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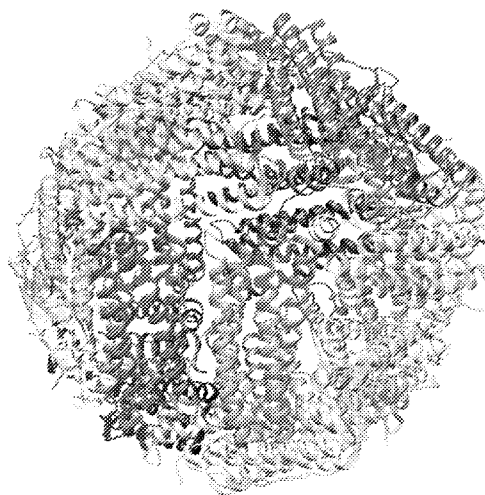
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(54) Title: FERRITIN-GOLD NANOPARTICLE CONJUGATES FOR PHOTODYNAMIC CANCER THERAPY

Figure 1A



(57) Abstract: The present invention provides a composition comprising a Ferritin-metal nanoparticle bioconjugate, wherein the Ferritin-metal nanoparticle bioconjugate comprises at least one metal nanoparticle and at least one Ferritin or a variant or a fragment thereof conjugated to the at least one metal nanoparticle; and wherein the Ferritin-metal nanoparticle bioconjugate specifically targets at least one cell expressing a transferrin receptor 1 (TfR1), and methods of making and use thereof.

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TITLE OF THE INVENTION
FERRITIN-GOLD NANOPARTICLE CONJUGATES FOR PHOTODYNAMIC
CANCER THERAPY

5 CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to and the benefit of U.S. Provisional Application No. 63/510,770, filed June 28, 2023, the disclosure of which is hereby incorporated by reference herein in its entirety.

10 BACKGROUND OF THE INVENTION

Ferritin is a protein naturally found in humans and many other life forms. The biological role of the protein is to sequester iron within the protein shell, limiting iron's interaction with extra and intra-cellular fluid, preventing the toxic formation of reactive oxygen species. Cells, however, need iron for proper function, hence ferritin's
15 role is to protectively shepherd iron throughout the body within the protein cage and release as necessary. As the metabolic processes of cancer cells are faster than healthy cells, they use more iron than a typical cell. To capture and include more iron, cancer cells over express receptors specific to iron transporting proteins, TfR1, which internalize iron into the cell. Thus, cancer cells can be targeted by developing therapies which
20 directly interact with these receptors, i.e.- using iron containing ferritin as a trojan horse. There are still outstanding issues that prevent a more complete understanding of the photochemistry of Ftn. Effective cancer therapies which specifically target cancer cells while leaving healthy cells unharmed are desired to treat a condition which kills ten million world-wide per year.

25 Thus, there is a need in the art for improved compositions and methods for the application of ferritin (Ftn) photochemistry driven iron sequestration to the treatment of cancer. This invention satisfies this unmet need.

SUMMARY OF THE INVENTION

30 The present invention relates to, in part, a composition comprising a Ferritin-metal nanoparticle bioconjugate, wherein the Ferritin-metal nanoparticle

bioconjugate comprises at least one metal nanoparticle and at least one Ferritin or a variant or a fragment thereof conjugated to the at least one metal nanoparticle; and wherein the Ferritin-metal nanoparticle bioconjugate specifically targets at least one cell expressing a transferrin receptor 1 (TfR1).

5 In one embodiment, the at least one Ferritin or a variant or a fragment thereof is selected from the group consisting of a human H-type Ferritin or a variant or a fragment thereof, horse spleen Ferritin or a variant or a fragment thereof, and any combination thereof.

10 In one embodiment, wherein the at least one metal nanoparticle is selected from the group consisting of a metal nanorod, metal nanosphere, metal nanostar, and any combination thereof. In one embodiment, the at least one metal nanoparticle is selected from the group consisting of a gold nanoparticle, iron nanoparticle, copper nanoparticle, and any combination thereof. In one embodiment, the at least one metal nanoparticle further comprises an oxide coating. In one embodiment, the oxide coating is selected
15 from the group consisting of a Fe₂O₃ coating, SiO₂ coating, and any combination thereof.

 In one embodiment, the at least one Ferritin or a variant or a fragment thereof is conjugated to the at least one metal nanoparticle via a linker In one embodiment, the linker comprises N-succinimidyl S-acetylthioacetate or a derivative thereof.

20 In one embodiment, the Ferritin-metal nanoparticle bioconjugate has an average hydrodynamic diameter of between 5 nm to 1,000 nm. In one embodiment, the Ferritin-metal nanoparticle bioconjugate has an average hydrodynamic diameter of between 10 nm to 300 nm. In one embodiment, the Ferritin-metal nanoparticle bioconjugate absorbs light having a wavelength of 200 nm to 1100 nm. In one
25 embodiment, the Ferritin-metal nanoparticle bioconjugate releases at least one iron ion when irradiated with light having a wavelength of between 200 nm to 1100 nm.

 In one embodiment, the metal nanoparticle is a gold nanorod, and the bioconjugate has an average width of between 5 nm to 20 nm and an average length of between 30 nm to 70 nm.

In one embodiment, the metal nanoparticle is a gold nanostar, and the bioconjugate comprises between 3 to 7 spikes, and wherein the spikes have an average length of between 20 nm to 150 nm.

In one embodiment, the metal nanoparticle is a gold nanosphere, and
5 the bioconjugate has a surface area of between 50 nm² to 900 nm².

In one embodiment, the Ferritin-metal nanoparticle bioconjugate further comprises a targeting domain.

In one embodiment, the composition further comprises a therapeutic agent. In one embodiment, the at least one cell expressing a TfR1 is a cell overexpressing
10 a TfR1. In one embodiment, the cell overexpressing a TfR1 is a cancer cell.

The present invention further relates to, in part, a method of treating a disease or disorder associated with the level or activity of a TfR1 in a subject in need thereof, wherein the method comprises: administering to the subject the composition of the present invention, and irradiating the subject at a wavelength of between about 200
15 nm to about 1100 nm.

In one embodiment, the disease or disorder is selected from the group consisting of cancer, prostate cancer, breast cancer, Alzheimer's disease, and any combination thereof.

In one embodiment, the present invention relates to a method of inhibiting
20 proliferation of at least one cell expressing a TfR1, wherein the method comprises: administering to the subject the composition of the present invention, and irradiating the subject at a wavelength of between 200 nm to 1100 nm.

In one embodiment, the present invention relates to a method of inducing ferroptosis of at least one cell expressing a TfR1, wherein the method comprises:
25 administering to the subject the composition of the present invention, and irradiating the subject at a wavelength of between 200 nm to 1100 nm.

In one embodiment, the irradiation selectively kills or inhibits the at least one cell expressing a TfR1. In one embodiment, the at least one cell expressing a TfR1 is a cancer cell.

30

In one embodiment, the present invention relates to a method of making a Ferritin-metal nanoparticle bioconjugate, comprising the steps of: providing a metal nanoparticle; and dispersing the metal nanoparticle in a solution comprising Ferritin.

In one embodiment, the solution comprising Ferritin is a buffered solution.

5

BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description of various embodiments of the invention will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings illustrative embodiments. It should be understood, however, that the invention is not limited to the precise arrangements and instrumentalities of the embodiments shown in the drawings.

Figure 1, comprising Figure 1A and Figure 1B, depict spherical 3D ribbon structures. Figure 1A depicts Wild-type H-type ferritin (HFtn). The model was generated using the UCSF Chimera software package, PDB code 3AJ0. Figure 1B depicts horse spleen ferritin (HSFtn). The model was generated using the UCSF Chimera software package, PDB code 2W00.

Figure 2 depicts a subunit ribbon structure of wild-type HFtn. Model generated using the UCSF Chimera software package, PDB code 3AJ0.

Figure 3, comprising Figure 3A and Figure 3B, depicts relevant single-phase models. Figure 3A depicts the hexagonal unit cell of Fh proposed by Michel et al., showcases a polyhedral representation where the yellow bonded atoms form a cubane-like structure consisting of four edge-shared Fe octahedra. Figure 3B depicts the ideal Fh structure along the c-axis, showing the central FeO₄ tetrahedra surrounded by 12 FeO₆ octahedra.

Figure 4, comprising Figure 4A through Figure 4F, depicts laboratory-based biomineralization synthetic strategies for Ftn. Figure 4A depicts oxidation-based biomineralization of Ftn. Figure 4B depicts reduction-based biomineralization of Ftn. Figure 4C depicts two-step reduction-based biomineralization of Ftn. Figure 4D depicts oxidation and deposition-based biomineralization of Ftn. Figure 4E depicts pH-dependent disassembly-reassembly based biomineralization of Ftn. Figure 4F depicts outer surface

reduction- based biomineralization of Ftn.

Figure 5 depicts a schematic representation of the synthesis of CuFtn.

Figure 6, comprising Figure 6A through Figure 6D, depicts transmission electron microscopy (TEM) images. Figure 6A depicts purified and concentrated stained CuFtn heterostructures with 2% PTA where dark spheres are Cu(OH)₂ nanoparticle cores and light spheres are protein cages. Figure 6B depicts purified and concentrate unstained CuFtn heterostructures where dark spheres are copper cores. Figure 6C depicts particle size analysis of CuFtn inner core diameter from TEM photographs using ImageJ. Figure 6D depicts diffused electron diffraction rings of the CuFtn from SAED analysis.

Figure 7, comprising Figure 7A and Figure 7B, depicts TEM image of CuCtrl unstained with different magnifications. Figure 7A depicts 100 nm resolution. Figure 7B depicts 50 nm resolution.

Figure 8 depicts a Tauc plot for the direct allowed transition of CuFtn and CuCtrl.

Figure 9 depicts JCPDS XRD peak positions of Cu(OH)₂ (Deep blue, JCPDS 13-420), CuO (Black, JCPDS 54-937), XRD spectra of Cu(OH)₂ (Deep blue, Commercial sample at 25 °C), Cu(OH)₂ (Purple, Commercial sample treated with UV-ozone at 100 °C), CuO (Black, Commercial sample at 25 °C), CuCtrl (Light blue, 25 °C), CuCtrl (Blue, Sample treated with UV-ozone at 100 °C).

Figure 10 depicts dynamic light scattering (DLS) Particle size analysis of CuFtn and apoFtn.

Figure 11, comprising Figure 11A and Figure 11B, depicts absorbance spectra. Figure 11A depicts yellow color PAR complex formed (λ_{max} = 414 nm). Figure 11B depicts red color Cu(II)- PAR complex formed (λ_{max} = 510 nm).

Figure 12, comprising Figure 12A and Figure 12B, depicts UV-Vis spectra. Figure 12A depicts UV-Vis absorbance spectrum of different [Cu(II)] = 0, 1, 10, 25, 50 and 100 μ M, apoFtn and CuFtn with PAR(100 μ M). Figure 12B depicts UV-Vis absorbance spectrum of CuFtn with PAR (80 mM) in Tris-HCl (0.1 M, pH 8.2) and sodium potassium tartrate (32 mM) under SSR for 120 min exposure (without compensation of degraded PAR).

Figure 13, comprising Figure 13A and Figure 13B, depicts UV-Vis spectra. Figure 13A depicts UV-Vis absorbance spectrum of Cu(II) ion release studies from CuFtn during exposure to SSR. Inset shows the $\frac{[PAR-Cu(II)]}{[PAR-Cu(II)]_0}$ vs time plot under SSR for 120 min. Figure 13B depicts UV-Vis absorbance spectrum of Cu(II) ion release studies from CuFtn under dark conditions. PAR absorbance. Inset shows the $\frac{[PAR-Cu(II)]}{[PAR-Cu(II)]_0}$ vs time plot under dark for 120 min. PAR and PAR-Cu(II) complex absorb at $\lambda = 414$ nm and $\lambda = 510$ nm, respectively.

Figure 14, comprising Figure 14A and Figure 14B, depicts data for the reduction of Cr(VI). Figure 14A depicts reduction of Cr(VI) in the presence of CuFtn under SSR, control reactions under dark conditions and with apoFtn. Figure 14B depicts the variation of [Cr(VI)] in the presence of CuFtn under SSR with long-pass filters $\lambda \geq 355$ nm, $\lambda \geq 330$ nm, $\lambda \geq 300$ nm, and Full SSR for t=0 and t=120 min.

Figure 15, comprising Figure 15A and Figure 15B, depicts spectroscopical data for the reduction of Cr(VI). Figure 15A depicts UV-Visible absorbance spectra of Cr(VI) reduction at $\lambda = 372$ nm under full SSR for 120 min. Figure 15B depicts wavelength-dependent Cr(VI) reduction variation at $\lambda = 372$ nm for 120 min under $\lambda \geq 355$ nm, $\lambda \geq 330$ nm, $\lambda \geq 300$ nm long pass filters and full SSR.

Figure 16, comprising Figure 16A and Figure 16B, depicts spectroscopical data for the reduction of Cr(VI). Figure 16A depicts UV-Visible absorbance spectra of Cr(VI) reduction at $\lambda = 267$ and 372 nm under full SSR for 120 min with CuFtn (45 μ M) in Tris-HCl (0.1 M, pH 8.2), PAR(80 mM), sodium potassium tartrate (32 mM), and Cr(VI) (0.020 mM)). Figure 16B depicts Cr(VI) reduction at $\lambda = 372$ nm for 120 min under full SSR with and without PAR (80 mM) in the medium.

Figure 17, comprising Figure 17A and Figure 17B, depicts spectroscopical data. Figure 17A depicts UV-Visible absorbance spectra of Cr(VI) reduction at $\lambda = 267$ and 372 nm under full SSR for 120 min with CuFtn (45 μ M) in Tris-HCl (0.1 M, pH 8.2), PAR(80 mM), sodium potassium tartrate (32 mM), and Cr(VI) (0.020 mM)). Figure 17B depicts Cr(VI) reduction at $\lambda = 372$ nm for 120 min under full SSR with and without PAR (80 mM) in the medium.

Figure 18, comprising Figure 18A and Figure 18B, depicts the proposed mechanism of Cr(VI) reduction to Cr(III) by CuFtn illumination. Figure 18A depicts Cr(VI) ions migrate to CuFtn core across the protein shell and get reduced directly by excited electrons from the CuFtn core excitation. Figure 18B depicts photochemical
5 excitation of the Cu(OH)₂ core transfers electrons via tunneling across the CuFtn shell to reduce the Cr(VI) ions.

Figure 19, comprising Figure 19A through Figure 19D, depicts data towards the investigation of location of Cu component. Figure 19A depicts total [Cr] obtained from the Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-
10 OES) studies of multicycle Cr reduction by CuFtn before and after dialysis. Figure 19B depicts total [Cu] obtained from the ICP-OES studies of multicycle Cr reduction by CuFtn before and after dialysis. Figure 19C depicts diagram showing the CuFtn after dialysis if Cr(III) would have deposited in the inner core. Figure 19D depicts CuFtn after dialysis if the electron transfer occurred through the protein shell and Cr(III) formed
15 outside the CuFtn (in the solution).

Figure 20, comprising Figure 20A and Figure 20B, depicts Cr degradation and reduction data. Figure 20A depicts chromate degradation control studies of CuFtn and Ftn with hole-scavenging agent tartrate at SSR. Figure 20B depicts Cr(VI)/Cr(III) reduction potential and Conduction band edges of Cu(OH)₂, CuO and Fh.

Figure 21, comprising Figure 21A through Figure 21C, depicts data for AuNR. Figure 21A depicts TEM images of AuNR with a varying aspect ratio. Figure 21B depicts UV-Visible absorbance spectra of each AuNR solution with varying longitudinal absorbance at 500-750 nm range. Figure 21C depicts AuNR samples from left to right showing distinct colors ranging from brownish red through green to purplish
25 red.

Figure 22 depicts the localized surface plasmon resonance (LSPR) process, in which free conduction electrons of the AuNPs undergo oscillation due to the interaction with the incoming light.

