_{DDT} of 1.00), the Net X-ray profile shows the presence of a high concentration of silver, located preferentially in the core of the nanoparticles, **Figure 5G**. Such a profile creates a rapid increment of the Ag/S atomic ratio from a S enriched surface to a Ag enriched core, giving as a result a very small region where the matrix composition could be Ag₂S, decreasing the NIR fluorescence of these nanoparticles, as shown in **Figure 5H**. On the contrary, the frequency distribution of the Ag/S ratio does not permit us to observe any maximum close to 2, which would indicate the presence of a thin Ag₂S region surrounded by structural defects, which would render Ag₂S nanoparticles with poor luminescent properties as observed in **Figure 5I**.

The optimized (from synthesis with χ_{DDT} of 0.60 with largest QY and luminescence lifetime) Ag₂S nanoparticles were tested as NIR-II *in vivo* imaging contrast agents. To do that, ligand exchange reaction is carried out to substitute the hydrophobic capping agents by hydrophilic, thiol bearing molecules for their stabilization in aqueous media. **Figure 6A** shows the ligand exchange process. In the first step, carboxylic acid terminated MUA ligands are introduced on the nanoparticles' surface, thereby acquiring negative charges which is confirmed by Zeta-potential measurements giving a value of ~ -27 mV. The hydrodynamic diameter of the MUA-functionalized nanoparticles is 14 ± 7 nm in PBS. After that, carboxylic groups were used as anchoring points, allowing for the linkage of PEG-NH₂ molecules (5 kDa) through the amino terminated group, performing a coupling reaction mediated by EDC and sulfo-NHS. The products of this reaction are PEG-modified nanoparticles with a hydrodynamic diameter of 20 ± 9 nm and a Zeta-potential of -16 mV, which is still negative due to the presence of remaining carboxylate groups on the surface of the nanoparticles that did not react with PEG, as seen in **Figure 6**. After the ligand exchange reaction, the luminescence lifetime decreased as evident in **Figure 6C**, when these nanoparticles were coated with PEG and transfer to PBS, the lifetime suffered a reduction from 1200 ns down to 379 ns. The observed reduction of the lifetime can be associated to the environment assisted multiphoton relaxation processes, when the nanoparticles are dispersed in water, as it was previously observed.²⁴ For the shake of comparison, we also measured the luminescence decay time of commercially available Ag₂S-PEG nanoparticles in PBS (see **Figure 6C**). Note that our "optimized"

Ag₂S nanoparticles showed a significantly increased luminescence decay time, revealing the effective reduction of non-radiative decays due to the improved Ag and S ratio in the final matrix.

5 In order to demonstrate the potential of the “optimized” Ag₂S nanoparticles for in vivo imaging, 150 µL of a solution with a nanoparticle concentration of 1 mg/mL were injected in 1-month-old CD1 female mice via retro-orbital injection. The anesthetized mouse was illuminated with a low power 808 nm laser diode providing an on-target laser power density as low as 45 mW/cm². The luminescence generated by the Ag₂S nanoparticles
10 was registered by an InGaAs infrared camera. In order to avoid any contribution of laser reflected light to the fluorescence image, a 1000 nm long-pass filter was used. In Figure 7A, an optical infrared fluorescence merged image of the mouse, as obtained 5 minutes after the injection of the nanoparticles, is shown. A clear luminescence signal is detected at the abdominal area of the mouse which is attributed to a fast accumulation of
15 nanoparticles in the liver. Although the highest intensity is detected from the liver, a detailed inspection of the sub-abdominal region reveals also clear fluorescence contrast. The zoom included in Figure 7B reveals the presence of nanoparticles in the bloodstream. In particular, vessels of the lower limbs are evident in the amplified fluorescence image. Indeed, it is clear that, even by using this low excitation power
20 density and low magnification optics, the optimized Ag₂S nanoparticles are bright enough to provide an image of the vessel anatomy with a sub-millimeter spatial resolution (see intensity profile in Figure 7C). In order to elucidate the biodistribution of the injected nanoparticles, the animal were sacrificed 1 hour after injection and the presence of Ag₂S in the different organs was elucidated from their fluorescence images.
25 Figure 7D shows the fluorescence image corresponding to the skeleton, indicating the partial accumulation of Ag₂S nanoparticles within bones. Figure 7E includes the infrared fluorescence images corresponding to the liver, spleen, and lungs. Only these organs provided any relevant infrared fluorescence. This fact indicates that 1-hour post-injection the Ag₂S nanoparticles were accumulated at bones, lungs, liver, and spleen. Finally,
30 Figure 7F shows an amplified fluorescence image of the bone structure of a limb. Note how the accumulation of Ag₂S nanoparticles at bones opens the way of obtaining high-resolution images of the skeleton by infrared fluorescence imaging. At this point we would like to stress that the accumulation of Ag₂S nanoparticles at bone structures could not only be used for advanced in vivo imaging of bone structure, but it can be also used for
35 therapeutic effects. Note that Ag₂S nanoparticles, are also known to be excellent

luminescent nanothermometers. This means that they could be potentially used to measure intra-bone temperature. This, in turns, becomes essential to achieve full control over photothermal therapies of bones, that have been recently demonstrated to be of high efficacy to promote bone regeneration.

5

Example 2. Preparation of Ag₂S dots and superdots.

Synthesis of Ag₂S dots. Ag/Ag₂S heterodimers (hereafter Ag₂S dots) were prepared as follows: 3 mmol of silver nitrate was poured into a round bottom flask containing 10 mL of octadecylamine at 160 °C under gentle stirring and N₂ atmosphere. After 10 minutes, the mixture acquired a metallic blue color. At this stage, 1.5 mmol of L-cysteine was added to the mixture as sulphur source. As result, the colour of the mixture turned from blue to black indicating the formation of the heterodimer (30 minutes). Then, the sample was cooled down and the product of the reaction was dispersed in 40 mL of CHCl₃. This dispersion was centrifuged at 21,000 g for 30 minutes and the precipitate was collected and dispersed in CHCl₃ at a concentration of 1 mg/mL. The Ag₂S dots coexists with Ag nanoparticles that appear in the solution as side-products as demonstrated in **Fig.8**.

Ultrafast laser irradiation of Ag₂S dots. The above-described synthesis of Ag₂S dots yields silver nanoparticles (NPs) as a side product. The presence of these NPs is due to the high redox potential of silver ions under the reaction temperature. To eliminate them, a CHCl₃ dispersion of the previously synthesized Ag₂S dots at a concentration of 1 mg/mL was irradiated with a Ti:Sapphire femtosecond amplifier operating at 808 nm. Laser pulses trigger the transformation of Ag NPs into AgCl particles, which are unstable in the colloidal stability and easy to remove (see **Fig. 9**). The laser provides pulses with a repetition rate of 1 kHz and tunable pulse widths in the 50-550 femtosecond range. The real-time NIR-II emission was continuously registered during the irradiation process by a fibre-coupled spectrometer.

Physical characterization of Ag₂S dots before and after ultrafast laser irradiation.

Fig. 1a depicts a high-angle annular dark-field imaging scanning transmission electron microscopy (HAADF-STEM) image of the as-synthesized Ag₂S dots. They present an elliptical shape with an average size of 9.5±1.0 nm (see **Fig. 10**). Two well-differentiated regions can be distinguished: a high electrodense core located eccentrically and a less

electrodense area that constitutes the major part of the NP. The high-resolution STEM of a typical particle reveals a crystalline structure, as seen in **Fig. 11b**, with lattice fringe $d_{111} = 2.30 \text{ \AA}$ in the more electrodense part, in accordance with those of cubic Ag phase (JCPDS 04-0783). The less electrodense area exhibits a lattice fringe of $d_{104} = 2.37 \text{ \AA}$ that matches with the monoclinic Ag_2S (JCPDS No. 14-0072). The less electrodense area in **Fig. 11b** is postulated to be Ag_2S , as the density of Ag_2S (7.2 g/cm^3) is lower than that of Ag (10.505 g/cm^3). This assumption is corroborated by the energy dispersive X-ray spectroscopy (EDS) analysis shown in **Fig. 11c, d and e**. Here, we can observe that, while the NP core is rich in Ag, the S is mainly located in the outer part of the NPs. The anisotropic distribution of elements is depicted in the net X-ray intensity profiles shown in **Fig. 11g** as obtained from the magnified STEM micrograph shown in **Fig. 11f**. In **Fig. 11g**, we can observe that the maximum of the X-ray intensity assigned to the Ag atoms arises from the electrodense region, while the X-ray intensity signal attributed to the S atoms becomes maximum in the less electrodense area. The elemental analysis of a Ag_2S dot shows a Ag:S ratio of 74:26. All these results support the presence of a metallic Ag region within the Ag_2S matrix.

After ultrafast illumination with 50 fs pulses for 90 min (9 W/cm^2), the average diameter of the NPs increased from $9.5 \pm 1.0 \text{ nm}$ to $12.3 \pm 1.0 \text{ nm}$, while the size of the metallic Ag core remained constant at around 5.1 nm , see **Fig. 11h**. High-resolution STEM of a representative Ag_2S dot after ultrafast laser irradiation (**Fig. 11i**) reveals, again, two well differentiated crystalline regions, an electrodense one with lattice fringe of $d_{111} = 2.30 \text{ \AA}$, which agrees with metallic Ag, and a less electrodense area with a lattice fringe of $d_{110} = 2.50 \text{ \AA}$ of the Ag_2S . The presence of a 1-nm-thick shell around the NPs can also be observed in this figure. Based on the analysis of the corresponding crystal lattices, the conjunction interfaces between the metallic and the semiconductor part consist of the (110) plane of Ag_2S and (111) of Ag, with a lattice mismatch of 8%. EDS images (**Figs. 11j-11m**) revealed that the resulting NPs are composed by Ag and S with an identical distribution as that observed in the as-synthesized NPs. Interestingly, EDS analysis detected the presence of Cl atoms distributed around the laser-irradiated Ag_2S dots (**Fig. 11l**). This stems from the formation of a AgCl shell. The presence of the AgCl shell is also evidenced in the net X-ray intensity profiles shown in **Fig. 11o** as obtained from the magnified STEM micrograph shown in **Fig. 11n**. The elemental analysis of a laser-treated Ag_2S dot shows a Ag:S:Cl ratio of 69:24:7. Again, the excess of Ag would indicate the conservation of the silver-rich core characteristic of the Ag_2S dots. To provide

a complete chemical and structural characterization of the samples, **figures 12 and 13** include X-ray diffraction (XRD) patterns, X-ray absorption near edge structure (XANES) spectra, energy-dispersive X-ray (EDS) spectra and High-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) images of the Ag₂S dots and superdots. These additional data support the conclusions extracted from **Fig. 11** that the as-synthesized dots are composed of two phases, a monoclinic Ag₂S phase and a cubic Ag phase. Further, when these nanoparticles are irradiated with an ultrafast laser, the chemical composition and structure of the nanoparticles changes and the resulting nanoparticles exhibit a new AgCl phase that would cover the nanoparticles forming a thin shell as observed in Fig. 11i.

Optical transformation of Ag₂S dots into superdots.

The structural changes induced in the Ag₂S dots by ultrafast laser pulses, summarized in **Fig. 11**, are accompanied by a dramatic modification of their optical properties that is noticeable even by naked eye. **Fig. 14a** shows the optical image of a colloidal dispersion of as Ag₂S dots in CHCl₃ before and after ultrafast laser irradiation (50 fs, 90 min, 9 W/cm²) that, as explained in the previous section, leads to the formation of a AgCl shell around the Ag₂S dots. Ultrafast laser irradiation induces an evident colour change in the dispersion, which becomes progressively more transparent. The absorption spectra of the dispersions before and after irradiation are shown in **Fig. 14b**. This figure evidences that the irradiation with 50 fs laser pulses leads to a 35% decrease in the absorption coefficient in the 400-800 nm range. Data included in **Fig. 15** reveal that this change takes place progressively during ultrafast laser irradiation. The absorption spectrum of the as-synthesized Ag₂S dots presents a shoulder around 400 nm, which is attributed to the plasmonic band of sub-100 nm Ag NPs and that is significantly reduced upon laser treatment. Besides modulating the absorption properties, ultrafast laser irradiation enhances the fluorescence brightness of the Ag₂S dots, as shown in the NIR-II fluorescence images in **Fig. 14c**, which correspond to a colloidal dispersion of dots before and after ultrafast laser irradiation (50 fs, 90 min, 9 W/cm²). The emission of the as-synthesized Ag₂S dots is barely detectable with our NIR-II imaging system, in contrast to the high brightness observed after the irradiation process. NIR-II brightness enhancement also takes place progressively during ultrafast irradiation as observed in **Fig. 14d**, which shows the time evolution of the NIR-II emission of Ag₂S dots during ultrafast laser irradiation. Inset in **Fig. 14d** reveals that such NIR-II brightness

enhancement is achieved without producing any relevant change in the spectral shape of the emission. This result proves that the chemical nature of the fluorophore does not change upon irradiation. **Fig. 14e** shows the fluorescence quantum yield (QY) of different colloidal dispersions of Ag₂S dots in CHCl₃ after ultrafast laser irradiation (50 fs, 9 W/cm²) for different illumination times. The QY increases monotonously with the irradiation time for treatment durations shorter than 40 min. Longer irradiations do not lead to any further improvement in the QY. As-synthesized dots show a QY of 0.13% that increases up to 10.7 % after 50 min irradiation with 50 fs pulses. **Fig. 16** includes some representative excitation and emission spectra used for the calculations of the QY values included in **Fig. 14e**. This constitutes an 80-fold enhancement, validating our referring to the laser-irradiated Ag₂S dots as superdots. This QY enhancement is accompanied by a substantial increase in the fluorescence lifetime from 200 ns up to 2.1 μ s, as shown in **Fig. 14f**. This lifetime increase follows the same trend with the irradiation time observed for the QY in **Fig. 14e** and it is independent of the dot concentration in the 0.1-1 mg/mL range. This fact rules out the possible influence of fluorescence self-absorption in our lifetime measurements.

Required conditions and mechanisms of dot-to-superdot transformation.

The ultrafast laser-induced dot-to-superdot transformation depends critically on multiple experimental variables. **Fig. 17a** shows the time evolution of the NIR-II fluorescence intensity generated by a colloidal dispersion of dots in CHCl₃ during ultrafast laser irradiation at different irradiation power densities. Curves included in **Fig. 17a** are obtained while maintaining the pulse duration (50 fs), repetition rate (1 kHz), and average power (0.6 W) of the irradiation laser. For irradiation power densities below 3 W/cm², which seems to be a threshold value, no apparent improvement in the luminescence properties is observed. For irradiation power densities between 3 W/cm² and 8 W/cm², the NIR-II fluorescence intensity increases with the irradiation time. In this range of power densities, the slope of the intensity vs time curve ($\eta = dI/dt$) depends strongly on the irradiation pulse energy (E_p). In fact, we find that $\eta \propto E_p^2$, which suggests that the dot-to-superdot transformation is triggered by a two-photon absorption process. For irradiation power densities between 8 and 9 W/cm² the NIR-II fluorescence intensity increases initially with irradiation time reaching a stable value for long irradiation times. Finally, irradiation power densities higher than 10 W/cm² lead to a decrease in the emitted

intensity for long irradiation times that could be attributed to degradation of the sample. This effect is highlighted when the irradiation power density is close to 100 W/cm^2 . Under these conditions, an initial increase in the emission intensity is followed by an abrupt reduction, rendering a non-luminescent and completely transparent dispersion that indicates the complete degradation of the sample. When the fluorescence intensity of the irradiated solution is represented as a function of the irradiation power density, the existence of an optimum irradiation power density close to 9 W/cm^2 becomes evident (see Fig. 18).