Figure 23 depicts the mechanism of how hot electrons move from a metal
30 nanorod to a semiconductor through a S.B at their interface. The direction of the wave vector k_0 , the electric field E_0 .

Figure 24, comprising Figure 24A through Figure 24D, depicts representative TEM images and NIR plasmon spectra. Figure 24A depicts UV-Visible absorbance spectra of AuNR-CTAB, which shows a weak transverse band at 520 nm and a sharp longitudinal band at 800 nm. Figure 24B depicts unstained TEM image of AuNR-CTAB. Figure 24C depicts unstained TEM image of AuNR-HFtn bioconjugates. Figure 24D depicts stained TEM image of AuNR-HFtn bioconjugates.

Figure 25, comprising Figure 25A and Figure 25B, depicts TEM images of HFtn. Figure 25A depicts a TEM image of HFtn stained with 1% PTA. The white color outer coating represents the protein shell whereas the black spheres are the Fh inner core. Figure 25B depicts TEM image of unstained HFtn. The black color spheres represent the Fh inner core of HFtn.

Figure 26, comprising Figure 26A through Figure 26C, depicts TEM and UV-Vis data for AuNr. Figure 26A depicts a TEM image of an unstained CTAB capped AuNR, Figure 26B depicts a TEM image of an unstained AuNR-HFtn bioconjugate. Figure 26C depicts a UV-Visible spectrum of AuNR-HFtn, CTAB capped AuNR(AuNR-CTAB), AuNR-Tris HCl and pure HFtn.

Figure 27, comprising Figure 27A through Figure 27D, depicts TEM and aspect ratio data for AuNR. Figure 27A depicts TEM images of unstained AuNR. Figure 27B depicts TEM images of unstained AuNR-HFtn bioconjugates. Figure 27C depicts aspect ratio distribution of AuNR calculated from ImageJ based on TEM images of unstained AuNR. Figure 27D depicts aspect ratio distribution of AuNR-HFtn calculated from ImageJ based on TEM images of unstained AuNR-HFtn.

Figure 28, comprising Figure 28A and Figure 28B, depicts structure and data for CTAB. Figure 28A depicts chemical structure of CTAB. Figure 28B depicts Attenuated Total Reflectance – Fourier Transform Infrared Spectroscopy (ATR-FTIR) spectra of CTAB, CTAB-AuNR, HFtn and AuNR-HFtn bioconjugates.

Figure 29 depicts Z.P values of AuNR-CTAB, AuNR-HFtn bioconjugates, and pure HFtn, extracted from the DLS studies.

Figure 30 depicts a calibration curve established with L-cysteine.

Figure 31, comprising Figure 31A and Figure 31B, depicts data for iron release of AuNR-HFtn. Figure 31A depicts UV-Vis absorbance spectrum of AuNR-HFtn

conjugate system exposed to NIR Illuminator at 850 nm for 60-, 120-, 180-, and 300-min. Absorbance at $\lambda = 562$ nm indicates the unique absorbance peak of Fe(II)-Fz complex. Figure 31B depicts release [Fe(II)] calculated from absorbance data at $\lambda = 562$ nm in 60-, 120-, 180-, and 300- min time intervals.

5 Figure 32, comprising Figure 32A and Figure 32B, depicts an iron release study conducted at 850 nm with the IR illuminator for 120 min. Figure 32A depicts Fe release study controls; AuNR-HFtn bioconjugates under NIR exposure at 850 nm with tartrate and Fz (AuNR-HFtn(L)), AuNR-HFtn bioconjugates at dark with tartrate and Fz (AuNR-HFtn(D)), AuNR-HFtn bioconjugates under NIR exposure at 850 nm without
10 tartrate and with Fz (noTart), HFtn under NIR exposure at 850 nm without AuNR, with tartrate and Fz (noAuNR), and apoHFtn under NIR exposure at 850 nm without AuNR, with tartrate and Fz (noAuNR-apoHFtn). Figure 32B depicts Fe release study controls under NIR exposure at 850 nm with Tartrate and Fz; AuNR-HFtn bioconjugates, AuNR-HSFtn, AuNR(SiO₂)-HFtn, and AuNR-HFtn(DTNB).

15 Figure 33, comprising Figure 33A through Figure 33D, depicts TEM images of AuNR-HFtn bioconjugates. Figure 33A depicts a TEM image of AuNR-HFtn. Figure 33B depicts a TEM image of AuNR-HSFtn. Figure 33C depicts a TEM image of AuNR(SiO₂)-HFtn. Figure 33D depicts a TEM image of AuNR-HFtn(DTNB).

 Figure 34, comprising Figure 34A and Figure 34B, depicts data for iron
20 release experiments. Figure 34A depicts the concentration of iron released during the Iron release control studies carried out under laser exposure for 60 min. Figure 34B depicts the concentration of iron released during the Iron release control studies carried out in different exposure times (30, 60, and 120 min).

 Figure 35, comprising Figure 35A through Figure 35C, depicts TEM
25 images and UV-Vis data. Figure 35A depicts a TEM image of reshaped unstained AuNR exposed to laser for 30 min. Figure 35B depicts a TEM image of welded unstained AuNR exposed to laser for 30 min. Figure 35C depicts the UV-Visible spectrum of the AuNR-HFtn conjugates exposed to laser for 0, 5, 10, 20, and 30 min. The longitudinal broad peak at 850 nm has been repositioned as a narrow peak around 800 nm

30 Figure 36, comprising Figure 36A and Figure 36B, depicts controls for Cr reduction. Figure 36A depicts the Cr(VI)reduction controls carried out under an IR

illuminator at 850 nm for 120 min. Figure 36B depicts the Cr(VI) reduction control study carried out under laser for 60 min.

Figure 37, comprising Figure 37A and Figure 37B, depicts Fh absorbance data. Figure 37A depicts a bandgap excitation diagram of Fh with 2.6 eV bandgap energy. Figure 37B depicts a UV-Visible absorbance spectra of the AuNR, HFtn, and Fh.

Figure 38, comprising Figure 38A and Figure 38B, depicts plasmon-induced resonance energy transfer (PIRET) diagrams. Figure 38A depicts PIRET mechanism. Figure 38B depicts a plasmonic hot electron transfer (PHETr) and plasmonic hot electron tunneling (PHETu) mechanism.

Figure 39 depicts a schematic of hybrid plasmonic system that will be investigated. The system consists of an AuNR on the exterior of the Ftn protein cage. Within the protein cage (shown here as a cutaway) is Fe(O)OH, which is a small band gap semiconductor. The plasmonic AuNRs (or AuNSs) sensitizes the photoexcitation of the Fe(O)OH to NIR.

Figure 40 depicts the reaction of SATA with primary amines introducing protected thiols. The deprotection with hydroxylamine leaves the -SH residue free for subsequent chemical reactions.

Figure 41 depicts the reaction involved with the DTNB capping with thiol containing molecules.

Figure 42 depicts a schematic of the AuNR/Ftn architecture. Excitation of the AuNR at its surface plasmon resonance (SPR) at 810 nm leads to the formation of aqueous Fe^{2+} . CB and VB denote the conduction and valence band, respectively, of the Fe(O)OH semiconductor core. Reduction of the Fe^{3+} in the core Fe(O)OH particle is likely reduced by hot electrons formed during the SPR excitation of the AuNR.

Figure 43, comprising Figure 43A and Figure 43B, depicts preliminary results. Figure 43A depicts a TEM of AuNR (dark rods) conjugated to HFtn (light gray circles). Figure 43B depicts an absorbance spectrum of AuNR prior to (black) and after conjugation to HFtn (red).

Figure 44, comprising Figure 44A through Figure 44D, depicts preliminary results for cell viability studies. Figure 44A depicts PC-3 Cell viability studies after 2- and 24-hr incubation times, columns labeled (L) were exposed to NIR for

24 hours while (D) were kept in the dark. Figure 44B depicts bright field microscopy of cells prior to treatment. Figure 44C depicts after dark incubation with AuNR/HFtn. Figure 44D depicts after exposure to NIR. Contrast in microscopy images was enhanced post capture to increase visibility of cells and the scale bar is 100 μm on all images.

5 Figure 45, comprising Figure 45A through Figure 45D, depicts UV-visible spectrum. Figure 45A depicts a UV-Visible spectrum of DMEM F12 culture medium. Figure 45B depicts a UV-Visible spectrum of DMEM F12 and various AuNR-HFtn conjugates with different [AuNR] ([AuNR]=49.1, 24.5, 12.3 and 4.9 μM). Figure 45C depicts the AuNR absorbance of the AuNR-HFtn ([AuNR]=49.1 and 4.9 μM) at 850 nm
10 in the DMEM F12 medium with varying time intervals ($t=30, 120, 360, 720$ min). Figure 45D depicts the HFtn absorbance of the AuNR-HFtn ([AuNR]=49.1 and 4.9 μM) at 280 nm in the DMEM F12 medium with varying time intervals ($t=30, 120, 360, 720$ min).

 Figure 46, comprising Figure 46A through Figure 46C, depicts UV-Vis and TEM data for AuNR-CTAB. Figure 46A depicts UV-Vis spectrum of the AuNR-
15 CTAB with two distinct LSPR absorbance band positions. The transverse band is shown at 520 nm, and the tunable longitudinal band position at 800 nm. Figure 46B depicts stained TEM image of AuNR-HFtn bioconjugate. As shown in the image the cheerio-like HFtn is attached to the AuNR. The outer protein shell is visible with the inner dense Fh core. Figure 46C depicts unstained TEM of AuNR-HFtn. The black inner core spheres
20 are aligned closely to the AuNR.

 Figure 47, comprising Figure 47A and Figure 47B, depicts hydroxycoumarin and emissive data. Figure 47A depicts the chemical reaction of 7-hydroxycoumarin formation. Figure 47B depicts the fluorescence absorbance of AuNR-HFtn at 455 nm emission under 850 nm NIR exposure (AuNR-HFtn (L)) and in the dark
25 (AuNR-HFtn(D)) 455 nm emission is due to the fluorescence intensity of umbelliferone formed with OH radicals.

 Figure 48, comprising Figure 48A through Figure 48D, depicts PC3 cell viability studies. Figure 48A depicts PC3 cell viability studies carried out with AuNR-HFtn and AuNR-apoHFtn bioconjugate systems kept for 2 h of incubation period with
30 PC3 cancer cell line. Figure 48B depicts PC3 cell viability studies carried out with AuNR-HFtn and AuNR-apoHFtn bioconjugate systems kept for 24 h of incubation period

with PC3 cancer cell line. Figure 48C depicts PC3 cell viability studies carried out with AuNR-HFtn bioconjugate systems kept for 2 h and 24 h of incubation period with PC3 cancer cell line. For each experiment, the reaction was completed for two AuNR concentrations ($[AuNR]=1.39$ and $174.5 \mu M$) under dark and light conditions. Figure 48D depicts PC3 cell viability studies carried out with AuNR-HFtn and AuNR-apoHFtn bioconjugate systems incubated for 24 h and without incubating with any systems as untreated PC3 cancer cells (denoted as 'control') The reaction was completed only for $[AuNR]=174.5 \mu M$ under dark and light conditions.

Figure 49, comprising Figure 49A through Figure 49C, depicts microscope images of PC3 cancer cells. Figure 49A depicts microscopic images of PC3 cancer cells without any bioconjugate systems. Figure 49B depicts PC3 cancer cells incubated for 24 h with AuNR-HFtn. Figure 49C depicts PC3 cancer cells incubated for 24 h with AuNR-HFtn and exposed to NIR light for 24 h.

Figure 50, comprising Figure 50A through Figure 50C, depicts data of AuNS. Figure 50A depicts UV-Vis spectra of AuNS in TX-100 with various longitudinal peak positions. Figure 50B depicts unstained image of AuNS in TX-100. Figure 50C depicts spike lengths of AuNS (Longitudinal band position at $\lambda = 810 \text{ nm}$).

Figure 51, comprising Figure 51A through Figure 51E, depicts unstained TEM images show the diverse spike distribution of AuNS. Figure 51A depicts 3-branched AuNS. Figure 51B depicts 4-branched AuNS. Figure 51C depicts 5-branched AuNS. Figure 51D depicts 6-branched AuNS. Figure 51E depicts 7-branched AuNS.

Figure 52, comprising Figure 52A and Figure 52B, depicts unstained TEM images. Figure 52A depicts AuNS with intermediate branches in the main branches. Figure 52B depicts AuNS with intermediate branches in the main branches.

Figure 53, comprising Figure 53A through Figure 53D, depicts TEM and UV-Visible spectra of bioconjugates. Figure 53A depicts a stained TEM image of the HFtn. Figure 53B depicts stained TEM image of AuNS-HFtn bioconjugates. The AuNS and the HFtn attached to the tips and the other surfaces of the AuNS have been shown. Figure 53C depicts unstained image of AuNS-HFtn. The HFtn attached to the spikes and trapped inside AuNS cages has been clearly shown. Figure 53D depicts UV-Vis spectra

of pure HFtn and AuNS-HFtn in Tris HCl (pH=7.4). ~40 nm redshift was observed when AuNS is transferred to HFtn in Tris HCl from TX-100.

Figure 54, comprising Figure 54A through Figure 54F, depicts unstained TEM images.

5 Figure 55, comprising Figure 55A through 55F, depicts unstained TEM images of AuNS-HFtn bioconjugates. Dark spheres attached to AuNS represent the Fh inner core of the HFtn.

 Figure 56, comprising Figure 56A and Figure 56B, depicts ATR-FTIR spectra. Figure 56A depicts TR-FTIR spectra of the AuNS-HFtn bioconjugates in Tris HCl and TX-100. Figure 56B depicts ATR-FTIR spectra of the AuNS-HFtn
10 bioconjugates in Tris HCl and TX-100 enlarged from 750-1750 cm^{-1} spectral range representing the amide I and II regions of the HFtn protein.

 Figure 57, comprising Figure 57A and Figure 57B, depicts data of NIR exposure. Figure 57A depicts UV-Vis absorbance peak of Fe(II)-Fz complex formation at
15 $\lambda=532$ nm for different 850 nm NIR exposure times and for controls (reaction mixture except for AuNS and HFtn). Figure 57B depicts [Fe(II)] released from the AuNS-HFtn bioconjugate system under 850 nm NIR exposure during 2,5, and 24 h exposure times.

 Figure 58, comprising Figure 58A and Figure 58B, depicts data for L-cysteine. Figure 58A depicts UV-Visible absorbance spectra of L-cysteine with different
20 concentrations in mM. (0.0625, 0.125, 0.250, 0.500, 1.000, and 2.000 mM). Figure 58B depicts a calibration curve generated for the identification of free thiol. The plot illustrates the [L-cysteine] vs. the UV-Visible absorbance at 412 nm.

 Figure 59, comprising Figure 59A through Figure 59E, depicts data for AuNSp. Figure 59A depicts a TEM image of unstained AuNSp-Citrate. Figure 59B
25 depicts an unstained TEM of AuNP in Tris HCl(pH=7.4) Figure 59C depicts an unstained TEM image of AuNSp-HSFtn. Figure 59D depicts a stained image of AuNSp-HSFtn. Figure 59E depicts a stained TEM image of AuNSp-HSFtn with fused AuNSps.

 Figure 60, comprising Figure 60A through Figure 60D, depicts TEM data for AuNSp. Figure 60A depicts a TEM image of unstained chemically synthesized
30 AuNP. Figure 60B depicts surface area distribution of the AuNSp. Figure 60C depicts a

TEM image of unstained AuNSp-HSFtn. Figure 60D depicts surface area of the AuNSp-HSFtn.

Figure 61 depicts hydrodynamic diameters of the AuNSp-Citrate and AuNSp-HSFtn.

5 Figure 62, comprising Figure 62A and Figure 62B, depicts UV-Vis spectra of AuNSp. Figure 62A depicts UV-Visible spectra of chemically synthesized citrate capped AuNSp (AuNSp-Citrate), AuNP in pH=7.4 Tris HCl buffer (AuNSp-Tris HCl), HSFtn in pH=7.4 Tris HCl buffer (HSFtn-Tris HCl) and AuNSp -HSFtn, respectively. Figure 62B depicts the magnified area of the UV-Visible spectra at 400-800 nm. The
10 peak redshift of the AuNSp-Tris HCl and AuNSp-HSFtn. with respect to AuNP-Citrate (520 nm) is 165 nm and 12 nm, respectively.

Figure 63 depicts the chemical reactions and conditions involved with the modification of primary amine in HSFtn with SATA to obtain solvent-exposed thiol groups for the effective AuNP attachment.

15 Figure 64 depicts the chemical reactions and conditions involved with the reaction between thiol-enhanced SATA-HSFtn with Ellman's reagent. The formation of 2-nitro-5-thiobenzoic acid shows visible absorbance at 412 nm ($\epsilon = 13,600 \text{ M}^{-1}\text{cm}^{-1}$).

Figure 65 depicts Cr(VI) reduction from the AuNSp-HSFtn during exposure to SSR with long pass filter $\geq 475 \text{ nm}$ and under dark.

20 Figure 66 depicts the relationship between the amount of SATA conjugation to HSFtn and SATA to HSFtn molar ratios in the reaction mixture [HSFtn] = $1.12 \mu\text{M}$. The number of SATA conjugated per HSFtn reached a plateau at ~ 14 as the [SATA] was increased.

25 Figure 67, comprising Figure 67A and Figure 67B, depicts data for iron release. Figure 67A depicts cumulative Fe(II) release from different reaction mixtures during exposure to SSR with long pass filter $\geq 475 \text{ nm}$. Figure 67B depicts Fe(II) release from the AuNSp-HSFtn during exposure to SSR with long pass filter $\geq 475 \text{ nm}$ with varying the [AuNP] ($= 57.6, 115.2, 230.4$ and $460.8 \mu\text{M}$).

30 Figure 68 depicts UV-Visible absorbance spectra of the AuNP-Citrate and Fh.

Figure 69 depicts UV-Visible absorbance spectra of the AuNP-Citrate and Fh.

Figure 70, comprising Figure 70A through Figure 70F, depicts stained TEM images. Figure 70A depicts stained TEM images of HSFTn. The cheerio-like structures represent the HSFTn protein. It consists of a dark spherical Fh inner core and a white outer shell. Figure 70B depicts a stained TEM image of photochemically grown AuNPs attached on HSFTn. The cheerio-like structures represent the HSFTn protein, and the dark spheres attached to the HSFTn are AuNPs. Figure 70C depicts an unstained TEM image of AuNS-HFTn. The dark spheres attached to the AuNS are the Fh core of HFTn. Figure 70D depicts an unstained TEM image of CTAB capped AuNR (AuNR-CTAB). Figure 70E depicts an unstained TEM image of Fe₂O₃ coated Janus AuNRs. Figure 70F depicts an unstained TEM image of SiO₂ coated AuNRs.

Figure 71 depicts a schematic diagram of a single-beam UV-Vis spectrophotometer.

Figure 72 depicts a schematic diagram of the ICP-OES.

Figure 73 depicts a schematic diagram of DLS.

Figure 74 depicts a schematic diagram representing the ATR process inside the single bounce diamond ATR apparatus used herein.

Figure 75 depicts a schematic representation of Bragg's law conditions.

Figure 76 depicts a schematic diagram of powder x-ray diffraction (PXRD).

DETAILED DESCRIPTION

This invention details mechanistic aspects of the photochemistry of ferritin (Ftn) and bioconjugates that consisted of Ftn linked to gold nanoparticles (AuNPs). The photochemistry repurposed Ftn from its role in biological systems as an iron-sequestration protein to potential applications in photocatalysis and nanobiomedicine.

The present invention harnesses two benign nanoparticulates into one treatment which selectively targets cancer cells and is activated by low flux near infrared light. Activation of the Ferritin-gold conjugates by low flux near infrared light results in release of iron which induces ferroptosis, a process that is effective in killing cancerous

cells. The use of this invention offers a therapeutic strategy that effectively treats at least cancers with overexpressed TrF1 receptors, such as prostate and breast cancers, while minimizing side effects commonly associated with conventional treatments.

5 This invention conjugates human ferritin protein to gold nanorods, to direct the gold nanorods to cancer cells. Once the Ferritin-gold nanorod structure is internalized, near infrared light is exposed to the area inducing photodynamic therapy. Photothermal treatments with gold nanorods cause extreme temperature changes in gold nanorods, effectively killing cells. The invention described herein, ferritin with gold nanorods, harnesses a lower flux light which induces an electron transfer event from the
10 gold nanorod to the sequestered iron within the ferritin protein. This iron is then released from the protein to the intracellular fluid, resulting in a series of cascading reactions which generate reactive oxygen species and induce ferroptosis. While cancer cells are dependent on excess iron compared to healthy cells, this treatment floods the cell with more iron than is sustainable and effectively kills the cell. Results demonstrate from the
15 invention show that when the Ferritin-gold conjugates are exposed to low flux near infrared light, iron is released and reactive oxygen species are generated. Experiments with a prostate cell line (PC-3) established that the treatment is internalized into the cells and is effective, killing >30% of the cells relative to controls.

This invention selectively targets cancer cells relative to healthy cells,
20 decreases the side effects during cancer treatments which plague current technologies like chemotherapy and decreases the near infrared light flux necessary to harness the cancer killing effects of gold particles.

Definitions

25 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

30 As used herein, each of the following terms has the meaning associated with it in this section.

The articles “a” and “an” are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

5 The term “about” will be understood by persons of ordinary skill in the art and will vary to some extent depending on the context in which it is used. As used herein when referring to a measurable value such as an amount, a temporal duration, and the like, the term “about” is meant to encompass variations of $\pm 20\%$ or $\pm 10\%$, more preferably $\pm 5\%$, even more preferably $\pm 1\%$, and still more preferably $\pm 0.1\%$ from the specified value, as such variations are appropriate to perform the disclosed methods.

10 The terms “patient,” “subject,” “individual,” and the like are used interchangeably herein, and refer to any animal, or cells thereof whether in vitro or in situ, amenable to the methods described herein. In certain non-limiting embodiments, the patient, subject or individual is a human.

The term “biological tissue” as used herein refers to a collection of
15 interconnected cells and extracellular matrix that perform a similar function or functions within an organism. Examples of biological tissues include, but are not limited to, connective tissue, muscle tissue, nervous tissue (of the brain, spinal cord, and nerves), epithelial tissue, organ tissue, cancer tissue, and any combination thereof. Connective tissue includes fibrous tissue like fascia, tendon, ligaments, heart valves, bone, and
20 cartilage. Muscle tissue includes skeletal muscle tissue, smooth muscle tissue, such as esophageal, stomach, intestinal, bronchial, uterine, urethral, bladder, and blood vessel tissue, and cardiac muscle tissue. Epithelial tissue includes simple epithelial tissue, such as alveolar epithelial tissue, blood vessel endothelial tissue, and heart mesothelial tissue, and stratified epithelial tissue.

25 The term “abnormal” when used in the context of organisms, tissues, cells or components thereof, refers to those organisms, tissues, cells or components thereof that differ in at least one observable or detectable characteristic (e.g., age, treatment, time of day, etc.) from those organisms, tissues, cells or components thereof that display the “normal” (expected) respective characteristic. Characteristics which are normal or
30 expected for one cell or tissue type, might be abnormal for a different cell or tissue type.

A “disease” is a state of health of an animal wherein the animal cannot

maintain homeostasis, and wherein if the disease is not ameliorated then the animal's health continues to deteriorate.

In contrast, a "disorder" in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal's state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal's state of health.

"Cancer," as used herein, refers to the abnormal growth or division of cells. Generally, the growth and/or life span of a cancer cell exceeds, and is not coordinated with, that of the normal cells and tissues around it. Cancers may be benign, pre-malignant or malignant. Cancer occurs in a variety of cells and tissues, including, but not limited to, the oral cavity (e.g., mouth, tongue, pharynx, etc.), digestive system (e.g., esophagus, stomach, small intestine, colon, rectum, liver, bile duct, gall bladder, pancreas, etc.), respiratory system (e.g., larynx, lung, bronchus, etc.), bones, joints, skin (e.g., basal cell, squamous cell, meningioma, etc.), breast, genital system, (e.g., uterus, ovary, prostate, testis, etc.), urinary system (e.g., bladder, kidney, ureter, etc.), eye, nervous system (e.g., brain, etc.), head and neck, endocrine system (e.g., thyroid, etc.), soft tissues (e.g., muscle, fat, etc.), and hematopoietic system (e.g., lymphoma, myeloma, leukemia, acute lymphocytic leukemia, chronic lymphocytic leukemia, acute myeloid leukemia, chronic myeloid leukemia, etc.).

As used herein, the term "diagnosis" refers to the determination of the presence of a disease or disorder. In some embodiments of the present invention, methods for making a diagnosis are provided which permit determination of the presence of a particular disease or disorder.

A "therapeutic" treatment is a treatment administered to a subject who exhibits signs or symptoms of a disease or disorder, for the purpose of diminishing or eliminating those signs or symptoms.

As used herein, "treating a disease or disorder" means reducing the severity and/or frequency with which a sign or symptom of the disease or disorder is experienced by a subject.

A disease or disorder is "alleviated" if the severity of a sign or symptom of the disease or disorder, the frequency with which such a sign or symptom is experienced

by a subject, or both, is reduced.

The term “derivative” refers to a small molecule that differs in structure from the reference molecule, but retains the essential properties of the reference molecule. A derivative may change its interaction with certain other molecules relative to the reference molecule. A derivative molecule may also include a salt, an adduct, tautomer, isomer, or other variant of the reference molecule.

The term “tautomers” are constitutional isomers of organic compounds that readily interconvert by a chemical process (tautomerization).

The term “isomers” or “stereoisomers” refer to compounds, which have identical chemical constitution, but differ with regard to the arrangement of the atoms or groups in space.

As used herein, the term “nanoparticle” refers to particles having a particle size on the nanometer scale, less than 1 micrometer. For example, the lipid nanoparticle may have a particle size up to about 50 nm. In another example, the lipid nanoparticle may have a particle size up to about 10 nm. In another example, the lipid nanoparticle may have a particle size up to about 6 nm. As used herein, “nanoparticle” refers to a number of nanoparticles, including, but not limited to, nanoclusters, nanocapsules, core-shell nanocapsules, nanovesicles, micelles, block copolymer micelles, lamellae shaped particles, polymersomes, dendrimers, and other nano-size particles of various other small fabrications that are known to those of skill in the art. The shapes and compositions of nanoparticles may be guided during condensation of atoms by selectively favoring growth of particular crystal facets to produce spheres, rods, wires, discs, cages, core-shell structures and many other shapes. The definitions and understandings of the entities falling within the scope of nanocapsule are known to those of skill in the art, and such definitions are incorporated herein by reference and for the purposes of understanding the general nature of the subject matter of the present application. However, the following discussion is useful as a further understanding of some of these terms.

For example, the term “nanocapsule” refers to a vesicular system or hollow particle with a shell surrounding a core-forming space, which, in certain instances, can be used for transporting a payload on a nanoscale level. A nanocapsule may also be a nano-sized version of a container. The payload of the nanocapsule can be,

but is not limited to drugs, medicaments, pharmaceutical compositions, chemical compositions, therapeutic compositions, biological macromolecules, dyes, biological material, immunological compositions, nutritional compositions, vitamins, proteins, nucleic acids, antibodies and vaccines. Various materials may be used for producing such nanocapsules. Nanocapsule refers to a particle having a hollow core that is surrounded by a shell, such that the particle has a size of less than about 1000 nanometers. When a nanocapsule includes a bioactive component, the bioactive component is located in the core that is surrounded by the shell of the nanocapsule.

As used herein, the term “nanocage” refers to a nanocapsule, whereby the shell is not solid, as described for the nanocapsule, but has multiple holes or pores in its shell, thereby making it possible for the payload within the core of the nanocage to come into contact with the surrounding environment. These holes or pores may be regular or irregular in shape and/or spacing on the surface of the particle.

The term “micelle”, a useful article in the employment of a general aspect of the present invention, can generally be thought of as a small – on the order of usually nanometers in diameter – aggregate of amphiphilic linear molecules having a polar, or hydrophilic end and an opposite non-polar, or hydrophobic end. These linear molecules can be comprised of simple molecules, or polymeric chains. A micelle can also be referred to as an aggregate of surfactant molecules dispersed in a liquid colloid. A typical micelle in aqueous solution can form an aggregate with the hydrophilic “head” regions in contact with surrounding solvent, and the sequestering of the hydrophobic tail regions in the micelle center. Other and similar definitions, descriptions and understandings of micelles are also known to those of skill in the art and are incorporated herein by reference.

The term “polymersome” as used herein refers to a vesicle-type which is typically composed of block copolymer amphiphiles, i.e., synthetic amphiphiles that have an amphiphilicity similar to that of lipids. By virtue of their amphiphilic nature (having a more hydrophilic block (head) and a more hydrophobic block (tail)), the block copolymers are capable of self-assembling into a head-to-tail and tail-to-head bilayer structure similar to liposomes. Compared to liposomes, polymersomes have much larger molecular weights, with number average molecular weights typically ranging from 1000

to 100000, preferably of from 2500 to 50000 and more preferably from 5000 to 25000, are typically chemically more stable, less leaky, less prone to interfere with biological membranes, and less dynamic due to a lower critical aggregation concentration. These properties result in less opsonization and longer circulation times. The terms “more hydrophilic” and “more hydrophobic” as used in the context of the amphiphilic nature of the block copolymers are used in a relative sense. i.e., both can be either hydrophilic or hydrophobic, as long as the difference in polarity between the blocks is sufficient for the formation of polymersomes according to the present invention. In view of the creation of a cavity in which water may be incorporated, it is preferred for the more hydrophilic end of the polymer to be hydrophilic per se. Further, in view of the use as a therapeutic agent carrier, it is desired that hydrophobic and/or hydrophilic therapeutic agents can be incorporated into the polymersomes. In one embodiment, the hydrophobic end of the polymer is hydrophobic per se. The amphiphilic nature of the block copolymers is preferably realized in the form of a block copolymer comprising a block made up of more hydrophilic monomeric units (A) and a block made up of more hydrophobic units (B), the block copolymer having the general structure A_nB_m , with n and m being integers of from 5 to 5000, 10 to 1000, or 10 to 500. It is also conceivable that one or more further units or blocks are built-in, e.g., a unit C with an intermediate hydrophilicity so as to yield a terpolymer having the general structure $A_nC_pB_m$, with n and m being as defined above, and p being an integer of from 5 to 5000, preferably 10 to 1000, more preferably 10 to 500. Any of the blocks can itself be a copolymer, i.e., comprise different monomeric units of the required hydrophilic respectively hydrophobic nature. In one embodiment, the blocks themselves are homopolymeric. Any of the blocks, in particular the more hydrophilic block, may bear charges. The number and type of charges may depend on the pH of the environment. Any combination of positive and/or negative charges on any of the blocks is contemplated by the present invention.

The term “liposome” as used herein refers to an artificially prepared vesicle composed of a lipid bilayer. A liposome may be classified as a unilamellar vesicle or a multivesicular vesicle.

The term “lipid bilayer” as used herein refers to a membrane made of two layers of lipid molecules. The lipid bilayer may have a similar thickness as that of a

naturally existing bilayer, such as a cell membrane, a nuclear membrane, and a virus envelope. For example, the lipid bilayer may have a thickness of about 10 nm or less, for example, in a range of about 1 nm to about 9 nm, about 2 nm to about 8 nm, about 2 nm to about 6 nm, about 2 nm to about 4 nm, or about 2.5 nm to about 3.5 nm. The lipid
5 bilayer is a barrier that retains ions, proteins, and other molecules while also preventing them from diffusing into undesirable areas. The “lipid molecules” forming the lipid bilayer may comprise a molecule including a hydrophilic head and a hydrophobic tail. The lipid molecule may comprise from about 14 to about 50 carbon atoms. Examples of the lipid molecules which may form a lipid bilayer include phospholipids, lipids
10 conjugated to polyethylene glycol (PEG), cholesterol, or any combination thereof.