Fig. 17b demonstrates that ultrafast laser-induced dot-to-superdot transformation only occurs when the as-synthesized Ag_2S dots are dispersed in CHCl_3 . When they are dispersed in water, toluene or hexane, pulsed irradiation leads to a decrease in their NIR-II fluorescence that can be attributed to laser-induced thermal loading. The presence of silver is also critical for the dot-to-superdot transformation. We corroborated this fact by analysing the effect of ultrafast laser irradiation in a Ag_2S dot sample free of Ag NPs. Ultrafast laser irradiation of these Ag_2S dots does not improve their fluorescence, as evidenced in Fig. 17c. Thus, we conclude that ultrafast laser driven dot-to-superdot transformation requires not only the presence of CHCl_3 but also of Ag nanoparticles in the dispersion. Moreover, the dynamics of the dot-to-superdot transformation depends critically on the duration of the laser pulses. Fig. 17d shows the time evolution of the NIR-II fluorescence intensity generated by a dispersion of Ag_2S dots in CHCl_3 during irradiation with laser pulses of 50, 100, 200 and 550 fs. All the curves included in this figure were obtained with the same irradiation power and irradiation power density (0.6 W and 9 W/cm^2 , respectively). The efficiency of the dot-to-superdot transformation, estimated from the magnitude of the enhancement in the NIR-II emission, decreases as the pulse duration increases. In addition, CW laser irradiation does not lead to any improvement in the NIR-II fluorescence, indicating the absence of any dot-to-superdot transformation.

The experimental evidence shown in the figures allows us to provide a plausible explanation for the dot-to-superdot transformation. This is schematically shown in Fig. 17e. Due to the synthesis route used here, the as-prepared dispersions of Ag_2S dots also contain Ag NPs whose plasmon resonance is responsible for the extinction peak observed at around 400 nm. When excited by 808 nm ultrafast pulses, two-photon absorption by Ag NPs leads to the local induction of a high free electron density that

results in their coulomb explosion (step 1 in **Fig. 17e**). The key role of multiphoton excitation is supported by the requirement of ultrafast laser pulses that ensure high photon densities and, hence, allows for sequential absorption through virtual states of Ag NPs. In addition, we have confirmed the participation of two 808 nm photons in the sequential process. The Coulomb explosion of Ag NPs leads to an increment in the concentration of highly reactive Ag atoms in the solution, which react with CHCl_3 yielding silver chloride (AgCl): $\text{Ag}^+ + \text{Cl}^- \rightarrow \text{AgCl}$ (step 2 in **Fig. 17e**). The interaction of the laser-generated AgCl molecules with the surface of the Ag_2S dots results in the formation of a AgCl protective shell (steps 3 and 4 in **Fig. 17e**). Therefore, after ultrafast laser irradiation, the low-bandgap semiconductor Ag_2S (0.9 eV) is coated with an inorganic shell of higher-bandgap AgCl (5.13 eV) several monolayers thick, as seen in **Fig. 11i**. This protective shell strongly reduces the non-radiative transitions that involve the vibronic activation of solvent CHCl_3 molecules and prevents the formation of shallow or deep midgap states as surface traps that would provide non-radiative deexcitation pathways. The reduction in the non-radiative decay probabilities caused by this protective shell simultaneously explains the increment in the NIR-II QY and in the fluorescence lifetime. The decrease in the non-radiative decay probabilities is also evidenced by a reduction in the light-to-heat conversion efficiency when the dot-to-superdot transformation takes place. Both dots and superdots can be damaged under excessive irradiation doses, explaining why at high irradiation illumination power densities the initial enhancement in NIR-II luminescence is followed by a nonreversible quenching (see **Fig. 17a**). An arising question is why the multiphoton excitation and subsequent coulomb explosion takes place only in the colloidal Ag nanoparticles and not in the Ag cores. A plausible explanation is that the Ag core does not show any plasmonic resonance in the visible domain due to the presence of Ag_2S surrounding it. The spectral location of the plasmon resonance of Ag nanoparticles strongly depends on the dielectric constant/refractive index of the surrounding medium. [<https://doi.org/10.1021/ja300901e>] The very different dielectric constants/refractive indices of chloroform and Ag_2S could be behind this effect. By contrast, the Ag nanoparticles exhibit a plasmon resonance at 425 nm, which favours nonlinear multiphoton absorption. That would render to an enhancement of the local electric field that in turn facilitates the electron stripping from the Ag nanoparticles facilitating the ionization of the Ag nanoparticles and the subsequent Coulomb explosion, in a similar way as it was described previously. [Chem. Phys. Lett. 229, 333 (1994)]

***In vivo* NIR-II imaging with Ag₂S superdots.**

To test the potential application of Ag₂S superdots for *in vivo* NIR-II imaging, we transferred them from their original solvent (CHCl₃) to phosphate buffer saline (PBS) by means of a ligand exchange procedure described in **Methods**. The adhesion of hydrophilic and bifunctional HS-PEG-COOH ligands on the surface of Ag₂S superdots makes them stable in PBS without any sign of precipitation for at least 12 months. The average hydrodynamic diameter and Z-potential after the ligand exchange are 22 nm, and -25 mV, respectively. PEG coating and dispersion in PBS does not cause a significant reduction in the fluorescence decay time of Ag₂S superdots, which is close to 2 μ s (see **Fig. 19a**). The fact that Ag₂S superdots show the same decay time in CHCl₃ and in PBS reveals the effectiveness of the AgCl protective shell against the appearance of multiphonon relaxation events favoured by the vibration modes of water molecules. Hence, this makes it possible to retain the same QY $\approx$ 10% in both solvents. **Fig. 19a** also includes, for the sake of comparison, the fluorescence decay curve obtained for commercially available (Sinano Corp. China) PEG-coated Ag₂S dots also dispersed in PBS. The fluorescence lifetime of our PEG-coated Ag₂S superdots dispersed in PBS is almost 40 times longer than that of commercial Ag₂S dots in PBS (53 ns). Such a long fluorescence lifetime and high QY suggests the potential application of our PEGylated superdots for high-contrast, low-dose NIR-II *in vivo* imaging. To evaluate this possibility, we carried out a study to compare the performance of Ag₂S superdots with that of other well-established NIR-II fluorescent probes: commercial Ag₂S dots, neodymium-doped nanocrystals (LaF₃:Nd³⁺) and single-walled carbon nanotubes (SWNTs). The FDA-approved ICG dye was not included in this comparative study as preliminary experiments revealed the poor spectral overlap between its emission tail and the spectral response of our NIR-II imaging system. We subcutaneously injected 100 μ L of a PBS solution containing, in each case, Ag₂S superdots, Ag₂S dots, LaF₃:Nd³⁺ and SWNTs into four different mice. The NP concentration was set in all cases to 1.5 mg/mL, corresponding to a total injected NP dose of $\approx$ 5 mg/kg. **Fig. 19b** shows the *in vivo* NIR-II fluorescence images obtained in each case for 808 nm illumination power densities ranging from 227 down to 0.3 mW/cm². The superior performance of our Ag₂S superdots is evident. These results support the fact that the Ag₂S superdots synthesized in this invention show the highest NIR-II brightness among the NIR-II fluorescent probes evaluated here (see **Table 2**).

Comparison of NIR-II brightness.

NIR-II Probe	ϵ @800 nm [M ⁻¹ cm ⁻¹]	NIR-II QY	NIR-II Brightness (= ϵ QY)	NIR-II lifetime
SWCNTs (PBS)	$8 \cdot 10^6$	$5 \cdot 10^{-4}$	$4 \cdot 10^2$	< 1 ns
ICG (PBS)	$1.2 \cdot 10^5$	$4.5 \cdot 10^{-4}$	$5.4 \cdot 10^1$	< 1ns
Nd:LaF ₃ (PBS)	$2 \cdot 10^2$	0.5	$1 \cdot 10^2$	30 μ s
Ag ₂ S dots (CHCl ₃)	$4.7 \cdot 10^5$	0.0013	$1.1 \cdot 10^3$	70 ns
Ag ₂ S superdots (CHCl ₃)	$4.4 \cdot 10^5$	0.101	$4.4 \cdot 10^4$	2.1 μ s
Ag ₂ S dots (PBS)	$4.6 \cdot 10^5$	0.0008	$5.9 \cdot 10^2$	50 ns
Ag ₂ S superdots (PBS)	$4.4 \cdot 10^5$	0.1	$2.6 \cdot 10^4$	2 μ s

- 5 **Table 2.** Molar extinction coefficient at 800 nm, NIR-II fluorescence quantum yield (QY), NIR-II brightness and NIR-II fluorescence lifetime of the Ag₂S dots and superdots in both CHCl₃ and PBS. The data for other well-established NIR-II fluorescent probes are also included.

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At this point it should be noted that the depth of a subcutaneous injection is not fully controllable even when following the exact same protocol. Nevertheless, the inhomogenous thickness of mouse skin ($\pm 80 \mu\text{m}$ over $400 \mu\text{m}$) could lead to negligible variation in transmitted fluorescence when compared with the orders of magnitude experimentally observed (**Fig 19c**), which are caused by the superior brightness of Ag₂S superdots. The superior NIR-II brightness of Ag₂S superdots enables the acquisition of reliable fluorescence images even at the ultra-low irradiation power density of 0.3 mW/cm^2 . This not only allows *in vivo* imaging with cost-effective excitation sources but also ensures a negligible thermal loading during image acquisition. At such ultra-low illumination power densities, no fluorescence from any of the other three tested NIR-II nanoprobe could be registered. The improvement in image contrast achieved by using Ag₂S superdots is quantified in **Fig. 19c**, which shows the power density dependence of the signal-to-noise ratio (SNR) calculated from the fluorescence images shown in **Fig. 19b**. From **Fig. 19c**, we conclude that the brightness improvement achieved during

ultrafast laser irradiation reduces the minimum illumination density that can be used for *in vivo* imaging by almost two orders of magnitude. Such enhancement is also evident when comparing the illumination conditions used in this work to those reported in previous works dealing with Ag₂S dots, SWNTs and lanthanide-doped nanocrystals (see **Table 3**).

Table 3. Reported experimental conditions for *in vivo* imaging with NIR-II nanoprobes.

Fluorescent probe	Photoluminescence properties		Surface properties		<i>In vivo</i> imaging conditions			Ref.
	Excitation wavelength (nm)	Emission wavelength (nm)	Coating / Functionalization	Z-potential (mV)	Concentration	Volume (μL)	Power density (W/cm ²)	
SWNTs	808	1150	PL-PEG	N/A	0.26 mg/mL	200	0.13	1
SWNTs	808	1200	DSPE-mPEG	N/A	500 nM	200	0.14	2
NaYF ₄ :Yb ³⁺ , Er ³⁺	980	1525	HAS	-13.6	2 mg/mL	200	0.14	3
RE-doped core/multishell	800	1525	DSPE-PEG2000-COOH	N/A	5–100 nM	200	0.2	4
PbS/CdS/ZnS QDs	808	1270	MPA	-24.2	0.04 mg/mL	100	0.10	5
IR-1061	808	900-1300	DSPE-mPEG	N/A	0.27 mg/mL	200	0.14	6
Ag ₂ S	808	1100	DOX@PEG	N/A	1.5 mg/mL	200	0.45	7
Ag ₂ S	808	1200	PEG	N/A	1 mg/mL	50	0.126	8
Ag ₂ S	808	1200	6PEG	N/A	1.34 mg/mL	200	0.14	9
Ag ₂ S	808	1200	PEG	N/A	1.5 mg/mL	200	0.14	10
Ag ₂ S superdots	808	1220	PEG	-30 mV	0.15 mg/mL	100	0.05	This work

Note that data included in **Fig. 19c** are obtained from the analysis of *in vivo* fluorescence images of subcutaneous injections. To provide an accurate and realistic number of the minimum power density that we can use for *in vivo* imaging, *in vivo* images obtained after intravenous injection of Ag₂S superdots are also analysed. **Fig. 19d** shows the *in vivo* fluorescence images of an anesthetized mouse after intravenous injection of Ag₂S superdots as obtained for three representative 808 nm excitation densities. Images were acquired 30 min after injection, and the fluorescence images reveal accumulation of superdots at both liver and spleen. **Fig. 19e** shows the dependence of the signal-to-noise ratio (SNR, expressed in dB) of the *in vivo* images as a function of the excitation power

density. A linear relation between the SNR and excitation power density is obtained. For an excitation power density as low as 10mW/cm² the SNR of the *in vivo* image is above 10 dB. Note that, according to **Table 3**, the acquisition of high contrast *in vivo* images with laser power densities of 10 mW/cm² based on the use of Ag₂S superdots constitutes an advantage over previous results using Ag₂S dots for *in vivo* imaging. **Table 3** reveals that previous works dealing with *in vivo* imaging by using Ag₂S dots typically use power densities above 100 mW/cm², i.e. one order of magnitude larger the power density used in this invention for *in vivo* imaging based on Ag₂S superdots. The superior brightness of Ag₂S superdots also allow for achieving larger penetration depths into tissues.

In addition, the potential use of our Ag₂S superdots for *in vivo* visualization of vessels has been evaluated. Two CD1 female 3 month-old mice totally shaven were subjected to an intravenous tail injection with 100 µL of a 0.15 mg/mL dispersion of Ag₂S dots and superdots in PBS respectively. 30 seconds after injection, magnified fluorescence images of the right limbs of both mice was acquired. Results are included in **Figure 19f and 19g**. Both images were obtained under the same experimental conditions (50 mW/cm² 808 nm excitation power density, emission signal filtered by two 850 nm and one 1100 long pass filters and a camera exposure time of 5 seconds). As it can be observed, Ag₂S superdots enable blood vessel imaging with a much higher contrast than Ag₂S dots thanks to their superior emission brightness. Such improvement allows not only better imaging of femoral artery but also to the visualization of additional vessels. **Figure 19h** includes the intensity profiles, obtained with both Ag₂S dots and superdots, along the dashed lines included in the fluorescence images and that correspond to a cross section of a secondary vessel. The presence of this secondary vessels is clearly evidenced by Ag₂S superdots while they are impossible to observe when using Ag₂S dots. Indeed, the analysis of the intensity profile of the secondary vessel obtained with Ag₂S superdots reveal that they provide a sub-200 nm spatial resolution.