“Dendrimers” have descriptions, definitions, and understandings in the literature. For example, and without limitation and including other and similar definitions, descriptions and understandings in the art, the term dendrimer from the Greek word, “dendron”, for tree, can refer to a synthetic, three-dimensional molecule with
15 branching parts. Descriptions and understandings of dendrimers can be gleaned from Holister et al., Dendrimers, Technology White Papers nr. 6, pub. October 2003 by cientifica, as well as the other literature published by those skilled in the art on dendrimers, all of which are incorporated herein by reference.

The definitions, descriptions, and understandings of “nanovesicle” are
20 well known to those of skill in the art, and are incorporated herein by reference. For example, “nanovesicle” can refer to a variety of small sac, sac-like or globular structures capable of containing fluid or other material therein

“Pharmaceutically acceptable” refers to those properties and/or substances which are acceptable to the subject from a pharmacological/toxicological point of view
25 and to the manufacturing pharmaceutical chemist from a physical/chemical point of view regarding composition, formulation, stability, subject acceptance and bioavailability.

“Pharmaceutically acceptable carrier” refers to a medium that does not interfere with the effectiveness of the biological activity of the active ingredient(s) and is not toxic to the host to which it is administered.

30 As used herein, the term “pharmaceutically acceptable carrier” means a pharmaceutically acceptable material, composition or carrier, such as a liquid or solid

filler, stabilizer, dispersing agent, suspending agent, diluent, excipient, thickening agent, solvent or encapsulating material, involved in carrying or transporting a compound useful within the invention within or to the subject such that it may perform its intended function. Typically, such constructs are carried or transported from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be “acceptable” in the sense of being compatible with the other ingredients of the formulation, including the compound useful within the invention, and not injurious to the subject. Some examples of materials that may serve as pharmaceutically acceptable carriers include: sugars, such as lactose, glucose, and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose, and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil, and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol, and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; surface active agents; alginic acid; pyrogen-free water; isotonic saline; Ringer’s solution; ethyl alcohol; phosphate buffer solutions; and other non-toxic compatible substances employed in pharmaceutical formulations. As used herein, “pharmaceutically acceptable carrier” also includes any and all coatings, antibacterial and antifungal agents, and absorption delaying agents, and the like that are compatible with the activity of the compound useful within the invention, and are physiologically acceptable to the subject. Supplementary active compounds may also be incorporated into the compositions. The “pharmaceutically acceptable carrier” may further include a pharmaceutically acceptable salt of the compound useful within the invention. Other additional ingredients that may be included in the pharmaceutical compositions used in the practice of the invention are known in the art.

The term “pharmaceutically acceptable salt” refers to any pharmaceutically acceptable salt, which upon administration to the subject is capable of providing (directly or indirectly) a compound as described herein. Such salts preferably are acid addition salts with physiologically acceptable organic or inorganic acids. Examples of the acid addition salts include mineral acid addition salts such as, for

example, hydrochloride, hydrobromide, hydroiodide, sulphate, nitrate, phosphate, and organic acid addition salts such as, for example, acetate, trifluoroacetate, maleate, fumarate, citrate, oxalate, succinate, tartrate, malate, mandelate, methane sulphonate, and p-toluenesulphonate. Examples of the alkali addition salts include inorganic salts such as, for example, sodium, potassium, calcium and ammonium salts, and organic alkali salts such as, for example, ethylenediamine, ethanolamine, N,N-dialkylethanolamine, triethanolamine, and basic amino acids salts. However, it will be appreciated that non-pharmaceutically acceptable salts also fall within the scope of the invention since those may be useful in the preparation of pharmaceutically acceptable salts. Procedures for salt formation are conventional in the art.

The term “solvate” in accordance with this invention should be understood as meaning any form of the active compound in accordance with the invention in which the said compound is bonded by a non-covalent bond to another molecule (normally a polar solvent), including especially hydrates and alcoholates.

As used herein, the term “pharmaceutical composition” refers to a mixture of at least one compound of the invention with other chemical components and entities, such as carriers, stabilizers, diluents, dispersing agents, suspending agents, thickening agents, and/or excipients. The pharmaceutical composition facilitates administration of the compound to an organism. Multiple techniques of administering a compound exist in the art including, but not limited to, intravenous, oral, aerosol, parenteral, ophthalmic, pulmonary and topical administration.

As used herein, the terms “therapeutic compound”, “therapeutic agent”, “drug”, “active pharmaceutical”, and “active pharmaceutical ingredient” are used interchangeably to refer to chemical entities that display certain pharmacological effects in a body and are administered for such purpose. Non-limiting examples of therapeutic agents include, but are not limited to, hydrophilic therapeutic agents, hydrophobic therapeutic agents, antibiotics, antibodies, small molecules, anti-cancer agents, chemotherapeutic agents, immunomodulatory agents, RNA molecules, siRNA molecules, DNA molecules, gene editing agents, gene-silencing agents, CRISPR-associated agents (e.g., guide RNA molecules, endonucleases, and variants thereof), analgesics, vaccines, anticonvulsants; anti-diabetic agents, antifungal agents, antineoplastic agents, anti-

parkinsonian agents, anti-rheumatic agents, appetite suppressants, biological response modifiers, cardiovascular agents, central nervous system stimulants, contraceptive agents, dietary supplements, vitamins, minerals, lipids, saccharides, metals, amino acids (and precursors), nucleic acids and precursors, contrast agents, diagnostic agents, dopamine
5 receptor agonists, erectile dysfunction agents, fertility agents, gastrointestinal agents, hormones, immunomodulators, antihypercalcemia agents, mast cell stabilizers, muscle relaxants, nutritional agents, ophthalmic agents, osteoporosis agents, psychotherapeutic agents, parasympathomimetic agents, parasympatholytic agents, respiratory agents, sedative hypnotic agents, skin and mucous membrane agents, smoking cessation agents,
10 steroids, sympatholytic agents, urinary tract agents, uterine relaxants, vaginal agents, vasodilator, anti-hypertensive, hyperthyroids, anti-hyperthyroids, anti-asthmatics and vertigo agents. In certain embodiments, the one or more therapeutic agents are water-soluble, poorly water-soluble drug or a drug with a low, medium or high melting point. The therapeutic agents may be provided with or without a stabilizing salt or salts.

15 Some examples of active ingredients suitable for use in the pharmaceutical formulations and methods of the present invention include: hydrophilic, lipophilic, amphiphilic or hydrophobic, and that can be solubilized, dispersed, or partially solubilized and dispersed, on or about the nanocluster. The active agent-nanocluster combination may be coated further to encapsulate the agent-nanocluster combination and
20 may be directed to a target by functionalizing the nanocluster with, e.g., aptamers and/or antibodies. Alternatively, an active ingredient may also be provided separately from the solid pharmaceutical composition, such as for co-administration. Such active ingredients can be any compound or mixture of compounds having therapeutic or other value when administered to an animal, particularly to a mammal, such as drugs, nutrients,
25 cosmeceuticals, nutraceuticals, diagnostic agents, nutritional agents, and the like. The active agents described herein may be found in their native state, however, they will generally be provided in the form of a salt. The active agents described herein include their isomers, analogs and derivatives.

30 As used herein, the terms “targeting domain”, “targeting moiety”, or “targeting group” are used interchangeably and refer to all molecules capable of specifically binding to a particular target molecule and forming a bound complex as

described above. Thus, the ligand and its corresponding target molecule form a specific binding pair.

The term “antibody”, as used herein, refers to an immunoglobulin molecule which is able to specifically bind to a specific epitope of an antigen. Antibodies
5 can be intact immunoglobulins derived from natural sources, or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. The antibodies in the present invention may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, multiple chain antibodies, intact immunoglobulins, synthetic antibodies, recombinant antibodies, intracellular antibodies (“intrabodies”), Fv,
10 Fab, Fab’, F(ab)2 and F(ab’)2, as well as single chain antibodies (scFv), heavy chain antibodies, such as camelid antibodies, and humanized antibodies (Harlow et al., 1999, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird et al., 1988, Science
15 242:423-426).

The term “antibody fragment” refers to at least one portion of an intact antibody, or recombinant variants thereof, and refers to the antigen binding domain, e.g., an antigenic determining variable region of an intact antibody, that is sufficient to confer recognition and specific binding of the antibody fragment to a target, such as an antigen.

20 By the term “synthetic antibody” as used herein, is meant an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid
25 sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using synthetic DNA or amino acid sequence technology which is available and well known in the art.

A “humanized antibody” refers to a type of engineered antibody having its CDRs derived from a non-human donor immunoglobulin, the remaining
30 immunoglobulin-derived parts of the molecule being derived from one (or more) human immunoglobulin(s). In addition, framework support residues may be altered to preserve

binding affinity (see, e.g., 1989, Queen et al., Proc. Natl. Acad. Sci. USA, 86:10029-10032; 1991, Hodgson et al., Bio/Technology, 9:421). A suitable human acceptor antibody may be one selected from a conventional database, e.g., the KABAT database, Los Alamos database, and Swiss Protein database, by homology to the nucleotide and amino acid sequences of the donor antibody. A human antibody characterized by a homology to the framework regions of the donor antibody (on an amino acid basis) may be suitable to provide a heavy chain constant region and/or a heavy chain variable framework region for insertion of the donor CDRs. A suitable acceptor antibody capable of donating light chain constant or variable framework regions may be selected in a similar manner. It should be noted that the acceptor antibody heavy and light chains are not required to originate from the same acceptor antibody. The prior art describes several ways of producing such humanized antibodies (see for example EP-A-0239400 and EP-A-054951).

A “chimeric antibody” refers to a type of engineered antibody which contains a naturally-occurring variable region (light chain and heavy chains) derived from a donor antibody in association with light and heavy chain constant regions derived from an acceptor antibody.

The term “donor antibody” refers to an antibody (monoclonal, and/or recombinant) which contributes the amino acid sequences of its variable regions, CDRs, or other functional fragments or analogs thereof to a first immunoglobulin partner, so as to provide the altered immunoglobulin coding region and resulting expressed altered antibody with the antigenic specificity and neutralizing activity characteristic of the donor antibody.

The term “acceptor antibody” refers to an antibody (monoclonal and/or recombinant) heterologous to the donor antibody, which contributes all (or any portion, but in some embodiments all) of the amino acid sequences encoding its heavy and/or light chain framework regions and/or its heavy and/or light chain constant regions to the first immunoglobulin partner. In certain embodiments a human antibody is the acceptor antibody.

By the term “recombinant antibody” as used herein, is meant an antibody which is generated using recombinant DNA technology, such as, for example, an

antibody expressed by a bacteriophage or yeast expression system. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid
5 sequence has been obtained using recombinant DNA or amino acid sequence technology which is available and well known in the art.

An “antibody heavy chain,” as used herein, refers to the larger of the two types of polypeptide chains present in antibody molecules in their naturally occurring conformations, and which normally determines the class to which the antibody belongs.

10 An “antibody light chain,” as used herein, refers to the smaller of the two types of polypeptide chains present in antibody molecules in their naturally occurring conformations. Kappa (κ) and lambda (λ) light chains refer to the two major antibody light chain isotypes.

“CDRs” are defined as the complementarity determining region amino
15 acid sequences of an antibody which are the hypervariable regions of immunoglobulin heavy and light chains. See, e.g., Kabat et al., Sequences of Proteins of Immunological Interest, 4th Ed., U.S. Department of Health and Human Services, National Institutes of Health (1987). There are three heavy chain and three light chain CDRs (or CDR regions) in the variable portion of an immunoglobulin. Thus, “CDRs” as used herein refers to all
20 three heavy chain CDRs, or all three light chain CDRs (or both all heavy and all light chain CDRs, if appropriate). The structure and protein folding of the antibody may mean that other residues are considered part of the antigen binding region and would be understood to be so by a skilled person. See for example Chothia et al., (1989) Conformations of immunoglobulin hypervariable regions; Nature 342, p 877-883.

25 The terms “effective amount” and “pharmaceutically effective amount” refer to a sufficient amount of an agent to provide the desired biological result. That result can be reduction and/or alleviation of a sign, symptom, or cause of a disease or disorder, or any other desired alteration of a biological system. An appropriate effective amount in any individual case may be determined by one of ordinary skill in the art using
30 routine experimentation.

A “therapeutically effective amount” refers to that amount which provides

a therapeutic effect for a given condition and administration regimen. In particular, “therapeutically effective amount” means an amount that is effective to prevent, alleviate or ameliorate symptoms of the disease or prolong the survival of the subject being treated, which may be a human or non-human animal. Determination of a therapeutically effective amount is within the skill of the person skilled in the art.

As used herein, the term “stabilizers” refers to either, or both, primary particle and/or secondary stabilizers, which may be polymers or other small molecules. Non-limiting examples of primary particle and/or secondary stabilizers for use with the present invention include, e.g., starch, modified starch, and starch derivatives, gums, including but not limited to polymers, polypeptides, albumin, amino acids, thiols, amines, carboxylic acid and combinations or derivatives thereof. Other examples include xanthan gum, alginic acid, other alginates, benitoniite, veegum, agar, guar, locust bean gum, gum arabic, quince psyllium, flax seed, okra gum, arabinogalactin, pectin, tragacanth, scleroglucan, dextran, amylose, amylopectin, dextrin, etc., cross-linked polyvinylpyrrolidone, ion-exchange resins, potassium polymethacrylate, carrageenan (and derivatives), gum karaya and biosynthetic gum. Other examples of useful primary particle and/or secondary stabilizers include polymers such as: polycarbonates (linear polyesters of carbonic acid); microporous materials (bisphenol, a microporous poly(vinylchloride), micro-porous polyamides, microporous modacrylic copolymers, microporous styrene-acrylic and its copolymers); porous polysulfones, halogenated poly(vinylidene), polychloroethers, acetal polymers, polyesters prepared by esterification of a dicarboxylic acid or anhydride with an alkylene polyol, poly(alkylenesulfides), phenolics, polyesters, asymmetric porous polymers, cross-linked olefin polymers, hydrophilic microporous homopolymers, copolymers or interpolymers having a reduced bulk density, and other similar materials, poly(urethane), cross-linked chain-extended poly(urethane), poly(amides), poly(benzimidazoles), collodion, regenerated proteins, semi-solid cross-linked poly(vinylpyrrolidone).

The terms “coat,” “coated,” or “coating,” as used herein, refer to at least a partial coating of the organic liquid. One hundred percent coverage is not necessarily implied by these terms.

The term “label” when used herein refers to a detectable compound or

composition that is conjugated directly or indirectly to a probe to generate a “labeled” probe. The label may be detectable by itself (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition that is detectable (e.g., avidin-biotin). In some instances, primers can be labeled to detect a PCR product.

As used herein, the term “specific binding” refers to that binding which occurs between such paired species as enzyme/substrate, receptor/agonist, antibody/antigen, and lectin/carbohydrate which may be mediated by covalent or non-covalent interactions or a combination of covalent and non-covalent interactions. When the interaction of the two species produces a non-covalently bound complex, the binding which occurs is typically electrostatic, hydrogen-bonding, or the result of lipophilic interactions. Accordingly, “specific binding” occurs between a paired species where there is interaction between the two which produces a bound complex having the characteristics of an antibody/antigen or enzyme/substrate interaction. In particular, the specific binding is characterized by the binding of one member of a pair to a particular species and to no other species within the family of compounds to which the corresponding member of the binding member belongs. Thus, for example, an antibody preferably binds to a single epitope and to no other epitope within the family of proteins.

The term “specifically binds”, as used herein with respect to an antibody, is meant for an antibody which recognizes a specific antigen, but does not substantially recognize or bind other molecules in a sample. For example, an antibody that specifically binds to an antigen from one species may also bind to that antigen from one or more species. But, such cross-species reactivity does not itself alter the classification of an antibody as specific. In another example, an antibody that specifically binds to an antigen may also bind to different allelic forms of the antigen. However, such cross reactivity does not itself alter the classification of an antibody as specific. In some instances, the terms “specific binding” or “specifically binding,” can be used in reference to the interaction of an antibody, a protein, or a peptide with a second chemical species, to mean that the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the chemical species; for example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally. If an

antibody is specific for epitope “A”, the presence of a molecule containing epitope A (or free, unlabeled A), in a reaction containing labeled “A” and the antibody, will reduce the amount of labeled A bound to the antibody.

As used herein, the terms “peptide”, “polypeptide”, and “protein” are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein’s or peptide’s sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. “Polypeptides” include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or any combination thereof.

As used herein, “polynucleotide” includes cDNA, RNA, DNA/RNA hybrid, antisense RNA, ribozyme, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified to contain non-natural or derivatized, synthetic, or semi-synthetic nucleotide bases. Also, contemplated are alterations of a wild type or synthetic gene, including but not limited to deletion, insertion, substitution of one or more nucleotides, or fusion to other polynucleotide sequences.

“Isolated” means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not “isolated,” but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is “isolated.” An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

The term “nutritional composition” may be a food product intended for

human consumption, for example, a beverage, a drink, a bar, a snack, an ice cream, a dairy product, for example a chilled or a shelf-stable dairy product, a fermented dairy product, a drink, for example a milk-based drink, an infant formula, a growing-up milk, a confectionery product, a chocolate, a cereal product such as a breakfast cereal, a sauce, a soup, an instant drink, a frozen product intended for consumption after heating in a microwave or an oven, a ready-to-eat product, a fast food or a nutritional formula.

“Instructional material”, as that term is used herein, includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of the nucleic acid, peptide, and/or compound of the invention in the kit for identifying, diagnosing or alleviating or treating the various diseases or disorders recited herein. Optionally, or alternately, the instructional material may describe one or more methods of identifying, diagnosing or alleviating the diseases or disorders in a cell or a tissue of a subject. The instructional material of the kit may, for example, be affixed to a container that contains one or more components of the invention or be shipped together with a container that contains the one or more components of the invention. Alternatively, the instructional material may be shipped separately from the container with the intention that the recipient uses the instructional material and the components cooperatively.

Throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

Description

This invention details mechanistic aspects of the photochemistry of ferritin (Ftn) and bioconjugates that comprise Ftn linked to gold nanoparticles (AuNPs). The repurposed Ftn has unique and novel applications in photocatalysis and nanobiomedicine.

5 Compositions and Nanoparticles of Ferritin (Ftn)

 The present invention provides one or more compositions comprising at least one Ferritin-metal nanoparticle based bioconjugate. In one embodiment, the present invention provides one or more compositions, comprising a Ferritin-metal nanoparticle bioconjugate, wherein the Ferritin-metal nanoparticle bioconjugate comprises at least one
10 metal nanoparticle and at least one Ferritin or a variant or a fragment thereof conjugated to the at least one metal nanoparticle; and wherein the Ferritin-metal nanoparticle bioconjugate specifically targets at least one cell expressing a ferritin binding cell receptor. In one embodiment, the ferritin binding cell receptor is transferrin receptor 1 (TfR1).

15 In one embodiment, the Ferritin is selected from the group consisting of a human H-type Ferritin or a variant or a fragment thereof, horse spleen Ferritin or a variant or a fragment thereof, and any combination thereof.

 In one embodiment, the at least one metal nanoparticle is selected from the group consisting of a metal nanorod, metal nanosphere, metal nanostar, and any
20 combination thereof. In one embodiment, the at least one metal nanoparticle is selected from the group consisting of a gold nanoparticle, iron nanoparticle, copper nanoparticle, and any combination thereof. Throughout the present disclosure, any embodiment relating to a metal nanoparticle is applicable to a bioconjugate, and vice-versa.

 In one embodiment, the at least one metal nanoparticle comprises a metal
25 selected from the group consisting of scandium, titanium, vanadium, chromium, manganese, iron, cobalt, nickel, copper, zinc, yttrium, niobium, molybdenum, technetium, ruthenium, rhodium, palladium, silver, cadmium, tantalum, tungsten, osmium, iridium, platinum, and gold. In one embodiment, the at least one metal nanoparticle comprises gold.

In one embodiment, the at least one metal nanoparticle comprises a gold nanoparticle. In one embodiment, the at least one metal nanoparticle comprises a gold nanorod.

5 In one embodiment, the at least one metal nanoparticle has an average width of between 5 nm to 20 nm. In one embodiment, the at least one metal nanoparticle has an average width of between 5 nm to 10 nm. In one embodiment, the at least one metal nanoparticle has an average width of between 5 nm to 15 nm. In one embodiment, the at least one metal nanoparticle has an average width of between 10 nm to 15 nm. In one embodiment, the at least one metal nanoparticle has an average width of between 10
10 nm to 20 nm.

In one embodiment, the at least one metal nanoparticle has an average length of between 20 nm to 80 nm. In one embodiment, the at least one metal nanoparticle has an average length of between 30 nm to 65 nm. In one embodiment, the at least one metal nanoparticle has an average length of between 30 nm to 60 nm. In one
15 embodiment, the at least one metal nanoparticle has an average length of between 30 nm to 55 nm. In one embodiment, the at least one metal nanoparticle has an average length of between 30 nm to 50 nm. In one embodiment, the at least one metal nanoparticle has an average length of between 30 nm to 45 nm. In one embodiment, the at least one metal nanoparticle has an average length of between 30 nm to 40 nm. In one embodiment, the
20 at least one metal nanoparticle has an average length of between 35 nm to 70 nm. In one embodiment, the at least one metal nanoparticle has an average length of between 40 nm to 70 nm. In one embodiment, the at least one metal nanoparticle has an average length of between 45 nm to 70 nm. In one embodiment, the at least one metal nanoparticle has an average length of between 55 nm to 70 nm. In one embodiment, the at least one metal
25 nanoparticle has an average length of between 60 nm to 70 nm. In one embodiment, the at least one metal nanoparticle has an average length of between 65 nm to 70 nm.

In one embodiment, the at least one metal nanoparticle absorbs light having a wavelength of between 600 nm to 1300 nm. In one embodiment, the at least one metal nanoparticle absorbs light having a wavelength of between 600 nm to 1200 nm. In
30 one embodiment, the at least one metal nanoparticle absorbs light having a wavelength of between 600 nm to 1100 nm. In one embodiment, the at least one metal nanoparticle

absorbs light having a wavelength of between 600 nm to 1000 nm. In one embodiment, the at least one metal nanoparticle absorbs light having a wavelength of between 600 nm to 900 nm. In one embodiment, the at least one metal nanoparticle absorbs light having a wavelength of between 600 nm to 800 nm. In one embodiment, the at least one metal nanoparticle absorbs light having a wavelength of between 600 nm to 700 nm. In one embodiment, the at least one metal nanoparticle absorbs light having a wavelength of between 700 nm to 1000 nm. In one embodiment, the at least one metal nanoparticle absorbs light having a wavelength of between 700 nm to 900 nm.

In one embodiment, the at least one metal nanoparticle absorbs light having a wavelength of about 800 nm. In one embodiment, the at least one metal nanoparticle absorbs light having a wavelength of about 850 nm. In one embodiment, the at least one metal nanoparticle absorbs light having a wavelength of about 900 nm. In one embodiment, the at least one metal nanoparticle absorbs light having a wavelength of about 950 nm. In one embodiment, the at least one metal nanoparticle absorbs light having a wavelength of about 1000 nm. In one embodiment, the at least one metal nanoparticle absorbs visible light. In one embodiment, the at least one metal nanoparticle absorbs near infrared light. In one embodiment, the at least one metal nanoparticle absorbs a light having a wavelength which can penetrate human tissue.

In one embodiment, the at least one metal nanoparticle comprises a gold nanostar. In one embodiment, the at least one metal nanoparticle comprises 3 to 7 spikes. In one embodiment, the at least one metal nanoparticle comprises 3 spikes. In one embodiment, the at least one metal nanoparticle comprises 4 spikes. In one embodiment, the at least one metal nanoparticle comprises 5 spikes. In one embodiment, the at least one metal nanoparticle comprises 6 spikes. In one embodiment, the at least one metal nanoparticle comprises 7 spikes. In one embodiment, the at least one metal nanoparticle comprises at least 3 spikes. In one embodiment, the at least one metal nanoparticle comprises more than 7 spikes.

In one embodiment, the spikes have an average length of between 20 nm to 150 nm. In one embodiment, the spikes have an average length of between 25 nm to 150 nm. In one embodiment, the spikes have an average length of between 30 nm to 150 nm. In one embodiment, the spikes have an average length of between 35 nm to 150 nm.

In one embodiment, the spikes have an average length of between 40 nm to 150 nm. In
one embodiment, the spikes have an average length of between 45 nm to 150 nm. In one
embodiment, the spikes have an average length of between 50 nm to 150 nm. In one
embodiment, the spikes have an average length of between 55 nm to 150 nm. In one
5 embodiment, the spikes have an average length of between 60 nm to 150 nm. In one
embodiment, the spikes have an average length of between 65 nm to 150 nm. In one
embodiment, the spikes have an average length of between 70 nm to 150 nm. In one
embodiment, the spikes have an average length of between 75 nm to 150 nm. In one
embodiment, the spikes have an average length of between 80 nm to 150 nm. In one
10 embodiment, the spikes have an average length of between 85 nm to 150 nm. In one
embodiment, the spikes have an average length of between 90 nm to 150 nm. In one
embodiment, the spikes have an average length of between 95 nm to 150 nm. In one
embodiment, the spikes have an average length of between 100 nm to 150 nm. In one
embodiment, the spikes have an average length of between 110 nm to 150 nm. In one
15 embodiment, the spikes have an average length of between 120 nm to 150 nm. In one
embodiment, the spikes have an average length of between 130 nm to 150 nm. In one
embodiment, the spikes have an average length of between 140 nm to 150 nm. In one
embodiment, the spikes have an average length of between 20 nm to 100 nm. In one
embodiment, the spikes have an average length of between 25 nm to 100 nm. In one
20 embodiment, the spikes have an average length of between 30 nm to 100 nm. In one
embodiment, the spikes have an average length of between 35 nm to 100 nm. In one
embodiment, the spikes have an average length of between 40 nm to 100 nm. In one
embodiment, the spikes have an average length of between 45 nm to 100 nm. In one
embodiment, the spikes have an average length of between 50 nm to 100 nm. In one
25 embodiment, the spikes have an average length of between 55 nm to 100 nm. In one
embodiment, the spikes have an average length of between 60 nm to 100 nm. In one
embodiment, the spikes have an average length of between 65 nm to 100 nm. In one
embodiment, the spikes have an average length of between 70 nm to 100 nm. In one
embodiment, the spikes have an average length of between 75 nm to 100 nm. In one
30 embodiment, the spikes have an average length of between 80 nm to 100 nm. In one
embodiment, the spikes have an average length of between 85 nm to 100 nm. In one

embodiment, the spikes have an average length of between 90 nm to 100 nm. In one embodiment, the spikes have an average length of between 95 nm to 100 nm.

In one embodiment, the at least one metal nanoparticle comprises a gold nanosphere. In one embodiment, the at least one metal nanoparticle has a surface area of between 50 nm² to 900 nm². In one embodiment, the at least one metal nanoparticle has a surface area of between 100 nm² to 900 nm². In one embodiment, the at least one metal nanoparticle has a surface area of between 150 nm² to 900 nm². In one embodiment, the at least one metal nanoparticle has a surface area of between 200 nm² to 900 nm². In one embodiment, the at least one metal nanoparticle has a surface area of between 250 nm² to 900 nm². In one embodiment, the at least one metal nanoparticle has a surface area of between 300 nm² to 900 nm². In one embodiment, the at least one metal nanoparticle has a surface area of between 350 nm² to 900 nm². In one embodiment, the at least one metal nanoparticle has a surface area of between 400 nm² to 900 nm². In one embodiment, the at least one metal nanoparticle has a surface area of between 450 nm² to 900 nm². In one embodiment, the at least one metal nanoparticle has a surface area of between 500 nm² to 900 nm². In one embodiment, the at least one metal nanoparticle has a surface area of between 550 nm² to 900 nm². In one embodiment, the at least one metal nanoparticle has a surface area of between 600 nm² to 900 nm². In one embodiment, the at least one metal nanoparticle has a surface area of between 650 nm² to 900 nm². In one embodiment, the at least one metal nanoparticle has a surface area of between 700 nm² to 900 nm². In one embodiment, the at least one metal nanoparticle has a surface area of between 750 nm² to 900 nm². In one embodiment, the at least one metal nanoparticle has a surface area of between 800 nm² to 900 nm². In one embodiment, the at least one metal nanoparticle has a surface area of between 850 nm² to 900 nm².

In one embodiment, the at least one metal nanoparticle is linked to ferritin (Ftn). In one embodiment, the at least one metal nanoparticle is covalently attached to Ftn. In one embodiment, the at least one metal nanoparticle is covalently attached to Ftn through a metal-sulfur bond. In one embodiment, the at least one metal nanoparticle is linked to Ftn using a sulfhydryl functionality. In one embodiment, the at least one metal nanoparticle is linked to Ftn using a surface carboxylate. In one embodiment, the at least

one metal nanoparticle is linked to Ftn using a cysteine residue. In one embodiment, the at least one metal nanoparticle is linked to Ftn using a lysine residue.

In various aspects, the composition comprises: one or more polymers of the present invention and one or more stabilizers. In other aspects, the composition
5 comprises: one or more nanoparticles of the present invention and one or more stabilizers. In various embodiments, the stabilizer to nanoparticle weight ratio is less than 50%. In one embodiment, the stabilizer comprises a biocompatible polymer. Examples of stabilizers include, but are not limited to, biocompatible polymer, a biodegradable polymer, a multifunctional linker, starch, modified starch, and starch derivatives, gums,
10 including but not limited to polymers, polypeptides, albumin, amino acids, thiols, amines, carboxylic acid and combinations or derivatives thereof, citric acid, xanthan gum, alginic acid, other alginates, benitoniite, veegum, agar, guar, locust bean gum, gum arabic, quince psyllium, flax seed, okra gum, arabinoglactin, pectin, tragacanth, scleroglucan, dextran, amylose, amylopectin, dextrin, etc., cross-linked polyvinylpyrrolidone, ion-
15 exchange resins, potassium polymethacrylate, carrageenan (and derivatives), gum karaya and biosynthetic gum, polycarbonates (linear polyesters of carbonic acid); microporous materials (bisphenol, a microporous poly(vinylchloride), micro-porous polyamides, microporous modacrylic copolymers, microporous styrene-acrylic and its copolymers); porous polysulfones, halogenated poly(vinylidene), polychloroethers, acetal polymers,
20 polyesters prepared by esterification of a dicarboxylic acid or anhydride with an alkylene polyol, poly(alkylenesulfides), phenolics, polyesters, asymmetric porous polymers, cross-linked olefin polymers, hydrophilic microporous homopolymers, copolymers or interpolymers having a reduced bulk density, and other similar materials, poly(urethane), cross-linked chain-extended poly(urethane), poly(imides), poly(benzimidazoles),
25 collodion, regenerated proteins, semi-solid cross-linked poly(vinylpyrrolidone), monomeric, dimeric, oligomeric or long-chain, copolymers, block polymers, block copolymers, polymers, PEG, dextran, modified dextran, polyvinyl alcohol, polyvinylpyrrolidone

The compositions are formulated in a pharmaceutically acceptable
30 excipient, such as wetting agents, buffers, disintegrants, binders, fillers, flavoring agents and liquid carrier media such as sterile water, water/ethanol etc. The compositions should

be suitable for administration either by topical administration or injection or inhalation or catheterization or instillation or transdermal introduction into any of the various body cavities including the alimentary canal, the vagina, the rectum, the bladder, the ureter, the urethra, the mouth, etc. For oral administration, the pH of the composition is preferably in the acid range (e.g., 2 to 7) and buffers or pH adjusting agents may be used. The contrast media may be formulated in conventional pharmaceutical administration forms, such as tablets, capsules, powders, solutions, dispersion, syrups, suppositories etc.