The superior brightness of Ag₂S superdots allows *in vivo* video rate NIR-II imaging at minimum illumination power densities and administered doses. **Fig. 20a** shows the NIR-II images obtained at different times after intravenous administration of 100 µL of PEG-coated Ag₂S superdots dispersed in PBS at a concentration of 0.15 mg/mL. This leads to a total dose of 15 µg, corresponding to approximately 0.5 mg/kg. This is more than one order of magnitude smaller than the administered dose (6.6 mg/kg) reported for NIR-II video recording using conventional Ag₂S dots. The use of low administration doses for

in vivo imaging is beneficial because of different reasons. Firstly, in order to develop a cost-effective probe for NIR-II *in vivo* imaging, it is mandatory to reduce the amount of material required to achieve high contrast *in vivo* images. Secondly, the use of low administration doses guarantees the minimization of possible adverse (toxicity) effects.

5 Note that later in this work we demonstrate a negligible *in vivo* toxicity of our Ag₂S superdots even for administration doses one order of magnitude larger than those required for *in vivo* imaging. Finally, Ag₂S superdots shows a non-negligible photothermal conversion efficiency so that, in order to avoid undesirable heating during the acquisition of *in vivo* images the use of low administration doses and low illumination

10 densities becomes mandatory. Images included in **Fig. 20a** were obtained by using an 808 nm illumination power density of 10 mW/cm², one order of magnitude lower than the illumination power density employed for NIR-II video recording using SWNTs (140 mW/cm²)²⁹. The illumination power density here used is also much lower than the safety threshold at this wavelength (329 mW/cm²) established by the International Commission

15 on Non-ionizing Radiation Protection (ANSI Z136.1-2000). NIR-II video recording enabled us to track the *in vivo* biodistribution of our Ag₂S superdots. The time evolution of the NIR-II fluorescence intensity generated by the Ag₂S superdots at the liver, spleen, heart and femoral artery after intravenous injection is shown in **Fig. 20b**. For the first 3 min post-injection the PEG-coated superdots were mainly circulating and gathering in

20 the liver, spleen and heart. The fluorescence signal observed at the femoral artery is assigned to the presence of Ag₂S superdots in the bloodstream. By fitting this curve to a first-order exponential, we can estimate that the blood half-life of our Ag₂S superdots is close to 20 min. As seen in **Fig. 20a and 20b**, most of the Ag₂S superdots have been uptaken by the liver and spleen after 30 minutes. The presence of superdots in these

25 organs was corroborated by *ex vivo* NIR-II images taken 100 minutes after injection (**Fig. 20c**). The high accumulation rates of NPs in the liver and spleen, which has been widely reported is related to filtration mechanisms promoted by the reticuloendothelial system (RES).

Finally, we evaluated the *in vivo* biocompatibility of our Ag₂S superdots. We analyzed

30 the time evolution of weight, daily food intake and temperature of a group of four CD1 mice after intravenous administration of 300 µL of a dispersion of Ag₂S superdots in PBS at a concentration of 0.5 mg/mL. This constitutes a total administration dose of 150 µg (5 mg/kg), one order of magnitude higher than that used for NIR-II *in vivo* video recording. We compared the results obtained for mice injected with Ag₂S superdots with those

35 obtained for a control group of four mice, injected with 300 µL of PBS. Experimental data

included in **Fig. 20d-f** reveal that, even for such relatively high administration doses, no differences are found when comparing the measured parameters for both groups. This suggests a negligible *in vivo* toxicity of our Ag₂S superdots. These findings are consistent with the reported low toxicity of conventional Ag₂S dots and with the already demonstrated ability of AgCl coatings to minimize NP cytotoxicity. Furthermore, to test the long-term biocompatibility of Ag₂S superdots we performed a 28 days subchronic toxicological experiment complemented with histological analyses regarding splenic, hepatic, and renal tissue samples.

10 **Materials and Methods of example 2.**

Chemical Reagents.

Ethanol absolute, n-hexane (95%), Silver nitrate (99%), L-Cysteine (96%), sodium diethyldithiocarbamate (DDTC) (ACS reagent grade), CHCl₃ (99.6%), HS-PEG-COOH (2100 g/mol), and PBS tablets were purchased from Sigma-Aldrich (Germany) and used as received.

Ultrafast laser irradiation.

For ultrafast laser irradiation, a Ti:Sapphire amplified was used (Spitfire from Spectra-Physics) that was pumped by a Ti:Sapphire oscillator (Tsunami from Spectra-Physics) both operating at 808 nm wavelength. The amplifier provides pulses with a repetition rate of 1 kHz and tunable pulse widths in the 50-550 femtosecond range by fine adjustment of the compensating gratings. The irradiation average power was controlled by using a set of polarizers and a $\lambda/2$ waveplate (AQWP10M-980 from Thorlabs). A 45 cm focal length lens was used to focus the irradiation beam into a hermetically closed quartz cuvette containing the dispersion of Ag₂S dots in CHCl₃ dispersion. The Ag₂S concentration was set in all the irradiation experiments to 1 mg/mL. The focusing lens was mounted on a translation stage that allowed changing the lens-to-cuvette distance and, hence, the laser spot size in the solution. This, in turns, made possible to change the laser power density while keeping the average laser power constant. The NIR-II emission was continuously registered during the irradiation process by a fibre-coupled spectrometer with enhanced sensitivity in the 900-1700 nm spectral range (Ocean Optics NIRQUEST212). Real-time temperature measurements during irradiation were performed by means of a thermocouple placed into the solution. The distance between the thermocouple and the laser focal spot was set to 2 mm in order to avoid direct heating

of the thermocouple by the 808 nm laser beam.

Synthesis and characterization of Ag-NP-free Ag₂S dots (neat Ag₂S dots).

The synthesis of the Ag-NP-free Ag₂S dots was carried out by thermal decomposition of the precursor silver diethyldithiocarbamate (AgDDTC). The precursor was synthesized as follows: 0.025 mol of AgNO₃ were dissolved in 200 mL of bidistilled water produced from Milli-Q water. Later 0.025 mol of DDTC (diethyldithiocarbamate) were dissolved in 300 mL of bidistilled water and added to the above solution. The resulting yellow powder was filtered and dried at 60 in vacuum using a rotary evaporator. After that, 25 mg of AgDDTC was dispersed in 5 mL of 1-dodecanethiol and the mixture was stirred under vacuum for 30 minutes. After that, the solution was heated up to 200 °C for 1 hour. After this time, the solution was cooled down naturally. When the temperature of the mixture reached 25°C, 10 mL of ethanol was added and the solution was centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and the precipitate collected in 10 mL of CHCl₃.

15 *Ligand exchange procedure.*

In order to use the Ag₂S superdots for *in vivo* imaging, they were transferred from CHCl₃ to water by a ligand exchange reaction between the octadecylamine and HS-PEG-COOH (Mw of 2100 g/mol). 2 mg of Ag₂S superdots dispersed in 1 mL of CHCl₃ were mixed with 1 mg of HS-PEG-COOH and sonicated for 5 minutes to facilitate the ligand exchange. Afterwards, the CHCl₃ was gently evaporated until reaching a total volume of 500 µL. Then, 500 µL of absolute ethanol were added and the dispersion was sonicated for 5 minutes. This process was repeated 4 additional times in order to ensure the maximum removal of CHCl₃. The Ag₂S superdots dispersed in 1 mL of absolute ethanol were then concentrated to a volume of 100 µL and transferred dropwise to 900 µL of PBS, prepared by dissolving a tablet in 100 mL of Milli-Q water.

25 *Scanning transmission electron microscopy (STEM).*

For the STEM studies a JEOL ARM200 cF was used. The microscope is equipped with a Cs corrector in the condenser lens and it is attached with an OXFORD INCA detector of 100 mm² for XEDS analysis. The experiments were carried out at 80 KV as it was found that the sample were beam sensitive to higher acceleration voltages. Thus, and as a prior step, the stability was carefully checked in function of the different parameters

of the microscope. In order to get the highest possible intensity in the XEDS detector without affecting the sample, we determined that the optimum conditions were achieved by using a condenser lens aperture of 50 micrometres and a spot size of 6. In these conditions, the probe current is well above 120 pA. The camera length for the STEM
5 images was set to 6 cm so that the collection angles range is from 90 to 370 mrad.

Dynamic Light Scattering (DLS) and Z-potential determination.

The DLS and Z-potential measurements were performed using a Zetasizer Nano ZS instrument (Malvern Instruments, U.K.). The accumulation time was determined automatically for each sample. The sample concentration was adjusted to obtain 3000
10 cps. The acquired data was processed using the software provided by Malvern (Zetasizer software v7.03).

Quantum yield measurements.

The absolute photoluminescence quantum yield (QY) was measured with a calibrated spectrofluorometer (Edinburgh Instruments, FLS920) equipped with an integrating
15 sphere (Jobin-Yvon). A Xe lamp was used as the excitation source, filtered with a longpass filter (610 nm) and a monochromator (wavelength: 808 nm, bandwidth: 20 nm) and the detector has been a liquid nitrogen cooled NIR photo-multiplier tube (Hamamatsu, R5509-72). The QY was calculated by dividing the total number of emitted photons in the 900-1700 nm range by the total number of absorbed photons at 808 nm.

20 *Luminescence decay curves.*

Luminescence decay curves were obtained by exciting the colloidal suspensions of Ag₂S NCs by a OPO oscillator pumped by frequently doubled Nd:YAG laser (Lotis), which provides 8 ns pulses at a repetition rate of 10 Hz. The fluorescence intensity was detected with a Peltier-cooled photomultiplier tube with enhanced sensitivity in the NIR-
25 II (Hamamatsu R5509-73). The contribution of scattered laser radiation was removed by using two bandpass filters (Thorlabs FEL850) and a high brightness monochromator (Andor Shamrock 320). The time evolution of the fluorescence signal was recorded and averaged by a digital oscilloscope (Le Croy Waverunner 6000).

In vivo imaging.

30 NIR-II *in vivo* images were obtained in a homemade NIR-II system. A fiber-coupled diode

laser operating at 808 nm was used as excitation source (LIMO30-F200-DL808). The illumination power density was controlled by adjusting the diode current. The anesthetized mouse was placed on a homemade temperature controlled plate operating at 36 °C. The NIR-II fluorescence image was acquired with a Peltier cooled InGaAs camera (Xenics Xeva 320) cooled down to -40 °C. Two longpass filters (Thorlabs FEL850) were used to remove the background signal generated by the scattered laser radiation. At this points we should note that in vivo images were also acquired using an additional 1100 nm long-pass filter, following previous works revealing that the use of such filter could increase the resolution of the NIR-II fluorescence image. In our case, the use of such filter does not leave to any significant increment in the image resolution.

In vivo experiments were approved by the regional authority for animal experimentation of Comunidad de Madrid and were conducted in agreement with the Universidad Autónoma de Madrid (UAM) Ethics Committee, in compliance with the European Union directives 63/2010UE and Spanish regulation RD 53/2013. For this study, 15 CD1 female mice (8-14 weeks old, weighing 25-39 g) bred at the animal facility at UAM were used. Mice were anesthetized prior to the imaging experiments in an induction chamber with a continuous flow of 4% isoflurane (Forane, AbbVie Spain, S.L.U) in 100% oxygen until loss of righting reflex was confirmed and breathing rhythm was significantly slowed. Anesthesia was maintained throughout the experiments by means of facemask inhalation of 1.5% isoflurane and core body temperature was kept at 36 ± 1 °C, as measured with a rectal probe, using a heating pad.

To study the imaging capabilities of the Ag₂S superdots compared to other NIR-II probes, four mice were shaven and subcutaneously injected with 100 µL of a 1.5 mg/mL NP (Ag₂S superdots, Ag₂S dots, LaF₃:Nd³⁺ or SWNTs) dispersion in PBS.

To study the biodistribution of the Ag₂S superdots, mice received a small incision in the neck skin to expose the right jugular vein. A polyethylene tubing catheter was inserted 2 mm in the caudal direction to infuse 100 µL of solution containing 1.5 mg/mL PEG-coated Ag₂S superdots dispersed in PBS. Three hours after infusion, mice were euthanized by isoflurane overdose and organs (liver, spleen, heart and lungs) were collected to obtain ex-vivo NIR-II images.

One additional mouse was anesthetized in order to check the potential thermal loading induced by laser with and without fur. In this case, the temperature at the skin surface was measured with an infrared thermographic camera (Fluke iT10).

In vivo biocompatibility studies.

Four additional mice were intraperitoneally injected with 300 μ L of a 500 μ g/mL solution of Ag₂S superdots in PBS. Four mice intraperitoneally injected with 300 μ L of PBS were used as control group. The animals were housed in two separated cages in a room (23 $\pm$ 2°C) maintained on a 12/12 h light/dark cycle with free access to food and water. Weight, food intake and surface temperature, as measured by an infrared thermographic camera (FLIR E-40), were analyzed in the awake mice on a daily basis for 2 weeks.

Claims

1. A method for manufacturing Ag₂S nanoparticles comprising the following steps:
 - a. Mixing a given amount of silver with a solvent comprising a mixture based
5 on an i) organic long-chain molecule, comprising between 4 and 20 carbon atoms in length and comprising an amine group, and ii) DDT (1-dodecanethiol), or any other thiol based molecule with a boiling point of between 140 and 200°C, wherein the solvent mole fraction (χ_{DDT}) calculated as $\chi_{\text{DDT}} = (n_{\text{DDT}} / (n_{\text{DDT}} + \text{organic long-chain molecule (such as nOLA)}))$ is between 0.30 and 0.90;
10
 - b. submitting the reaction mixture of a) to a temperature between 80°C and 350°C in, preferably a closed atmosphere; and
 - c. collecting the nanoparticles.
- 15 2. The method of claim 1, wherein the solvent mole fraction (χ_{DDT}) is between 0.55 and 0.65.
3. The method of claim 1, wherein the solvent mole fraction (χ_{DDT}) is between 0.59 and 0.61.
- 20 4. The method of claim 1, wherein the solvent mole fraction (χ_{DDT}) is 0.60.
5. The method of any of claims 1 to 4, wherein silver can be provided in any form selected from the list consisting of: Ag(DDTC), silver nitrate, silver dihydrocarbyl
25 thiophosphate, silver dioctyl sulfosuccinate, silver thiobenzoate, silver acetate, silver dodecanoate, silver tetradecanoate and silver octadecenoate.
6. The method of any of claims 1 to 4, wherein the organic long-chain molecules are selected from the list consisting of octylamine, trioctylamine, dodecylamine,
30 octadecylamine, and OLA (oleylamine).
7. The method of claim 1, wherein the solvent mole fraction (χ_{DDT}) is 0.60, and the organic long-chain molecules is OLA (oleylamine).