The compounds, nanoparticles, or compositions of the invention can be formulated and administered to a subject, as now described. The invention encompasses the preparation and use of pharmaceutical compositions comprising the compound, nanoparticle, and/or compositions of the invention useful for the delivery of a therapeutic agent to a cell. The invention also encompasses the preparation and use of pharmaceutical compositions comprising the compound, nanoparticle, and/or compositions of the invention useful for the treatment of a disease or disorder.

Such a pharmaceutical composition may consist of the active ingredient alone, in a form suitable for administration to a subject, or the pharmaceutical composition may comprise the active ingredient and one or more pharmaceutically acceptable carriers, one or more additional ingredients, or some combination of these. The active ingredient may be present in the pharmaceutical composition in the form of a physiologically acceptable ester or salt, such as in combination with a physiologically acceptable cation or anion, as is well known in the art.

The pharmaceutical compositions useful for practicing the invention may be administered to deliver a dose of between about 0.01 ng/kg/day and 500 mg/kg/day.

In various embodiments, the pharmaceutical compositions useful in the methods of the invention may be administered, by way of example, systemically, parenterally, or topically, such as, in oral formulations, inhaled formulations, including solid or aerosol, and by topical or other similar formulations. In addition to the appropriate therapeutic composition, such pharmaceutical compositions may contain pharmaceutically acceptable carriers and other ingredients known to enhance and facilitate drug administration. Other possible formulations, such as nanoparticles, liposomes, resealed erythrocytes, and immunologically based systems may also be used

to administer an appropriate modulator thereof, according to the methods of the invention.

Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for ethical administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to animals, patients, and subjects of all sorts. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals and patients is well understood, and the ordinarily skilled veterinary pharmacologist can design and perform such modification with merely ordinary, if any, experimentation.

Pharmaceutical compositions that are useful in the methods of the invention may be prepared, packaged, or sold in formulations suitable for oral, rectal, vaginal, parenteral, topical, pulmonary, intranasal, buccal, intravenous, ophthalmic, intrathecal and other known routes of administration. Other contemplated formulations include projected nanoparticles, liposomal preparations, resealed erythrocytes containing the active ingredient, and immunologically-based formulations.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

The relative amounts of the active ingredient, the pharmaceutically acceptable carrier, and any additional ingredients in a pharmaceutical composition of the invention will vary, depending upon the identity, size, and condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, the composition may comprise between 0.1% and 100% (w/w) active ingredient.

In addition to the active ingredient, a pharmaceutical composition of the invention may further comprise one or more additional pharmaceutically active agents.

Controlled- or sustained-release formulations of a pharmaceutical composition of the invention may be made using conventional technology.

A formulation of a pharmaceutical composition of the invention suitable for oral administration may be prepared, packaged, or sold in the form of a discrete solid dose unit including, but not limited to, a tablet, a hard or soft capsule, a cachet, a troche, or a lozenge, each containing a predetermined amount of the active ingredient. Other formulations suitable for oral administration include, but are not limited to, a powdered or granular formulation, an aqueous or oily suspension, an aqueous or oily solution, or an emulsion.

A tablet comprising the active ingredient may, for example, be made by compressing or molding the active ingredient, optionally with one or more additional ingredients. Compressed tablets may be prepared by compressing, in a suitable device, the active ingredient in a free-flowing form such as a powder or granular preparation, optionally mixed with one or more of a binder, a lubricant, an excipient, a surface active agent, and a dispersing agent. Molded tablets may be made by molding, in a suitable device, a mixture of the active ingredient, a pharmaceutically acceptable carrier, and at least sufficient liquid to moisten the mixture. Pharmaceutically acceptable excipients used in the manufacture of tablets include, but are not limited to, inert diluents, granulating and disintegrating agents, binding agents, and lubricating agents. Known dispersing agents include, but are not limited to, potato starch and sodium starch glycolate. Known surface active agents include, but are not limited to, sodium lauryl sulphate. Known diluents include, but are not limited to, calcium carbonate, sodium carbonate, lactose, microcrystalline cellulose, calcium phosphate, calcium hydrogen phosphate, and sodium phosphate. Known granulating and disintegrating agents include, but are not limited to, corn starch and alginic acid. Known binding agents include, but are not limited to, gelatin, acacia, pre-gelatinized maize starch, polyvinylpyrrolidone, and hydroxypropyl methylcellulose. Known lubricating agents include, but are not limited to, magnesium stearate, stearic acid, silica, and talc.

Tablets may be non-coated or they may be coated using known methods to achieve delayed disintegration in the gastrointestinal tract of a subject, thereby providing sustained release and absorption of the active ingredient. By way of example, a material

such as glyceryl monostearate or glyceryl distearate may be used to coat tablets. Further by way of example, tablets may be coated using methods described in U.S. Pat. Nos. 4,256,108; 4,160,452; and 4,265,874 to form osmotically-controlled release tablets. Tablets may further comprise a sweetening agent, a flavoring agent, a coloring agent, a
5 preservative, or combinations of these in order to provide pharmaceutically elegant and palatable preparation.

Hard capsules comprising the active ingredient may be made using a physiologically degradable composition, such as gelatin. Such hard capsules comprise the active ingredient, and may further comprise additional ingredients including, for
10 example, an inert solid diluent such as calcium carbonate, calcium phosphate, or kaolin.

Soft gelatin capsules comprising the active ingredient may be made using a physiologically degradable composition, such as gelatin. Such soft capsules comprise the active ingredient, which may be mixed with water or an oil medium such as peanut oil, liquid paraffin, or olive oil.

15 Liquid formulations of a pharmaceutical composition of the invention which are suitable for oral administration may be prepared, packaged, and sold either in liquid form or in the form of a dry product intended for reconstitution with water or another suitable vehicle prior to use.

Liquid suspensions may be prepared using conventional methods to
20 achieve suspension of the active ingredient in an aqueous or oily vehicle. Aqueous vehicles include, for example, water and isotonic saline. Oily vehicles include, for example, almond oil, oily esters, ethyl alcohol, vegetable oils such as arachis, olive, sesame, or coconut oil, fractionated vegetable oils, and mineral oils such as liquid paraffin. Liquid suspensions may further comprise one or more additional ingredients
25 including, but not limited to, suspending agents, dispersing or wetting agents, emulsifying agents, demulcents, preservatives, buffers, salts, flavorings, coloring agents, and sweetening agents. Oily suspensions may further comprise a thickening agent.

Known suspending agents include, but are not limited to, sorbitol syrup, hydrogenated edible fats, sodium alginate, polyvinylpyrrolidone, gum tragacanth, gum
30 acacia, and cellulose derivatives such as sodium carboxymethylcellulose, methylcellulose, and hydroxypropylmethylcellulose. Known dispersing or wetting agents

include, but are not limited to, naturally-occurring phosphatides such as lecithin, condensation products of an alkylene oxide with a fatty acid, with a long chain aliphatic alcohol, with a partial ester derived from a fatty acid and a hexitol, or with a partial ester derived from a fatty acid and a hexitol anhydride (e.g. polyoxyethylene stearate, 5 heptadecaethyleneoxycetanol, polyoxyethylene sorbitol monooleate, and polyoxyethylene sorbitan monooleate, respectively). Known emulsifying agents include, but are not limited to, lecithin and acacia. Known preservatives include, but are not limited to, methyl, ethyl, or n-propyl-para-hydroxybenzoates, ascorbic acid, and sorbic acid. Known sweetening agents include, for example, glycerol, propylene glycol, sorbitol, 10 sucrose, and saccharin. Known thickening agents for oily suspensions include, for example, beeswax, hard paraffin, and cetyl alcohol.

Liquid solutions of the active ingredient in aqueous or oily solvents may be prepared in substantially the same manner as liquid suspensions, the primary difference being that the active ingredient is dissolved, rather than suspended in the 15 solvent. Liquid solutions of the pharmaceutical composition of the invention may comprise each of the components described with regard to liquid suspensions, it being understood that suspending agents will not necessarily aid dissolution of the active ingredient in the solvent. Aqueous solvents include, for example, water and isotonic saline. Oily solvents include, for example, almond oil, oily esters, ethyl alcohol, 20 vegetable oils such as arachis, olive, sesame, or coconut oil, fractionated vegetable oils, and mineral oils such as liquid paraffin.

Powdered and granular formulations of a pharmaceutical preparation of the invention may be prepared using known methods. Such formulations may be administered directly to a subject, used, for example, to form tablets, to fill capsules, or to 25 prepare an aqueous or oily suspension or solution by addition of an aqueous or oily vehicle thereto. Each of these formulations may further comprise one or more of dispersing or wetting agent, a suspending agent, and a preservative. Additional excipients, such as fillers and sweetening, flavoring, or coloring agents, may also be included in these formulations.

30 A pharmaceutical composition of the invention may also be prepared, packaged, or sold in the form of oil-in-water emulsion or a water-in-oil emulsion. The

oily phase may be a vegetable oil such as olive or arachis oil, a mineral oil such as liquid paraffin, or a combination of these. Such compositions may further comprise one or more emulsifying agents such as naturally occurring gums such as gum acacia or gum tragacanth, naturally-occurring phosphatides such as soybean or lecithin phosphatide, esters or partial esters derived from combinations of fatty acids and hexitol anhydrides such as sorbitan monooleate, and condensation products of such partial esters with ethylene oxide such as polyoxyethylene sorbitan monooleate. These emulsions may also contain additional ingredients including, for example, sweetening or flavoring agents.

Methods for impregnating or coating a material with a chemical composition are known in the art, and include, but are not limited to methods of depositing or binding a chemical composition onto a surface, methods of incorporating a chemical composition into the structure of a material during the synthesis of the material (i.e., such as with a physiologically degradable material), and methods of absorbing an aqueous or oily solution or suspension into an absorbent material, with or without subsequent drying.

As used herein, “parenteral administration” of a pharmaceutical composition includes any route of administration characterized by physical breaching of a tissue of a subject and administration of the pharmaceutical composition through the breach in the tissue. Parenteral administration thus includes, but is not limited to, administration of a pharmaceutical composition by injection of the composition, by application of the composition through a surgical incision, by application of the composition through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration is contemplated to include, but is not limited to, cutaneous, subcutaneous, intraperitoneal, intravenous, intramuscular, intracisternal injection, and kidney dialytic infusion techniques.

Formulations of a pharmaceutical composition suitable for parenteral administration comprise the active ingredient combined with a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. Such formulations may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold in unit dosage form, such as in ampules or in multi-dose containers containing a

preservative. Formulations for parenteral administration include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In one embodiment of a formulation for parenteral administration, the active ingredient is provided in dry (i.e., powder or granular) form for reconstitution with a suitable vehicle (e.g., sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition.

The pharmaceutical compositions may be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution may be formulated according to the known art, and may comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents, wetting agents, or suspending agents described herein. Such sterile injectable formulations may be prepared using a non-toxic parenterally-acceptable diluent or solvent, such as water or 1,3-butane diol, for example. Other acceptable diluents and solvents include, but are not limited to, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono- or di-glycerides. Other parentally-administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form, in a liposomal preparation, or as a component of a biodegradable polymer systems.

Compositions for sustained release or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

Formulations suitable for topical administration include, but are not limited to, liquid or semi-liquid preparations such as liniments, lotions, oil-in-water or water-in-oil emulsions such as creams, ointments or pastes, and solutions or suspensions. Topically-administrable formulations may, for example, comprise from about 1% to about 10% (w/w) active ingredient, although the concentration of the active ingredient may be as high as the solubility limit of the active ingredient in the solvent. Formulations for topical administration may further comprise one or more of the additional ingredients described herein.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for pulmonary administration via the buccal cavity. Such a formulation may comprise dry particles which comprise the active ingredient and which have a diameter in the range from about 0.5 to about 7 nanometers, and preferably from about 1 to about 6 nanometers. Such compositions are conveniently in the form of dry powders for administration using a device comprising a dry powder reservoir to which a stream of propellant may be directed to disperse the powder or using a self-propelling solvent/powder-dispensing container such as a device comprising the active ingredient dissolved or suspended in a low-boiling propellant in a sealed container. Preferably, such powders comprise particles wherein at least 98% of the particles by weight have a diameter greater than 0.5 nanometers and at least 95% of the particles by number have a diameter less than 7 nanometers. More preferably, at least 95% of the particles by weight have a diameter greater than 1 nanometer and at least 90% of the particles by number have a diameter less than 6 nanometers. Dry powder compositions preferably include a solid fine powder diluent such as sugar and are conveniently provided in a unit dose form.

Low boiling propellants generally include liquid propellants having a boiling point of below 65 °F at atmospheric pressure. Generally the propellant may constitute 50 to 99.9% (w/w) of the composition, and the active ingredient may constitute 0.1 to 20% (w/w) of the composition. The propellant may further comprise additional ingredients such as a liquid non-ionic or solid anionic surfactant or a solid diluent (preferably having a particle size of the same order as particles comprising the active ingredient).

Pharmaceutical compositions of the invention formulated for pulmonary delivery may also provide the active ingredient in the form of droplets of a solution or suspension. Such formulations may be prepared, packaged, or sold as aqueous or dilute alcoholic solutions or suspensions, optionally sterile, comprising the active ingredient, and may conveniently be administered using any nebulization or atomization device. Such formulations may further comprise one or more additional ingredients including, but not limited to, a flavoring agent such as saccharin sodium, a volatile oil, a buffering agent, a surface active agent, or a preservative such as methylhydroxybenzoate. The

droplets provided by this route of administration preferably have an average diameter in the range from about 0.1 to about 200 nanometers.

The formulations described herein as being useful for pulmonary delivery are also useful for intranasal delivery of a pharmaceutical composition of the invention.

5 Another formulation suitable for intranasal administration is a coarse powder comprising the active ingredient and having an average particle from about 0.2 to 500 micrometers.

Such a formulation is administered in the manner in which snuff is taken i.e. by rapid inhalation through the nasal passage from a container of the powder held
10 close to the nares. Formulations suitable for nasal administration may, for example, comprise from about as little as 0.1% (w/w) and as much as 100% (w/w) of the active ingredient, and may further comprise one or more of the additional ingredients described herein.

A pharmaceutical composition of the invention may be prepared,
15 packaged, or sold in a formulation suitable for buccal administration. Such formulations may, for example, be in the form of tablets or lozenges made using conventional methods, and may, for example, contain 0.1 to 20% (w/w) active ingredient, the balance comprising an orally dissolvable or degradable composition and, optionally, one or more of the additional ingredients described herein. Alternately, formulations suitable for
20 buccal administration may comprise a powder or an aerosolized or atomized solution or suspension comprising the active ingredient. Such powdered, aerosolized, or aerosolized formulations, when dispersed, preferably have an average particle or droplet size in the range from about 0.1 nanometers to about 2000 micrometers, and may further comprise one or more of the additional ingredients described herein.

25 A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for ophthalmic administration. Such formulations may, for example, be in the form of eye drops including, for example, a 0.1-1.0% (w/w) solution or suspension of the active ingredient in an aqueous or oily liquid carrier. Such drops may further comprise buffering agents, salts, or one or more other of
30 the additional ingredients described herein. Other ophthalmically-administrable

formulations which are useful include those which comprise the active ingredient in microcrystalline form or in a liposomal preparation.

As used herein, “additional ingredients” include, but are not limited to, one or more of the following: excipients; surface active agents; dispersing agents; inert
5 diluents; granulating and disintegrating agents; binding agents; lubricating agents; sweetening agents; flavoring agents; coloring agents; preservatives; physiologically degradable compositions such as gelatin; aqueous vehicles and solvents; oily vehicles and solvents; suspending agents; dispersing or wetting agents; emulsifying agents, demulcents; buffers; salts; thickening agents; fillers; emulsifying agents; antioxidants;
10 antibiotics; antifungal agents; stabilizing agents; and pharmaceutically acceptable polymeric or hydrophobic materials. Other “additional ingredients” which may be included in the pharmaceutical compositions of the invention are known in the art and described, for example in Genaro, ed., 1985, Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa.

15 Typically dosages of the compound of the invention which may be administered to an animal or patient, preferably a human, range in amount from about 0.01 mg to about 100 g per kilogram of body weight of the animal or patient. While the precise dosage administered will vary depending upon any number of factors, including, but not limited to, the type of animal and type of disease state being treated, the age of the
20 animal or patient and the route of administration. Preferably, the dosage of the compound will vary from about 0.01 mg to about 500 mg per kilogram of body weight of the animal or patient. The compound can be administered to an animal or patient as frequently as several times daily, or it can be administered less frequently, such as once a day, once a week, once every two weeks, once a month, or even less frequently, such as once every
25 several months or even once a year or less. The frequency of the dose will be readily apparent to the skilled artisan and will depend upon any number of factors, such as, but not limited to, the type and severity of the disease being treated, the type and age of the animal, patient, etc.

Administration of the compounds of the present invention or the
30 compositions thereof may be continuous or intermittent, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is

therapeutic or prophylactic, and other factors known to skilled practitioners. The administration of the agents of the invention may be essentially continuous over a preselected period of time or may be in a series of spaced doses. Both local and systemic administration is contemplated. The amount administered will vary depending on various factors including, but not limited to, the composition chosen, the particular disease, the weight, the physical condition, and the age of the mammal, and whether prevention or treatment is to be achieved. Such factors can be readily determined by the clinician employing animal models or other test systems which are well known to the art.

One or more suitable unit dosage forms having the therapeutic agent(s) of the invention, which, as discussed below, may optionally be formulated for sustained release, can be administered by a variety of routes including parenteral, including by intravenous and intramuscular routes, as well as by direct injection into the diseased tissue. For example, the therapeutic agent may be directly injected into the muscle. The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods well known to pharmacy. Such methods may include the step of bringing into association the therapeutic agent with liquid carriers, solid matrices, semi-solid carriers, finely divided solid carriers or combinations thereof, and then, if necessary, introducing or shaping the product into the desired delivery system.

When the therapeutic agents of the invention are prepared for administration, they are preferably combined with a pharmaceutically acceptable carrier, diluent or excipient to form a pharmaceutical formulation, or unit dosage form. The total active ingredients in such formulations include from 0.1 to 99.9% by weight of the formulation. A “pharmaceutically acceptable” is a carrier, diluent, excipient, and/or salt that is compatible with the other ingredients of the formulation, and not deleterious to the recipient thereof. The active ingredient for administration may be present as a powder or as granules; as a solution, a suspension or an emulsion.

Pharmaceutical formulations containing the therapeutic agents of the invention can be prepared by procedures known in the art using well known and readily available ingredients. The therapeutic agents of the invention can also be formulated as

solutions appropriate for parenteral administration, for instance by intramuscular, subcutaneous or intravenous routes.

The pharmaceutical formulations of the therapeutic agents of the invention can also take the form of an aqueous or anhydrous solution or dispersion, or alternatively
5 the form of an emulsion or suspension.

Thus, the therapeutic agent may be formulated for parenteral administration (e.g., by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose form in ampules, pre-filled syringes, small volume infusion containers or in multi-dose containers with an added preservative. The active
10 ingredients may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredients may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

15 It will be appreciated that the unit content of active ingredient or ingredients contained in an individual aerosol dose of each dosage form need not in itself constitute an effective amount for treating the particular indication or disease since the necessary effective amount can be reached by administration of a plurality of dosage units. Moreover, the effective amount may be achieved using less than the dose in the
20 dosage form, either individually, or in a series of administrations.

The pharmaceutical formulations of the present invention may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, and salts of the type that are well-known in the art. Specific non-limiting examples of the carriers and/or diluents that are useful in the pharmaceutical
25 formulations of the present invention include water and physiologically acceptable buffered saline solutions, such as phosphate buffered saline solutions pH 7.0-8.0.

In general, water, suitable oil, saline, aqueous dextrose (glucose), and related sugar solutions and glycols such as propylene glycol or polyethylene glycols are suitable carriers for parenteral solutions. Solutions for parenteral administration contain
30 the active ingredient, suitable stabilizing agents and, if necessary, buffer substances. Antioxidizing agents such as sodium bisulfate, sodium sulfite or ascorbic acid, either

alone or combined, are suitable stabilizing agents. Also used are citric acid and its salts and sodium Ethylenediaminetetraacetic acid (EDTA). In addition, parenteral solutions can contain preservatives such as benzalkonium chloride, methyl- or propyl-paraben and chlorobutanol. Suitable pharmaceutical carriers are described in Remington's

5 Pharmaceutical Sciences, a standard reference text in this field.

 The active ingredients of the invention may be formulated to be suspended in a pharmaceutically acceptable composition suitable for use in mammals and in particular, in humans. Such formulations include the use of adjuvants such as muramyl dipeptide derivatives (MDP) or analogs that are described in U.S. Patent Nos. 4,082,735;
10 4,082,736; 4,101,536; 4,185,089; 4,235,771; and 4,406,890. Other adjuvants, which are useful, include alum (Pierce Chemical Co.), lipid A, trehalose dimycolate and dimethyldioctadecylammonium bromide (DDA), Freund's adjuvant, and IL-12. Other components may include a polyoxypropylene-polyoxyethylene block polymer (Pluronic®), a non-ionic surfactant, and a metabolizable oil such as squalene (U.S. Patent
15 No. 4,606,918).

 Additionally, standard pharmaceutical methods can be employed to control the duration of action. These are well known in the art and include control release preparations and can include appropriate macromolecules, for example polymers, polyesters, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methyl
20 cellulose, carboxymethyl cellulose or protamine sulfate. The concentration of macromolecules as well as the methods of incorporation can be adjusted in order to control release. Additionally, the agent can be incorporated into particles of polymeric materials such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylenevinylacetate copolymers. In addition to being incorporated, these agents can also
25 be used to trap the compound in microcapsules.

 Accordingly, the composition of the present invention may be delivered via various routes and to various sites in a mammal body to achieve a particular effect (see, e.g., Rosenfeld et al., 1991; Rosenfeld et al., 1991a; Jaffe et al., supra; Berkner, supra). One skilled in the art will recognize that although more than one route can be
30 used for administration, a particular route can provide a more immediate and more effective reaction than another route. In one embodiment, the composition described

above is administered to the subject by subretinal injection. In other embodiments, the composition is administered by intravitreal injection. Other forms of administration that may be useful in the methods described herein include, but are not limited to, direct delivery to a desired organ (e.g., the eye), oral, inhalation, intranasal, intratracheal, intravenous, intramuscular, subcutaneous, intradermal, and other parental routes of administration. Additionally, routes of administration may be combined, if desired. In another embodiment, route of administration is subretinal injection or intravitreal injection.

The active ingredients of the present invention can be provided in unit dosage form wherein each dosage unit, e.g., a teaspoonful, tablet, solution, or suppository, contains a predetermined amount of the composition, alone or in appropriate combination with other active agents. The term “unit dosage form” as used herein refers to physically discrete units suitable as unitary dosages for human and mammal subjects, each unit containing a predetermined quantity of the compositions of the present invention, alone or in combination with other active agents, calculated in an amount sufficient to produce the desired effect, in association with a pharmaceutically acceptable diluent, carrier, or vehicle, where appropriate. The specifications for the unit dosage forms of the present invention depend on the particular effect to be achieved and the particular pharmacodynamics associated with the composition in the particular host.

These methods described herein are by no means all-inclusive, and further methods to suit the specific application will be apparent to the ordinary skilled artisan. Moreover, the effective amount of the compositions can be further approximated through analogy to compounds known to exert the desired effect.

It will be understood by those of skill in the art that numerous and various modifications can be made without departing from the spirit of the present disclosure. Therefore, it should be clearly understood that the forms disclosed herein are illustrative only and are not intended to limit the scope of the present disclosure.

Method of Treating

In one embodiment, the present invention provides a method of treating a disease or disorder associated with the level or activity of a Tfr1 in a subject in need

thereof, wherein the method comprises administering to the subject a composition of the present invention, and irradiating the subject at a wavelength of between about 200 nm to about 1100 nm.

5 In one embodiment, the disease or disorder is selected from the group consisting of cancer, prostate cancer, breast cancer, Alzheimer's disease, and any combination thereof.

In one embodiment, the present invention provides a method of inhibiting proliferation of at least one cell expressing a Tfr1, wherein the method comprises administering to the subject one or more of the compositions described herein, and
10 irradiating the subject at a wavelength of between 200 nm to 1100 nm.

In one embodiment, the subject is irradiated at a wavelength of 200 nm to 1300 nm. In one embodiment, the subject is irradiated at a wavelength of 250 nm to 1300 nm. In one embodiment, the subject is irradiated at a wavelength of 300 nm to 1300 nm. In one embodiment, the subject is irradiated at a wavelength of 350 nm to 1300 nm. In
15 one embodiment, the subject is irradiated at a wavelength of 400 nm to 1300 nm. In one embodiment, the subject is irradiated at a wavelength of 450 nm to 1300 nm. In one embodiment, the subject is irradiated at a wavelength of 500 nm to 1300 nm. In one embodiment, the subject is irradiated at a wavelength of 550 nm to 1300 nm. In one embodiment, the subject is irradiated at a wavelength of 600 nm to 1300 nm. In one
20 embodiment, the subject is irradiated at a wavelength of 650 nm to 1300 nm. In one embodiment, the subject is irradiated at a wavelength of 700 nm to 1300 nm. In one embodiment, the subject is irradiated at a wavelength of 750 nm to 1300 nm. In one embodiment, the subject is irradiated at a wavelength of 800 nm to 1300 nm. In one embodiment, the subject is irradiated at a wavelength of 850 nm to 1300 nm. In one
25 embodiment, the subject is irradiated at a wavelength of 900 nm to 1300 nm. In one embodiment, the subject is irradiated at a wavelength of 950 nm to 1300 nm. In one embodiment, the subject is irradiated at a wavelength of 1000 nm to 1300 nm. In one embodiment, the subject is irradiated at a wavelength of 1050 nm to 1300 nm.

In one embodiment, the subject is irradiated at a wavelength of 200 nm to
30 1100 nm. In one embodiment, the subject is irradiated at a wavelength of 250 nm to 1100 nm. In one embodiment, the subject is irradiated at a wavelength of 300 nm to 1100 nm.

In one embodiment, the subject is irradiated at a wavelength of 350 nm to 1100 nm. In one embodiment, the subject is irradiated at a wavelength of 400 nm to 1100 nm. In one embodiment, the subject is irradiated at a wavelength of 450 nm to 1100 nm. In one embodiment, the subject is irradiated at a wavelength of 500 nm to 1100 nm. In one
5 embodiment, the subject is irradiated at a wavelength of 550 nm to 1100 nm. In one embodiment, the subject is irradiated at a wavelength of 600 nm to 1100 nm. In one embodiment, the subject is irradiated at a wavelength of 650 nm to 1100 nm. In one embodiment, the subject is irradiated at a wavelength of 700 nm to 1100 nm. In one embodiment, the subject is irradiated at a wavelength of 750 nm to 1100 nm. In one
10 embodiment, the subject is irradiated at a wavelength of 800 nm to 1100 nm. In one embodiment, the subject is irradiated at a wavelength of 850 nm to 1100 nm. In one embodiment, the subject is irradiated at a wavelength of 900 nm to 1100 nm. In one embodiment, the subject is irradiated at a wavelength of 950 nm to 1100 nm. In one embodiment, the subject is irradiated at a wavelength of 1000 nm to 1100 nm. In one
15 embodiment, the subject is irradiated at a wavelength of 1050 nm to 1100 nm.

In one embodiment, the subject is irradiated with visible light. In one embodiment, the subject is irradiated with near-infrared (NIR) light.

In one embodiment, the present invention provides method of inducing ferroptosis of at least one cell expressing a TfR1, wherein the method comprises
20 administering to the subject one or more of the compositions described herein, and irradiating the subject at a wavelength of between about 200 nm to about 1100 nm.

Any therapeutic agent or any combination of therapeutic agents disclosed herein may be administered to a subject to treat a disease or disorder. The therapeutic agents herein can be formulated in any number of ways, often according to various
25 known formulations in the art or as disclosed or referenced herein.

In certain embodiments, the method of treating a disease or disorder comprises a “triggered” functionality. In other words, the system may remain inert in the body until specifically triggered. In some embodiments, the polymer is used advantageously in therapeutic applications such as to first target the polymer to a
30 specified location, and then trigger them into an activated state. Sometimes referred to as a “dual targeted delivery system,” this feature may minimize the side effects of systemic

therapeutic agents. For example, in some embodiments, upon delivering the polymer to a specific cell, a reagent, such as water, proton, acid, or protonated water, may be applied to the cell thereby causing the release of a therapeutic agent from the polymer. In some embodiments, this may provide a clinician the ability to control and visualize drug therapy noninvasively.

In some embodiments, the size of the compound or composition of the present invention allows for passive diffusion into cells. In some embodiments, where the compound or composition is on a smaller scale, the small size allows the compound or compositions to travel almost anywhere in the body where therapy may need to be performed. For example, in some embodiments, the method comprises compounds that act as a hydrolysis triggered therapeutic agent delivery and therapeutic agent release systems.

In one embodiment, the compound or composition undergoes uptake into cells. In one embodiment, the compound or composition undergoes uptake into cancer cells. In one embodiment, the compound or composition undergoes uptake into breast cancer cells. In one embodiment, the compound or composition undergoes uptake into liver cancer cells. In one embodiment, the compound or composition undergoes uptake into macrophage cells. In one embodiment, the compound or composition undergoes uptake into dendritic cells. For example, in one embodiment, the compound or composition can be coated with dextran to target the macrophage cells, since macrophages have dextran receptors. In various embodiments, the method further comprises allowing the compound or composition to accumulate in a region of the biological tissue, wherein the targeting domain facilitated accumulation of the compound, nanoparticle, or composition in the region. In one embodiment, the compound or composition localizes around the exterior of the nucleus.

In various aspects, the compound or composition of the present invention can be used alone or in combination with a therapeutic agent to deliver a therapeutic agent payload to a target cell. Often, the therapeutic agent may be released based on the degradation of, e.g., a controlled release biodegradable matrix and/or polymer.

The preferred dosage of the compound or nanoparticle will vary according to a number of factors, such as the administration route, the age, weight and species of

the subject, but in general containing in the order of from 1 $\mu\text{mol/kg}$ to 1 mmol/kg bodyweight of the compound or nanoparticle.

Administration may be topical, parenteral (e.g., intravenously, intraarterially, intramuscularly, interstitially, subcutaneously, transdermally, or intrasternally), or into an externally voiding body cavity (e.g., the gastrointestinal tract, rectum, bladder, uterus, vagina, nose, ears or lungs), peritoneally, orally, intradermal, ocular, in an animate human or non-human (e.g., mammalian, reptilian or avian) body.

Method of Making

10 The present invention further relates to, in part, a method of making a Ferritin-metal nanoparticle based bioconjugate. In one embodiment, the method of making a Ferritin-metal nanoparticle based bioconjugate comprises the steps of: providing a metal nanoparticle; and dispersing the metal nanoparticle in a solution comprising Ferritin.

15 The metal nanoparticle is any embodiment relating to a metal nanoparticle described elsewhere herein. In one embodiment, the solution comprising Ferritin is buffered.

EXPERIMENTAL EXAMPLES

20 The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

25 Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the present invention and practice the claimed methods. The following working examples therefore are not to be construed as limiting in any way the remainder of the disclosure.