8. The method of claim 7, wherein silver is provided in the form consisting of Ag(DDTC).
- 5 9. The method of any of claims 1 to 8, wherein the closed atmosphere is created by using a vacuum, preferably for about 10 min, to remove air and then filled with N₂.
- 10 10. The method of any of claims 1 to 9, wherein in step b) the mixture is heated to a temperature between 170°C and 210°C, still more preferably to a temperature about 190°C, under magnetic stirring, preferably with a heating rate of 20°C/min and under slow magnetic stirring.
- 15 11. The method of any of claims 1 to 10, wherein prior to step c) the reaction is cooled down by any suitable means, preferably by naturally cooling the reaction.
- 20 12. The method of any of claims 1 to 11, wherein in step c) the nanoparticles are collected by the addition of ethanol, which reduces the colloidal stability of the nanoparticles, preferably by adding about 10 mL of ethanol to the raw product and centrifuging at about 10,000 rpm for 10 min, and preferably repeating this process twice.
- 25 13. The method of any of claims 1 to 12, wherein the resultant product of step c) is dispersed in chloroform and stored.
- 30 14. A method for manufacturing pegylated Ag₂S nanoparticles comprising the following steps:
- a. adding a hydrophilic agent such as 11-mercaptoundecanoic acid (MUA), dihydrolipoic acid, 2,3 dimercaptosuccinic acid, mercaptopropionic acid, or cysteine, to a dispersion containing the Ag₂S/Ag nanoparticles as obtained from the method of any of claims 1 to 13, preferably in chloroform at room temperature;
 - b. Precipitating and collecting the product of step a);
 - c. Covering the precipitates of step b) with PEG, preferably PEG-NH₂, more preferably via EDC/NHS coupling; and
 - 35 d. Collecting the nanoparticles.

15. Ag₂S nanoparticles obtained or obtainable by the method of any of claims 1 to 13.
- 5 16. Ag₂S nanoparticles obtained or obtainable by the method of any of claims 7 or 8.
17. Ag₂S nanoparticles obtained or obtainable by the method of claim 14.
- 10 18. A composition comprising the nanoparticles of any of claims 15 to 17.
19. A composition as defined in claim 18 or the nanoparticles as defined in any of claims 15 to 17 for use as an in vivo imaging agent.
- 15 20. A composition as defined in claim 18 or the nanoparticles as defined in any of claims 15 to 17 for use as an in vitro imaging agent.
21. A method of manufacturing Ag₂S nanoparticles comprising the following steps:
- 20 a. Treating or irradiating Ag₂S nanoparticles as defined in any of claims 15 to 17 with an ultra-fast laser, wherein such treatment is performed by dispersing the Ag₂S nanoparticles in chloroform, dichloromethane, dichloroethane, trichloroethane or in any organic solvent comprising chlorine; and
- 25 b. Optionally collecting the nanoparticles.
22. The method of claim 21, wherein such treatment is performed by dispersing the Ag₂S nanoparticles in chloroform.
23. The method of claim 21 or 22, wherein after step a) or b) the nanoparticles are
- 30 further subjected to a ligand exchange reaction to generate hydrophilic Ag₂S nanoparticles, wherein the ion exchange reaction is carried out by mixing hydrophobic Ag₂S nanoparticles with an equivalent amount or an excess amount of a hydrophilic molecule comprising a thiol group so that the surface of the Ag₂S nanoparticles are functionalized by the hydrophilic group thus obtaining or
- 35 resulting in hydrophilic particles.

24. The method of any of claims 21 to 23, wherein the nanoparticles are irradiated or treated using an ultra-fast laser with a power density of less than $40\text{W}/\text{cm}^2$, with pulses of less than 500 femto-seconds, and at preferably a frequency of 1 kHz for preferably approximately 10 minutes.
25. The method of any of claims 21 to 23, wherein the nanoparticles are irradiated or treated using an ultra-fast laser with a power density of between 6 and $10\text{W}/\text{cm}^2$, with pulses of about 50 femto-seconds, and at preferably a frequency of 1 kHz for preferably approximately 10 minutes.
26. Ag_2S nanoparticles obtained or obtainable by the method of any of claims 21 to 25.
27. A composition comprising the nanoparticles of claim 26.
28. A composition as defined in claim 27 or the nanoparticles as defined in claim 26, for use as an in vivo imaging agent.
29. A composition as defined in claim 27 or the nanoparticles as defined in claim 26, for use as as an in vitro imaging agent.

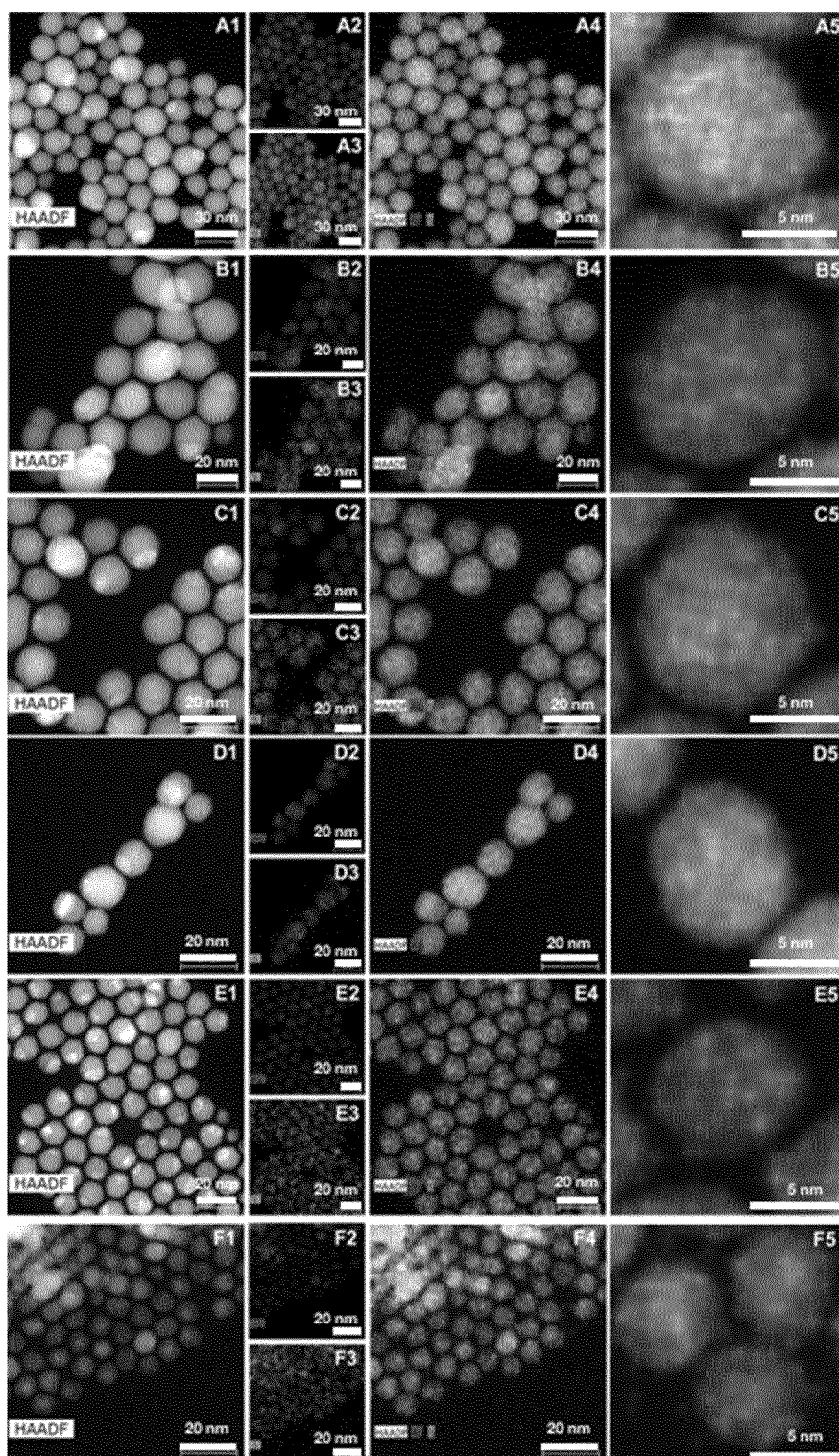
FIGURES

Fig. 1

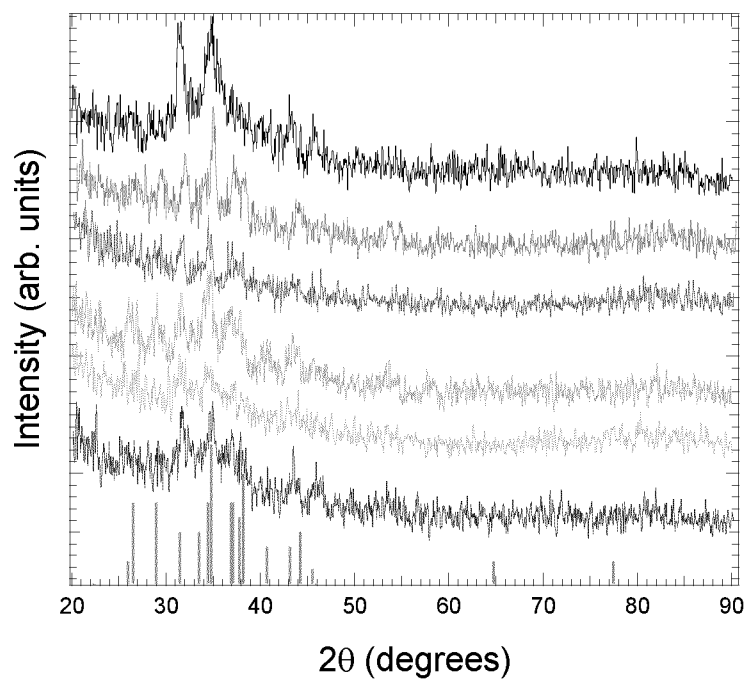


Fig. 2

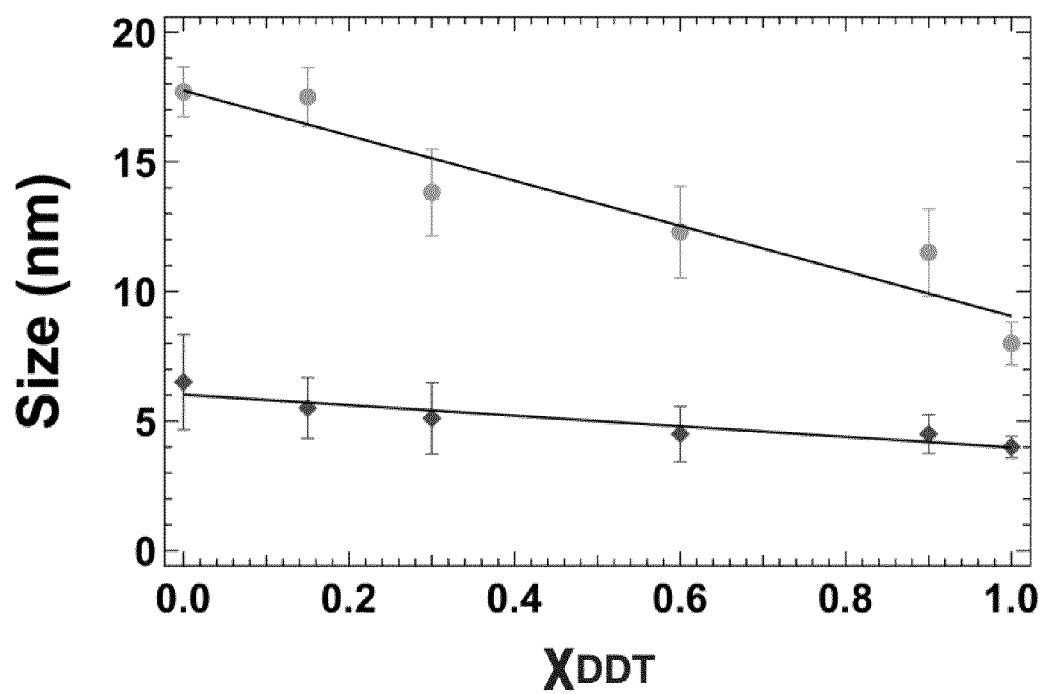


Fig. 3

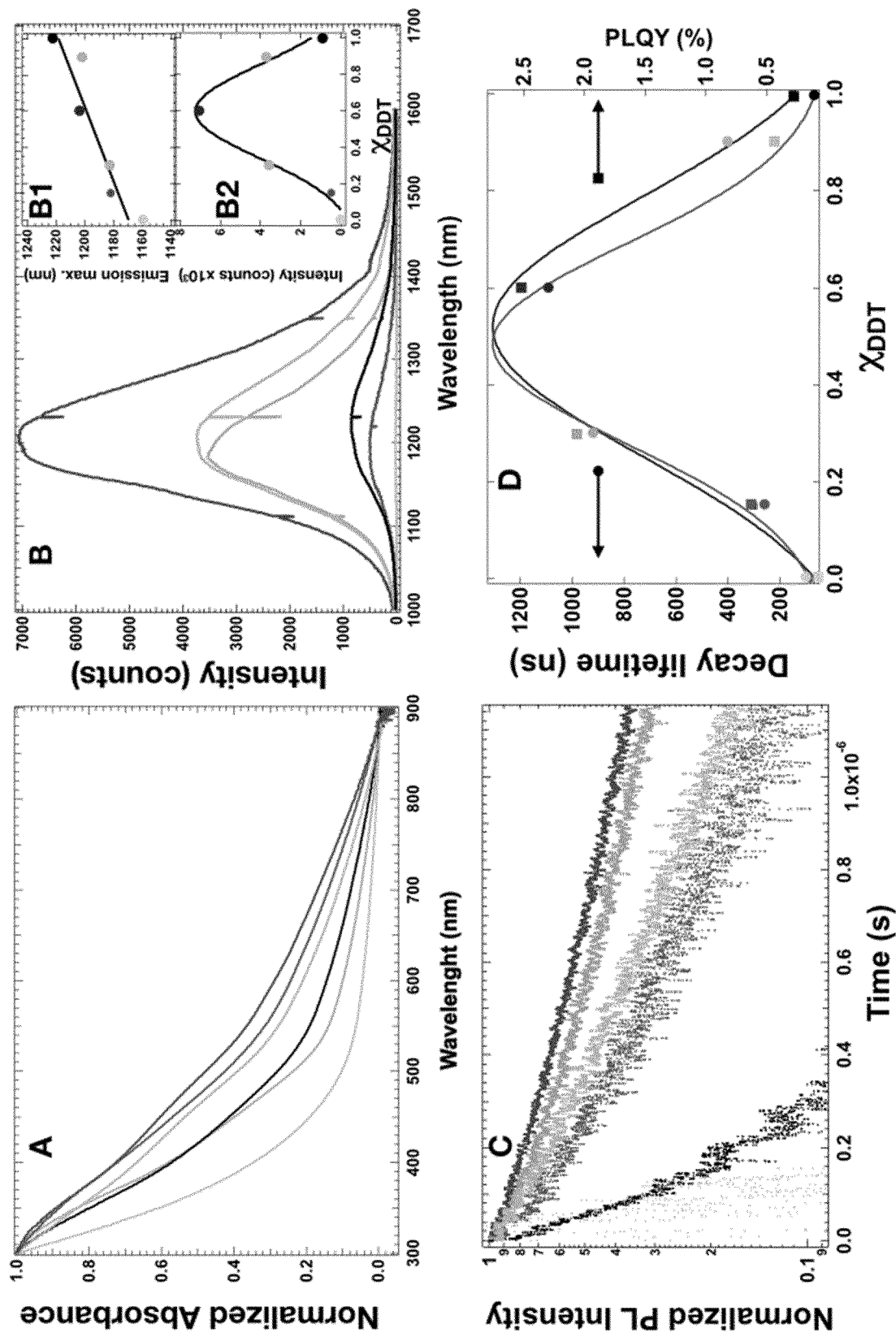


Fig. 4

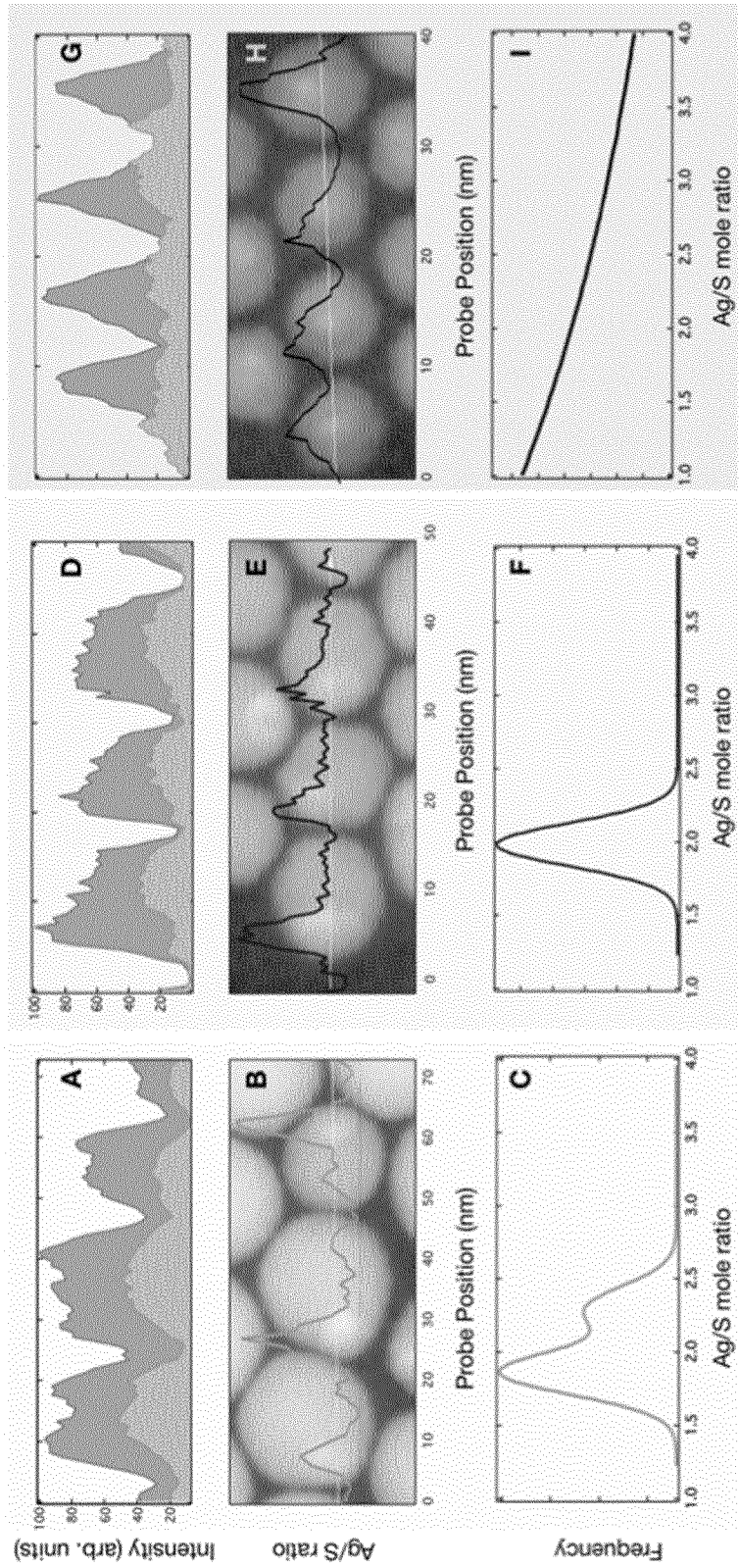


Fig. 5

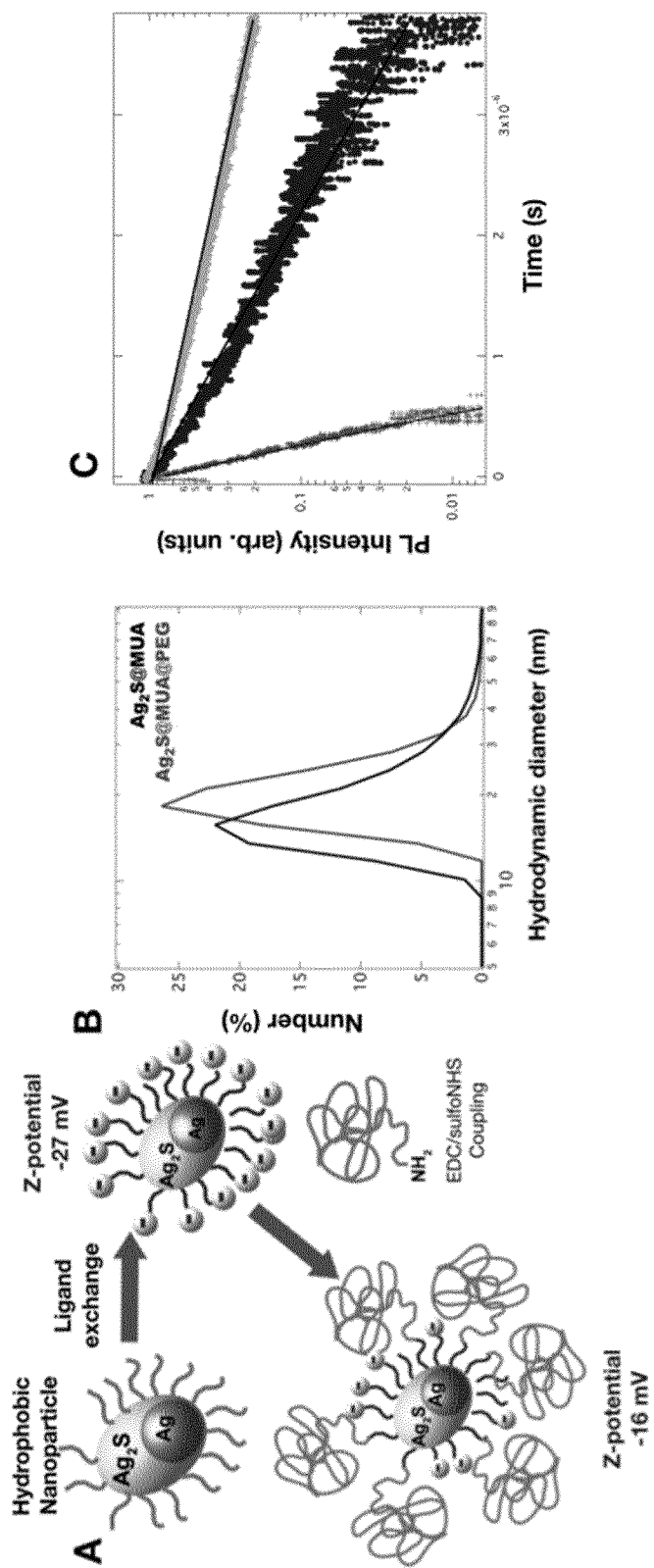


Fig. 6

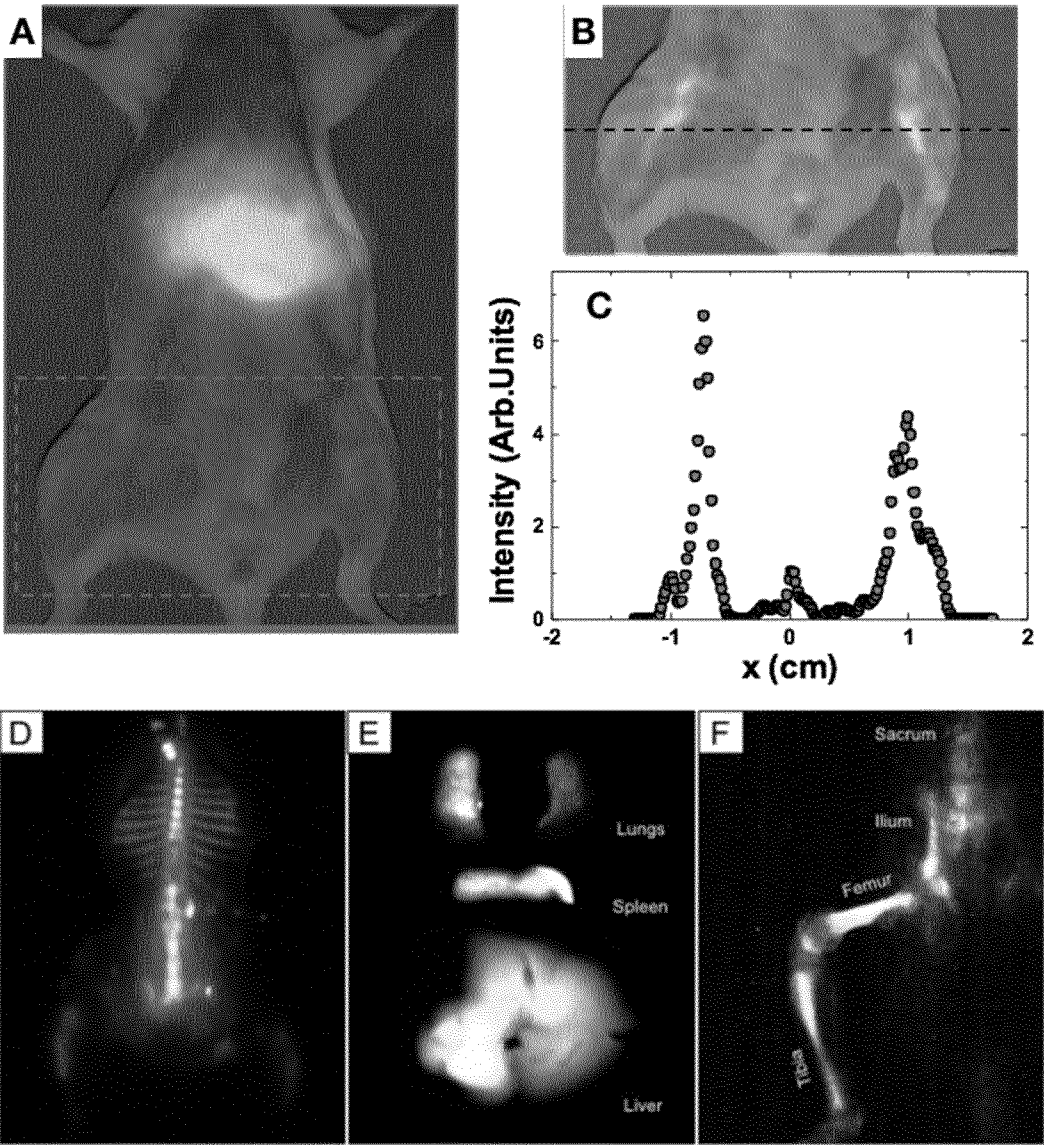


Fig. 7

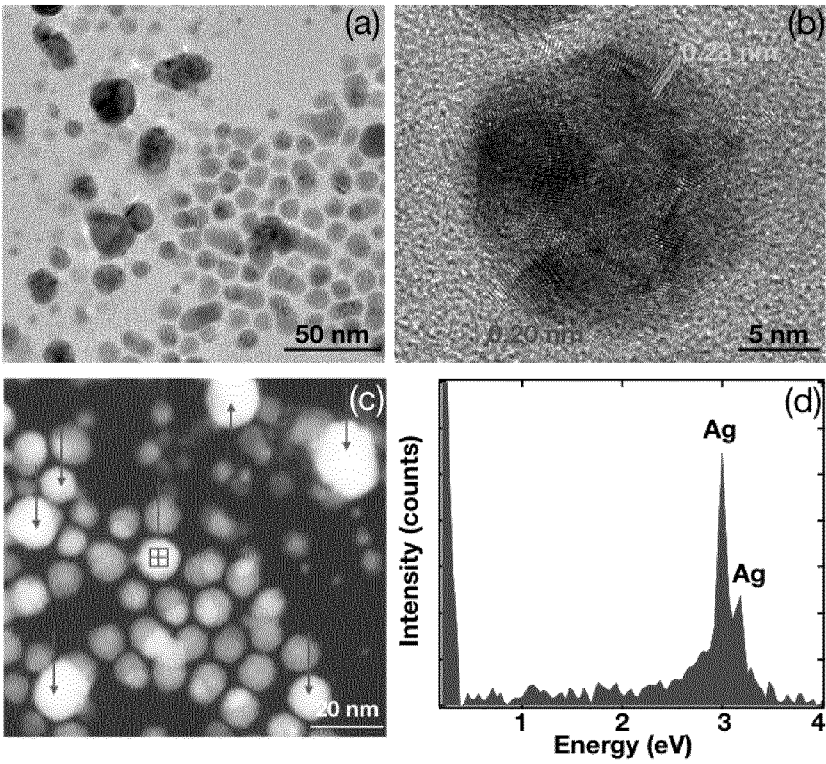


Fig. 8

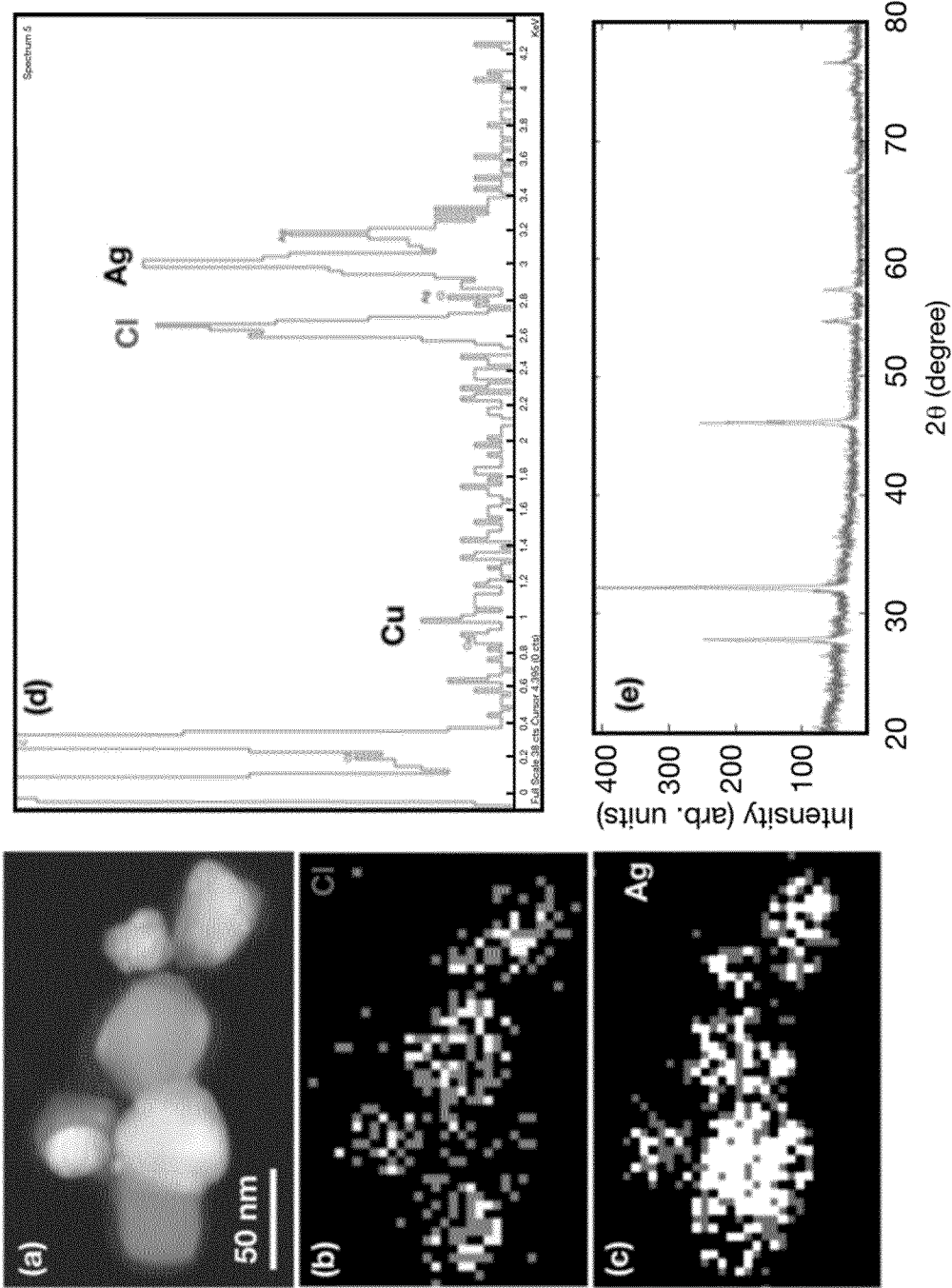


Fig. 9

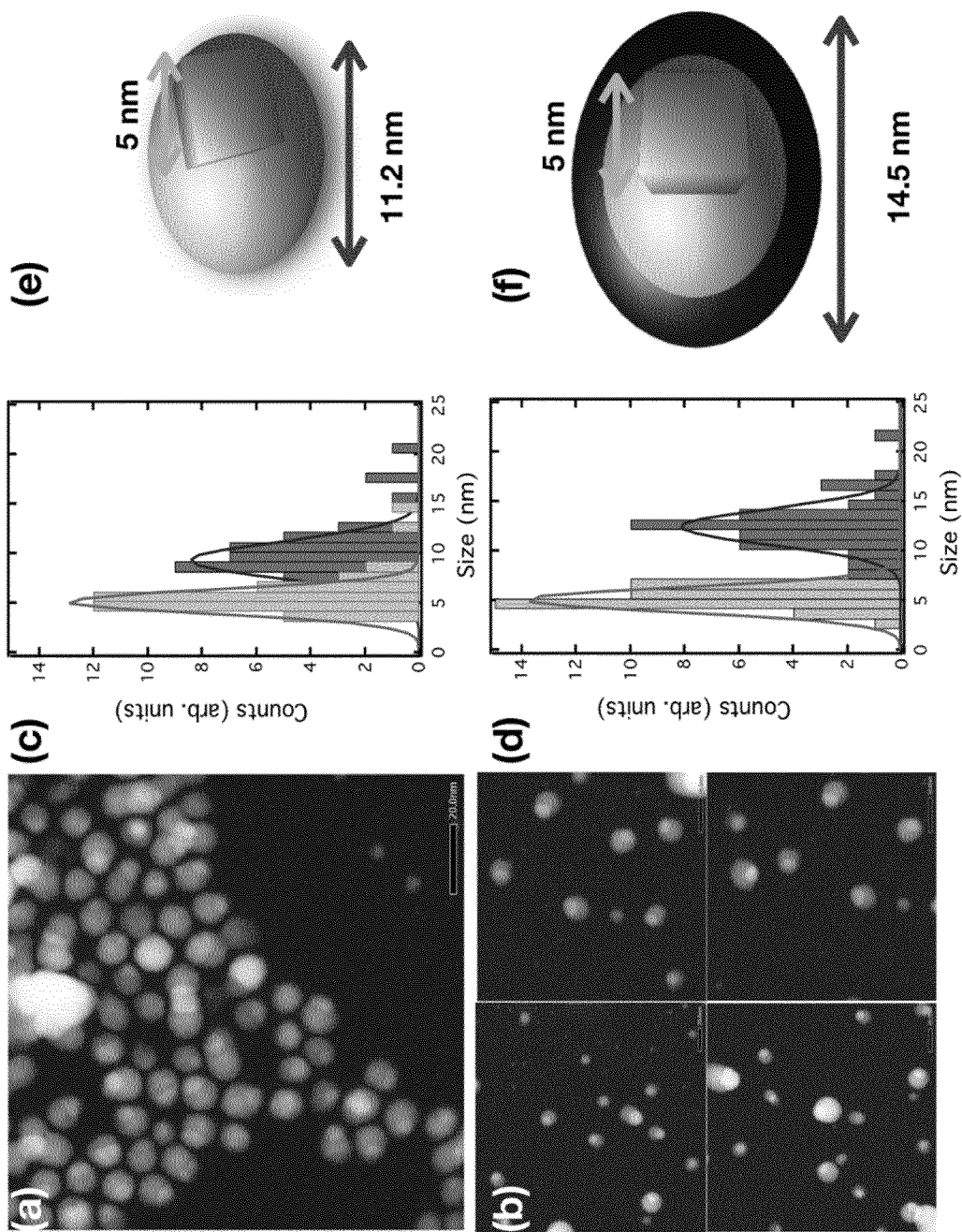


Fig. 10

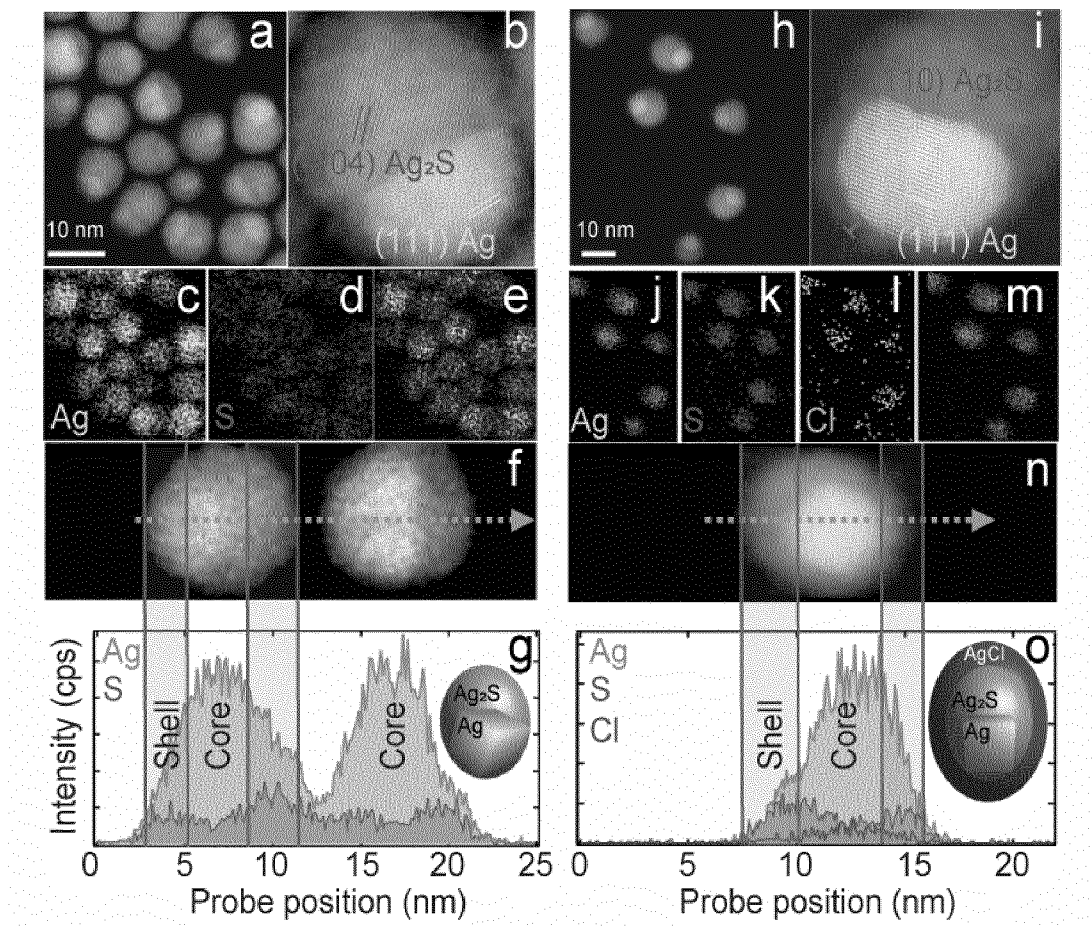


Fig. 11

12/20

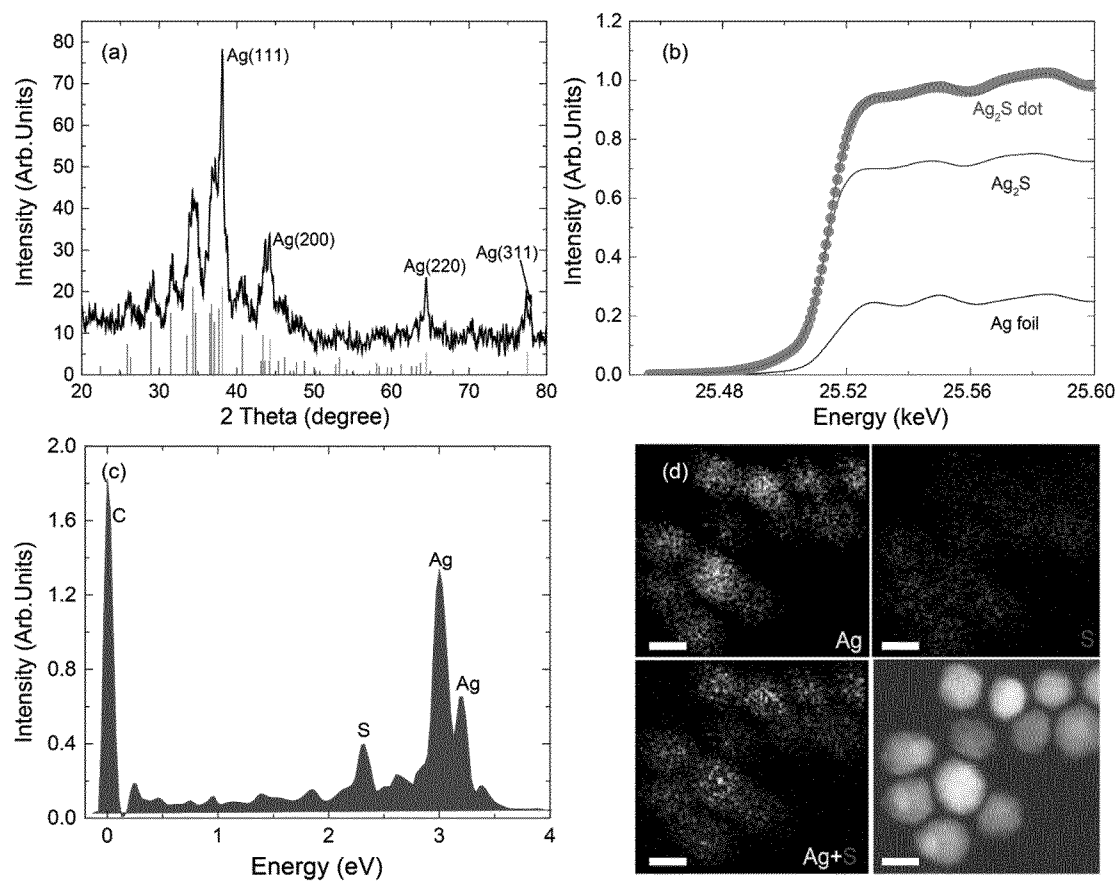


Fig. 12

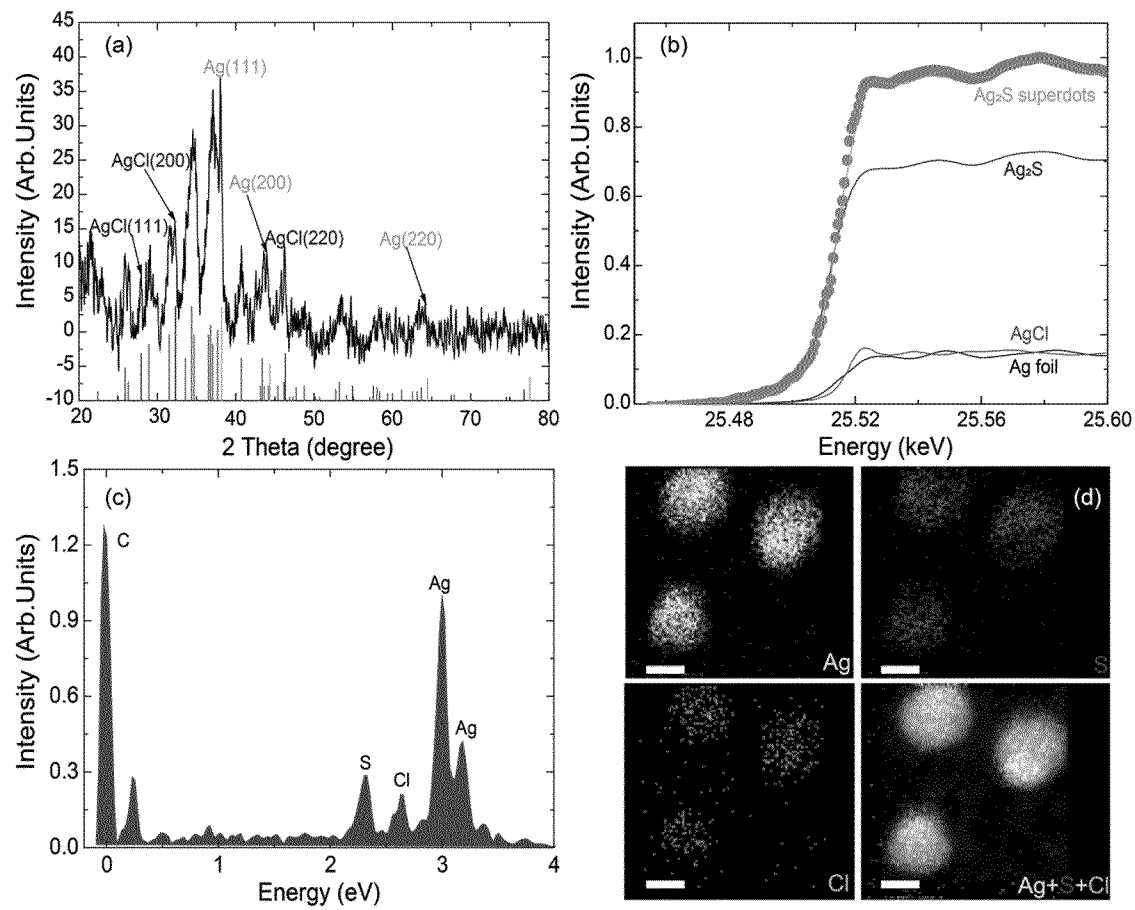


Fig. 13

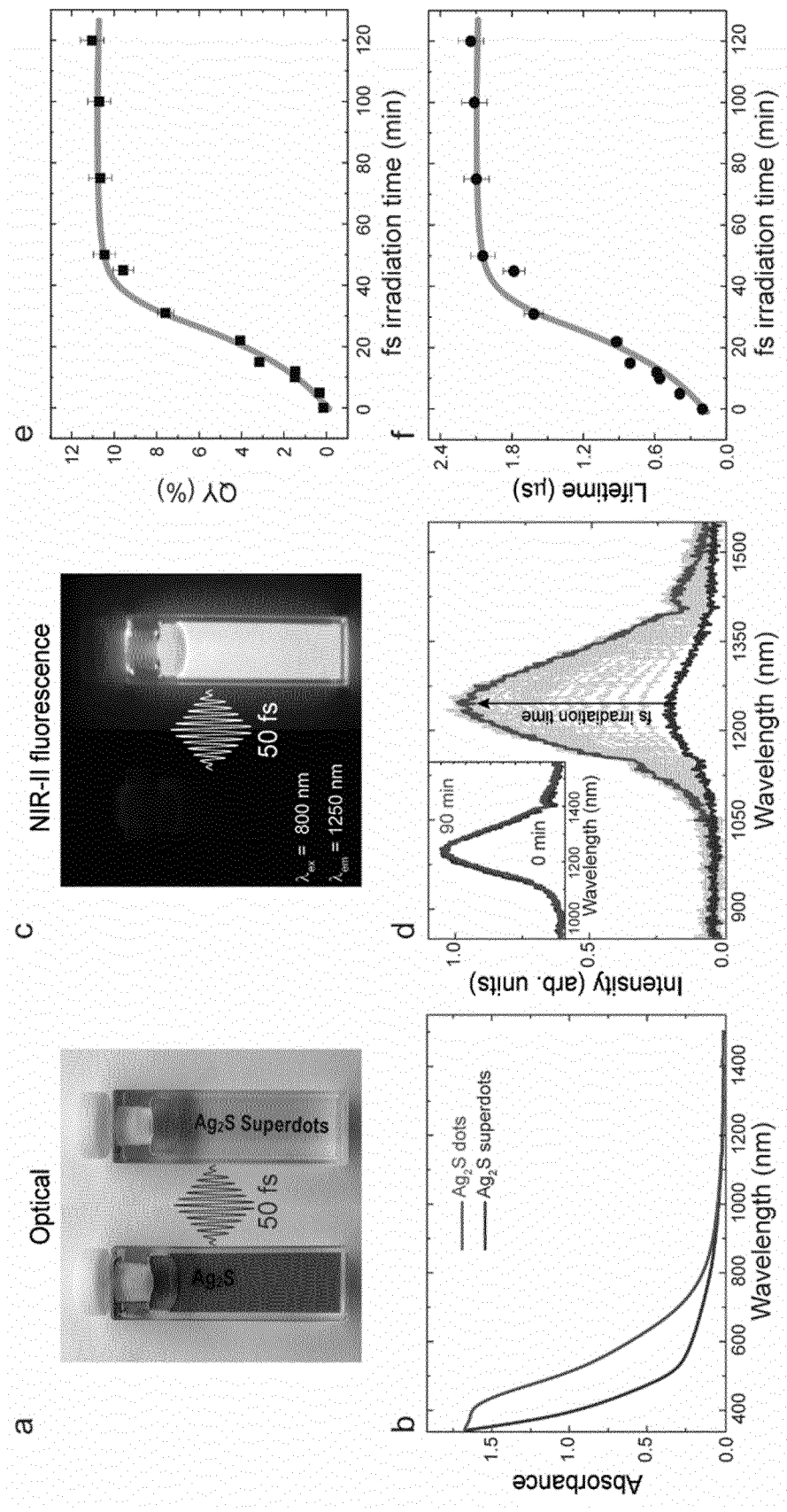


Fig. 14

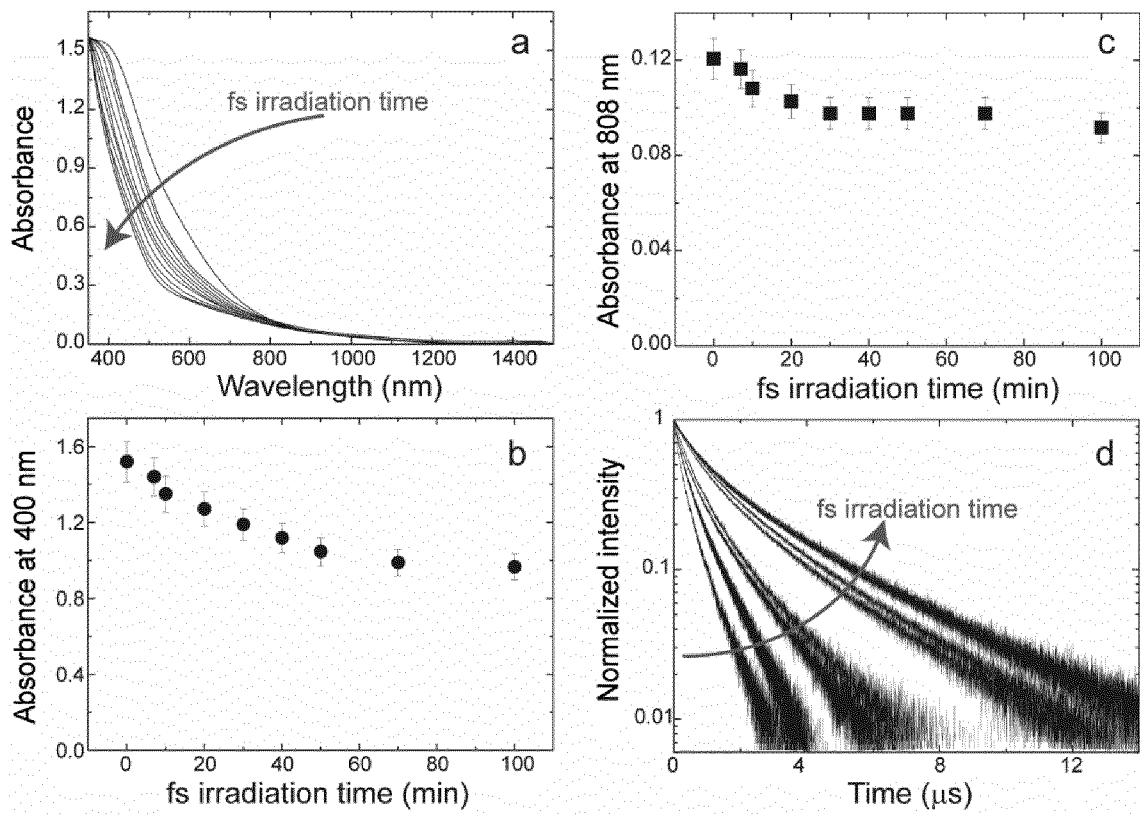


Fig. 15

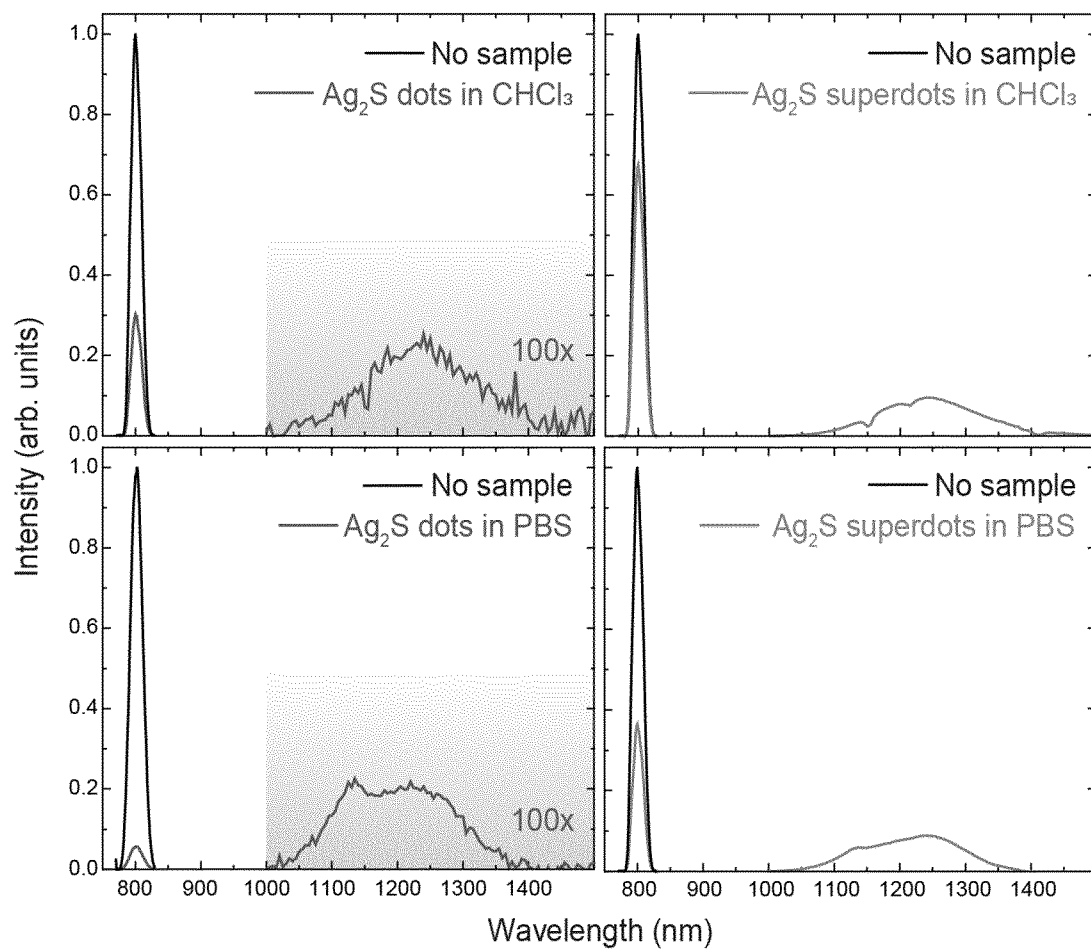


Fig. 16

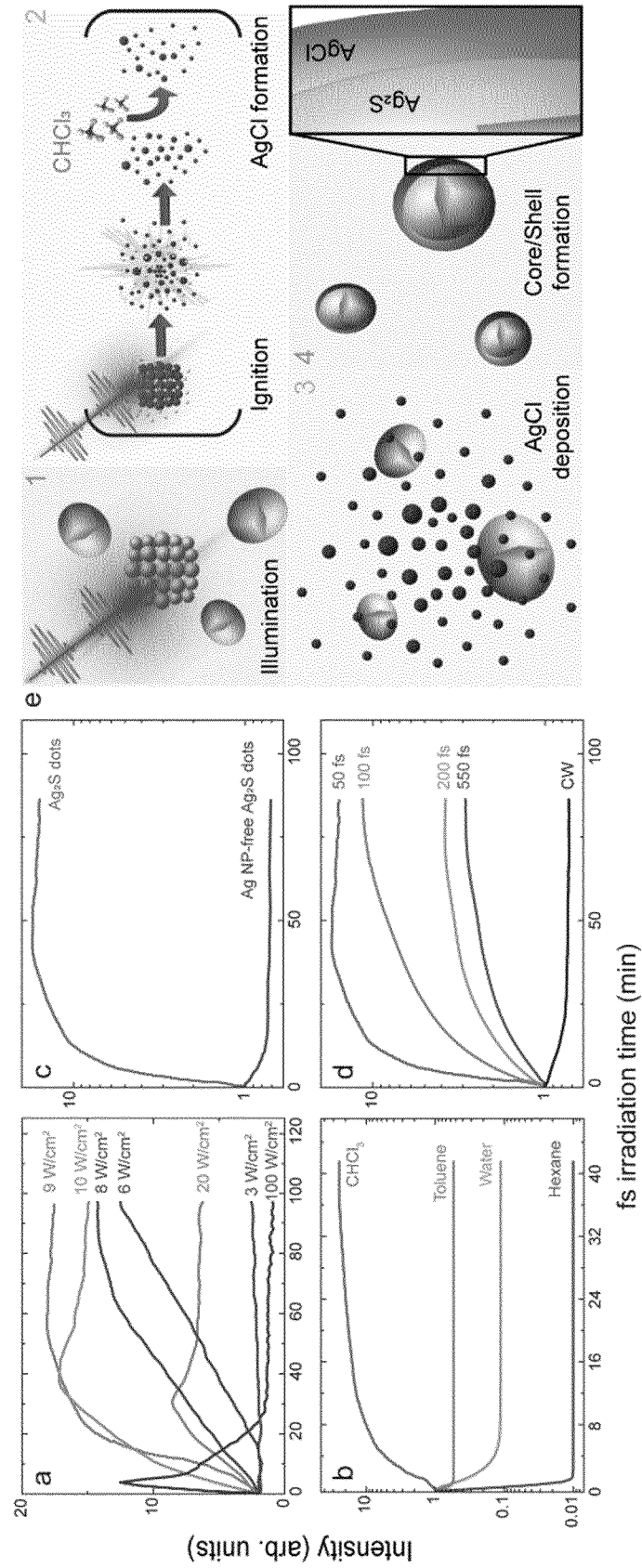
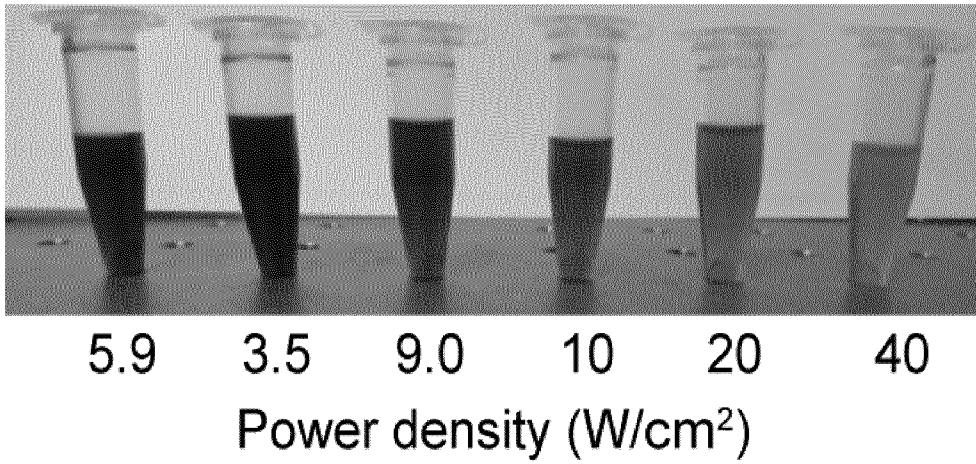


Fig. 17

a



b

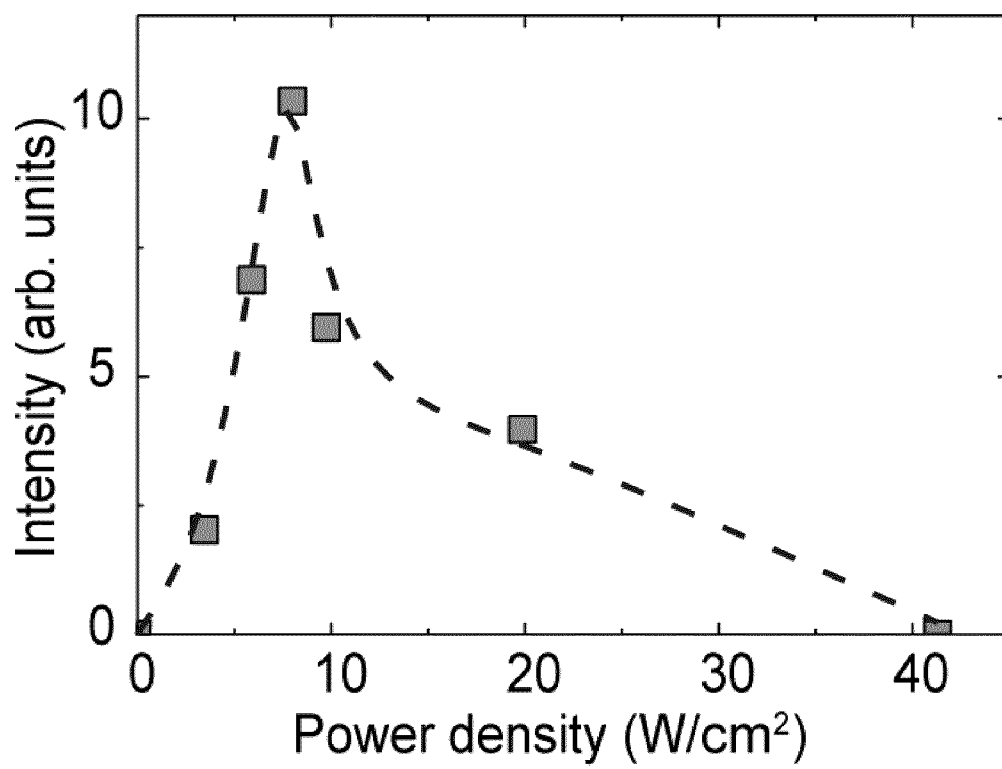


Fig. 18

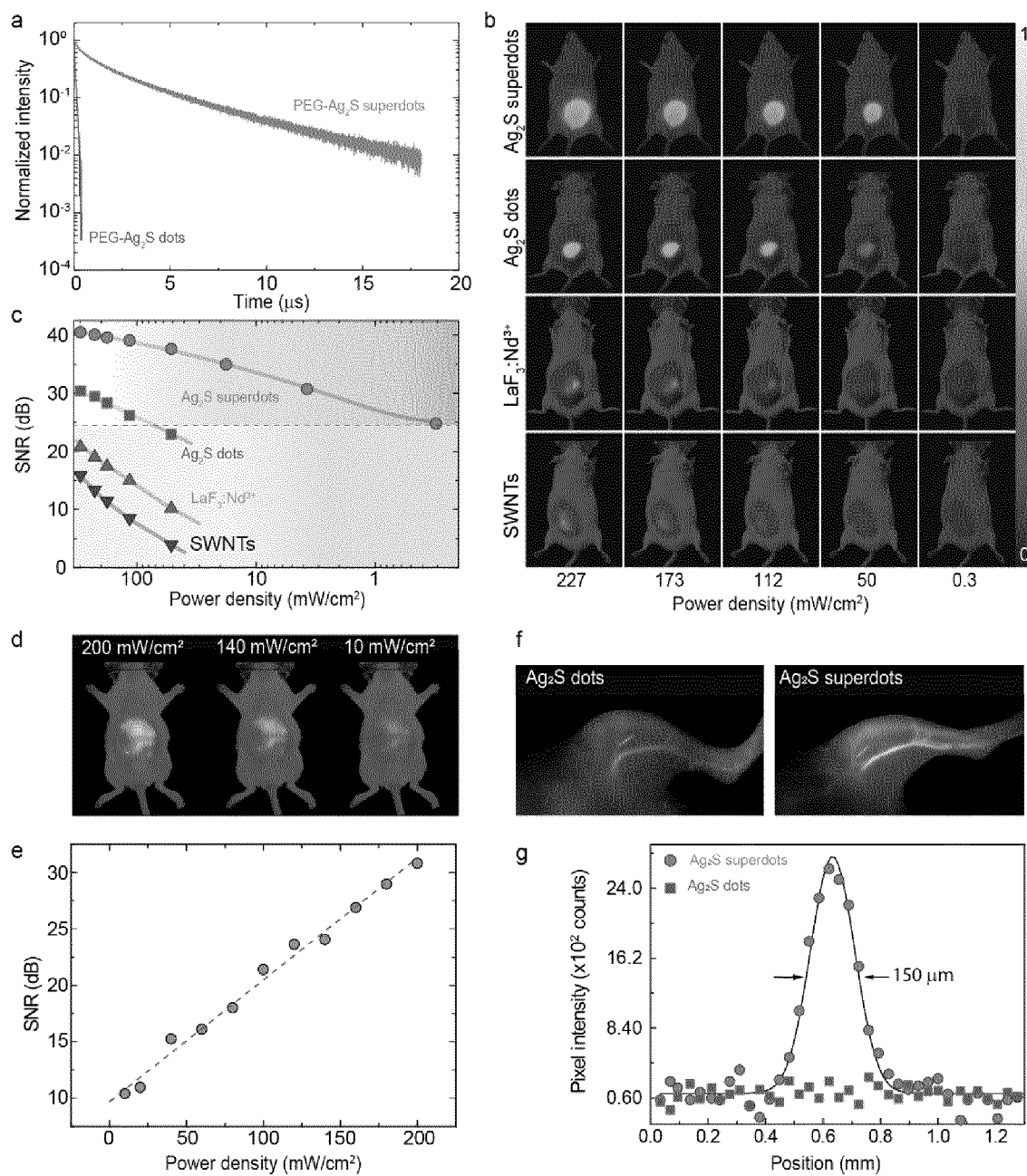


Fig. 19

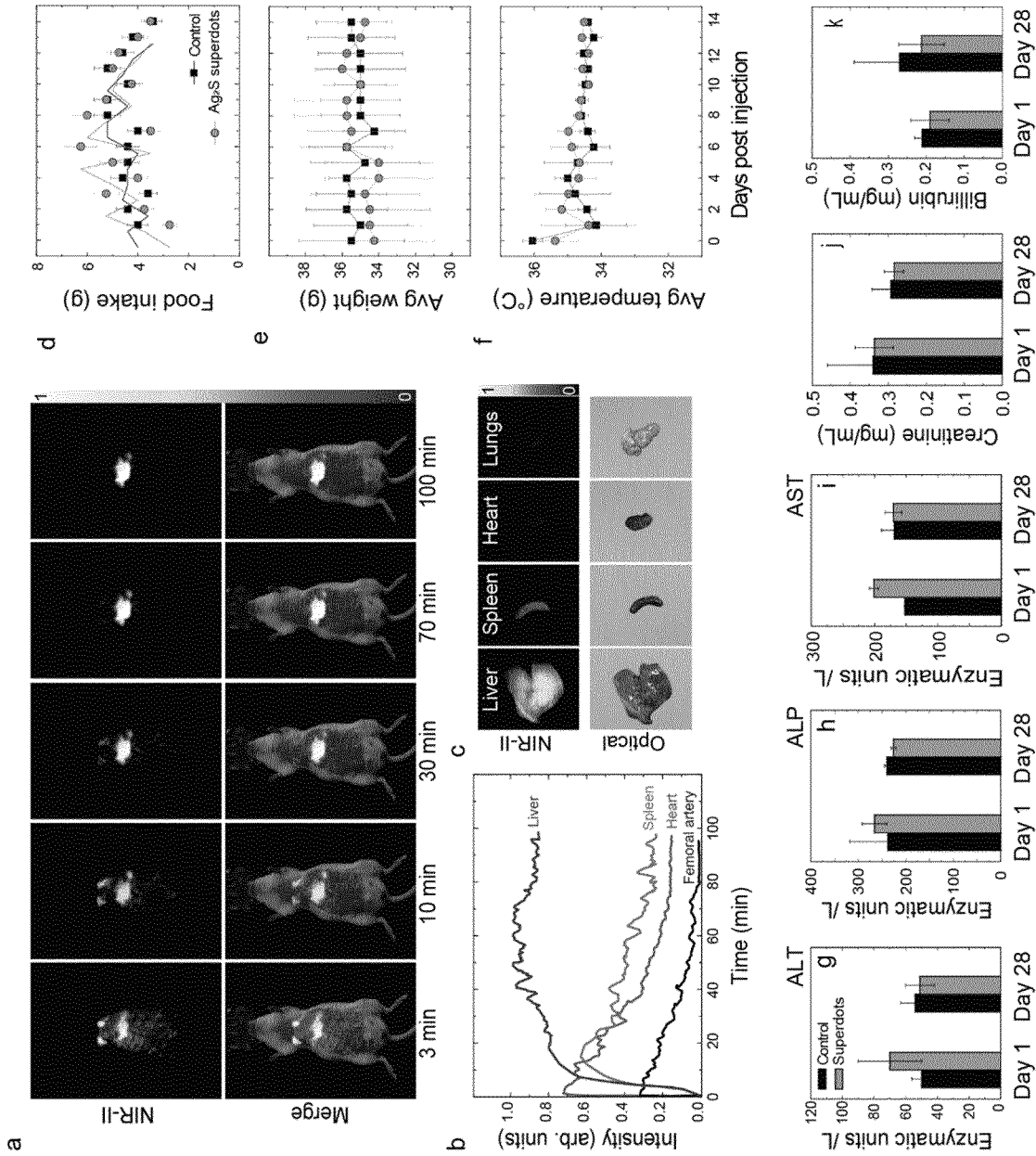


Fig. 20

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/050939

A. CLASSIFICATION OF SUBJECT MATTER		
INV. C01G5/00	B82Y15/00	B82Y30/00
G01N21/64	A61K49/00	C09K11/58
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)		
C01G B82Y C01B G01N A61K C09K		
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, CHEM ABS Data, 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	US 2016/083647 A1 (WANG QIANGBIN [CN] ET AL) 24 March 2016 (2016-03-24)	1-13,15,
A	paragraph [0005] - paragraph [0020];	16,18-20
	examples 1-6	14,17,
	-----	21-29
A	DATABASE WPI	
	Week 201750	1-29
	31 May 2017 (2017-05-31)	
	Thomson Scientific, London, GB;	
	AN 2017-40536W	
	XP002798659,	
	& CN 106 753 344 A	
	31 May 2017 (2017-05-31)	
	the whole document	

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☒ 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		Date of mailing of the international search report
15 April 2020		23/04/2020
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 Siebel, Eric

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2020/050939

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YAPING DU ET AL: "Near-Infrared Photoluminescent Ag ₂ S Quantum Dots from a Single Source Precursor", JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 132, no. 5, 10 February 2010 (2010-02-10), pages 1470-1471, XP055684943, US	1-13,15, 16,18-20
A	ISSN: 0002-7863, DOI: 10.1021/ja909490r 5th paragraph on page 1470, left column, Scheme 1 & Yaping Du ET AL: "Supporting Information Near-Infrared Photoluminescent Ag ₂ S Quantum Dots from a Single Source Precursor", 10 February 2010 (2010-02-10), XP055684944, Retrieved from the Internet: URL:https://pubs.acs.org/doi/suppl/10.1021/ja909490r/suppl_file/ja909490r_si_001.pdf [retrieved on 2020-04-09] the whole document	14,17, 21-29
X	----- YAN ZHANG, YEJUN ZHANG, GUOSONG HONG, WEI HE, KUN ZHOU, KAI YANG, FENG LI, GUANGCUN CHEN, ZHUANG LIU, HONGJIE DAI, QIANGBIN WANG: "Biodistribution, pharmacokinetics and toxicology of Ag ₂ S near-infrared quantum dots in mice.", BIOMATERIALS, vol. 34, 14 February 2013 (2013-02-14), pages 3639-3646, XP028985994, DOI: 10.1016/j.biomaterials.2013.01.089 page 3640, paragraph Materials and Methods	14,17
X	----- REMPEL S V ET AL: "The irradiation influence on the properties of silver sulfide (Ag ₂ S) colloidal nanoparticles", PHYSICS OF THE SOLID STATE, AMERICAN INSTITUTE OF PHYSICS, WOODBURY, NY, US, vol. 59, no. 8, 25 August 2017 (2017-08-25), pages 1629-1636, XP036306556, ISSN: 1063-7834, DOI: 10.1134/S1063783417080224 [retrieved on 2017-08-25] the whole document	26-29
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/050939

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
L	LU FENG ET AL: "Facile one-pot synthesis of monodispersed NIR-II emissive silver sulfide quantum dots", INORGANIC CHEMISTRY COMMUNICATIONS, ELSEVIER, AMSTERDAM, NL, vol. 106, 10 June 2019 (2019-06-10), pages 233-239, XP085741117, ISSN: 1387-7003, DOI: 10.1016/J.INOCHE.2019.06.013 [retrieved on 2019-06-10] the whole document -----	1-29
A	KAI CHENG ET AL: "High-Security Nanocluster for Switching Photodynamic Combining Photothermal and Acid-Induced Drug Compliance Therapy Guided by Multimodal Active-Targeting Imaging", ADVANCED FUNCTIONAL MATERIALS, vol. 28, no. 36, 1 September 2018 (2018-09-01), page 1803118, XP055684517, DE ISSN: 1616-301X, DOI: 10.1002/adfm.201803118 Scheme 1 -----	1-29

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2020/050939

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			EP 2716733 A1	09-04-2014
			JP 5815126 B2	17-11-2015
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