30

Example 1: Photochemistry of Biomineralized Copper Hydr(oxide) Nanoparticles in Horse Spleen Ferritin

The present invention relates to, in part, a mechanistic understanding of the underlying mechanisms involved in the photochemistry of Ferritin (Ftn) with relevance to photocatalysis. In particular, research was designed to determine whether the light-induced bandgap excitation of the semiconductor core of horse-spleen ferritin (HSFtn) resulted in electron transfer from the inorganic core to aqueous redox active reactant via electron transport through the 2 nm thick shell of HSFtn. To investigate this mechanistic pathway, 4-5 nm copper (hydr)oxide nanoparticles were mineralized within the internal volume of HSFtn (CuFtn). It was shown that, unlike the native iron oxyhydroxide-bearing (Ferrihydrite; Fh) Ftn, the visible light photoexcitation of the inorganic core of CuFtn (measured optical bandgap to be 3.65 eV) did not exhibit any release of redox-active metal cation from the HSFtn cage into solution. By photoexciting CuFtn in the presence of aqueous chromate (Cr(VI)) it was shown that the Cr(VI) underwent reduction to Cr(III) in solution. The research strategy eliminated the possibility that metal cations escaping from the HSFtn during photoexcitation could be responsible for Cr(VI) reduction. Hence, the research showed for the first time that electrons resulting from a photoexcited metal oxide core of Ftn could transfer through the protein shell to reduce an aqueous redox active reactant. The research also investigated the wavelength-dependent photochemistry of CuFtn to show that bandgap excitation was indeed responsible for the electrons that transfer across the protein shell.

Ftn Structure

A comprehensive overview of the fundamental aspects encompassing the structure and practical applications of Ftn are presented herein. Ftn is a globular protein which in its natural state, consists of a core material that has been shown to be similar to the iron oxyhydroxide phase, Fh. Ftn can also exist in the absence of the inorganic core material, and in this circumstance, it is referred to as apoferritin (apoFtn). The protein shell of Ftn is composed of 24 subunits (each about 20 kDa) that self-assemble under a wide range of conditions into the globular form. The outer diameter of the protein shell is ~12-13 nm, and the inner diameter of the protein is ~7-8 nm, where the thickness of the

protein shell is 2~2.5 nm (Mohanty, A. et al., 2022, ACS Bio & Med Chem Au, 2, 258). Figure 1A and Figure 1B exhibit the spherical 3D ribbon structure of wild-type HFtn and HSFtn, respectively.

In mammalian Ftn, the conformation of the apoFtn subunits consists of two antiparallel helix pairs (A, B, and C, D), a short alpha helix (E), and a long loop connecting B and C helices (BC loop) assembling into an approximately rhombic dodecahedron shape (space group F432) (Jutz, G. et al., 2015, Chem. Rev., 115, 1653) (Figure 2). The A-helix, C-helix of subunits, and BC loop form the outer surface, while the B-helix and D-helix assemble the inner surface of the Ftn (Harrison, P. M. et al., Ciba Foundation Symposium 51 – Iron Metabolism, pp. 19-40). Ftn is characterized by eight 3-fold channels and six 4-fold channels having pore sizes between ~3 and 5 Å. These different channels bridge the connectivity of the inner cavity to the external environment. In animal Ftn, the 3-fold channels are hydrophilic, and the 4-fold channels are hydrophobic (Lv, C. et al., 2014, Biochemistry, 53, 2232). Therefore, the three-fold channels enable most ions to diffuse into the cavity.

ApoFtn typically contains a high content of non-polar acidic residues such as glutamic acid (Glu) and aspartic acid (Asp), making the inner surface of the Ftn negatively charged (Chang, X. et al., 2023, Annu. Rev. Food Sci. Technol., 14, 113). This situation creates a favorable electrostatic gradient and dynamics through the pores/channels that enable the uptake of metal cations. Amino acid sequences of Ftn exhibit polymorphism within species. In humans, two fundamental subunits, H (~21 kDa) and L (~19 kDa), show different amino acid sequences, numbers, and spatial arrangements. The H and L subunits were originally assigned because of their predominant availability in the heart and liver, respectively. It is more common currently to refer to the same subunits as heavy (H) and light (L), respectively. Apart from the structure, these subunits perform different functions during the iron mineralization process. A dinuclear ferroxidase center comprises two iron-binding sites of conserved amino acid ligands Glu27, Tyrosine(Tyr) Tyr34, Glu62, Histidine(His) His65, Glu107, and Glutamine(Gln) Gln141, located in H subunits that enzymatically oxidize Fe(II) (Liao, X. et al., 2012, Free. Radic. Biol. Med., 53, 375). The Fe(II) transport efficacy, clearing the catalytic site on the H subunits, hydrolysis, nucleation, and core growth is

mediated by the L subunits (Koorts, A. M. et al., 2011, Acute Phase Proteins). In contrast, in the L subunits, some of these residues are substituted with various other residues (E27Y, E62K, H65G). However, both L and H subunits contain a putative nucleation site (at E61, E64, and E67 residues) that can perform slow oxidation of Fe(II).
5 Additional residues at E57 and E60 in L subunits enhance the Fh nucleation process. The differences in H and L subunits facilitate fast or slow iron metabolism, long-term iron storage, and/or quick release depending on the requirements of different tissues.

Various types of mammalian Ftn, such as HFtn (Li, L. et al., 2010, Proc. Natl. Acad. Sci., 107, 3505), HSFtn (Cerkez, E. B. et al., 2019, Environ. Sci. Nano, 6,
10 85), rat liver Ftn, amphibian Ftn having M (Middle) subunits like bullfrog Ftn (Tripathi, J. et al., 1994, Proteins Struct. Funct. Bioinforma., 18, 107), bacterioferritin (Rivera, M., 2017, Acc. Chem. Res., 50, 331), DNA-binding protein from starved cells (DPS) found in prokaryotes (Karas, V. O. et al., 2015, J. Bacteriol., 197, 3206), or mini-Ftn are widely studied to investigate structural and functional properties, 3D spatial arrangement and
15 amino acid sequences.

Fh and Biomineralization of Ftn Core

Fh holds significant importance in the present work as it has been shown to be the core material within laboratory-prepared iron loaded Ftn (Michel, F. M. et al.,
20 2010, Biochimica et Biophysica Acta – General Subjects, pp. 871-885). Fh, is a Fe(III)-oxyhydroxide material and despite extensive research, the structure, composition, and water content of Fh is still under some debate. In general, the intrinsic nanophase Fh, exists in two forms: a less-ordered two-line Fh, and a more-ordered six-line Fh. The number of lines refers to the number of reflections in the XRD pattern for that particular
25 material (Michel, F. M. et al., 2007, Science, 316, 1726). Originally, Drits et al. put forth a multiphase model to elucidate the structure of six-line Fh, which involved a hexagonal lattice with a space group of P31c and exclusively comprised of octahedral iron sites (Drits, V. A. et al., 1993, Clay Miner., 28, 185). More recent studies by Michel et al. contend that the structure of Fh, characterized by domain sizes ranging from 2 to 6 nm,
30 can be effectively represented by a single-phase model (Figure 3A and Figure 3B). This model corresponds to the hexagonal space group P63mc, with a unit cell exhibiting

average dimensions of $\sim a = 5.95 \text{ \AA}$ and $\sim c = 9.06$ The chemical formula for Fh is determined to be $\text{Fe}_{10}\text{O}_{14}(\text{OH})_2 (\text{Fe}_5\text{O}_8\text{H})$. This stoichiometry was later supported by neutron scattering measurements that indicated that Fh was associated with a $\text{Fe}_5\text{O}_8\text{H}$ stoichiometry (Harrington, R. et al., 2011, Environ. Sci. & Technol., 45, 9883). Although, it is mentioned that various other chemical formulas for Fh have been suggested based on the varying proportions of OH and H_2O in relation to Fe, including $2\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}$ (V., C. F., 1972, Proc. Int. Clay Conf. 1972 Madrid., 333), $\text{Fe}_2\text{O}_3 \cdot 3\text{H}_2\text{O}$ (Eggleton, R. A., 1988, Clays Clay Mine., 36, 111), FeOOH (Manceau, A. et al., 2014, Am. Mineral., 99, 102), and $\text{Fe}_{10}\text{O}_{14}(\text{OH})_2$ (Michel, F. M. et al., 2010, Proc. Natl. Acad. Sci., 107, 2787).

Various chemical formulas for Fh have been suggested based on the varying proportions of OH and H_2O in relation to Fe, including $2\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}$, $\text{Fe}_2\text{O}_3 \cdot 3\text{H}_2\text{O}$, FeOOH , and $\text{Fe}_{10}\text{O}_{14}(\text{OH})_2$. Neutron scattering measurements have confirmed that the core of Fh exhibits the $\text{Fe}_5\text{O}_8\text{H}$ stoichiometry, supporting the model proposed by Michel et al., which effectively describes the observed properties of Fh so far. According to the Michel model, the structure of Fh, characterized by domain sizes ranging from 2 to 6 nm, can be effectively represented by a single-phase model. This model corresponds to the hexagonal space group $\text{P6}_3\text{mc}$, with a unit cell exhibiting average dimensions of $\sim a = 5.95 \text{ \AA}$ and $\sim c = 9.06 \text{ \AA}$ (Figure 1). The chemical formula for Fh is determined to be $\text{Fe}_{10}\text{O}_{14}(\text{OH})_2 (\text{Fe}_5\text{O}_8\text{H})$.

By changing the physiological pH of the medium, the solubility of different materials inside Ftn can be adjusted to facilitate aggregation/ precipitation in the protein's nanocavity with controlled size and morphology. Typically, up to a maximum of 4500 iron atoms can be accommodated in Ftn and maintain colloidal stability (Macara, I. G. et al., 1973, Biochem. J., 135, 343). However, the loading capacity can be adjusted within the range according to the solution phase conditions. The diameter of the core inorganic Fh material depends on the loading. When the iron loading per protein cage is lower, the mineral form of Ftn resembles two-line Fh, and as the iron loading per cage increases, it transitions towards six-line Fh (Dehner, C. et al., 2013, JBIC J. Biol. Inorg. Chem., 18, 371).

While laboratory-prepared, iron-loaded Ftn has been addressed, it is mentioned that naturally occurring Ftn was first analyzed by electron microscopic

techniques. (X-ray and neutron scattering (XRNS) and single crystal X-ray diffraction (SCXRD)) (Chasteen, N. D. et al., 1999, J. Struc. Biol., 126, 182; Carta, D. et al., 2017, Mater. Chem. Phys., 113, 349). SCXRD and high-resolution transmission electron spectroscopic (HRTEM) studies indicate the diameter of the core material of wild-type Ftn to be 6-7 nm in diameter. According to Galvez et al., the HS Ftn core contains Fh with a magnetite surface (Galvez, N. et al., 2008, J. Am. Chem. Soc., 130, 8062). These workers used bright-field transmission electron microscopy (BFTEM), small-angle X-ray scattering (SAXS), and X-ray absorption near-edge spectroscopy (XANES) techniques to analyze the inner core material. However, recent research using electron energy loss spectroscopy (EELS) has demonstrated that electron beam damage can cause significant changes in Fh, including the reduction of Fe^{3+} to Fe^{2+} and the migration of iron between octahedral and tetrahedral sites. Hence, multiphase models for Fh structure may be a result of electron beam-induced alterations rather than representing its inherent structure.

Nature's primary purpose for Ftn is to sequester free Fe(II) as a relatively insoluble Fe(III) mineral iron (oxy)hydroxide phase to avoid the Fenton reaction in organisms. Such a reaction, if not controlled in organisms, generates harmful free radicals that can damage genetic material (Orino, K. et al., 2001, Biochem. J., 357, 241). While not of primary biological significance, but very relevant to the present work is that laboratory-based studies have shown that Ftn also has a distinctive affinity for nonferrous metal ions (Cd^{+2} , Zn^{+2} , Cu^{2+} , Co^{+2} , Mn^{+2} , and Mg^{+2}) and oxoanions (PO_4^{3-} , VO_4^{3-} , AsO_4^{3-} and MoO_4^{2-}) (Polanams, J. et al., 2005, Inorg. Chem., 44, 3203). Laboratory-based research has shown that various non-iron bearing cores can be mineralized within the hollow nanocavity under *in vitro* conditions. Figure 3 summarizes some of the biomineralization strategies used to mineralize inorganic metal (oxy)hydroxide in the Ftn cavity. Cobalt was mineralized in Ftn as cobalt oxide (Co(O)OH) by oxidative hydrolysis of Co(II) under H_2O_2 (Douglas, T., 2000, Inorg. Chem., 39, 1828) (Figure 4A), and X-ray absorption spectroscopy (XAS) was used to analyze a metastable oxyhydroxide phase of Manganese mineralized in HS Ftn, which closely corresponds to in γ and β - MnOOH phases (Mackle, P. et al., 1993, J. Am. Chem. Soc., 115, 8471). Meldrum et al. utilized the Ftn cavity to mineralize a ferrimagnetic iron oxide core (Fe_3O_4), also called "magnetoferritin," which could be used for biomedical imaging and cell labeling

(Meldrum, F. C. et al., 1992, Science, 257, 522). Copper (Figure 4B) and CuFe Prussian Blue nanoparticles (Galvez, N. et al., 2005, Dalt. Trans., 15, 2492) (Figure 4D) and precious materials like Platinum (Fan, J. et al., 2011, Biomaterials, 32, 1611), Palladium (Kanbak-Aksu, S. et al., 2012, Chem. Commun., 48, 5745), Iridium (Taher, M. et al., 5 2022, Angew. Chemie Int. Ed., 61, e202116623), Rhodium (Pekarik, V. et al., 2017, Dalt. Trans., 46, 13690) (Figure 4B), and quantum dots like PbS (Hennequin, B. et al., 2008, Adv. Mater., 20, 3592), CdSe (Xing, R. et al., 2009, Dalt. Trans., 10, 1710), CdS (Naito, M. et al., 2010, Angew. Chemie Int. Ed., 49, 7006), and CuS nanoparticles (Wang, Z. Z. Z. et al., 2016, ACS Nano, 10, 3453) were mineralized in the apoFtn cavity for 10 photocatalytic and energy applications. Amendola and coworkers developed Au-Fe alloy nanoparticles for safe clinical translations (Figure 4C). Kasyutich et al. showed that the Ag nanoparticles could be mineralized more successfully in bacterioferritins than other Ftn types due to conserved specific binding and nucleation sites of Ag(I) (Kasyutich, O. et al., 2010, J. Am. Chem. Soc., 132, 3621). Okuda et al. fabricated both nickel or 15 chromium hydroxide nanoparticles inside the Ftn cavity under precisely controlled pH and CO₂ dissolution (Okuda, M. et al., 2003, Biotechnol. Bioeng., 84, 187). Gadolinium ion (Gd³⁺) is a well-known contrast agent, which could shorten the spin-lattice relaxation time (T₁) and enhance the quality of the MRI images. Hence, Gd³⁺ encapsulated Ftn could be a beneficial bioconjugate for MRI scanning (Neburkova, J. et al., 2020, 20 Nanoscale Adv., 2, 5567). Bio-organic compounds like curcumin nanoparticles are well known for their antibacterial properties. Curcumin fabrication in Ftns could be useful for effective antibiotics (Figure 4E) (Chen, L. et al., 2014, Food Res. Int., 62, 1147; Pandolfi, L. et al., 2017, Biomacromolecules, 18, 3318). Moreover, Palladium and silver nanoparticles attached to the outer surface of Ftn have exhibited catalytic and enzymatic 25 properties (Figure 4E) (Peskova, M. et al., 2019, J. Colloid Interface Sci., 537, 20-27).

Applications of Ftn

The mineralized apoFtn cavity can be utilized in vitro with a range of inorganic oxides, sulfides, and zero-valent metal nanoparticles for catalysis. The 30 electronic properties of single-walled carbon nanotubes (SWNTs) mainly depend on tube diameter and chirality. Jeong et al. used cobalt-filled apoFtn as a wet catalyst for

synthesizing SWNTs with a narrow diameter distribution (Jeong, G.-H. et al., 2005, J. Am. Chem. Soc., 127, 8238). A stable iron–sulfur cluster mineralized in apoFtn was utilized as a model photocatalyst for photocatalytic hydrogen generation at neutral pH. The researchers observed a long-distance electron diffusion from photosensitizers to the catalyst through the protein shell (Chen, W. et al., 2019, Chem. Sci., 10, 2179). Fh core in Ftn was used for cytochrome c and viologens reduction (Nikandrov, V. V. et al., 1997, J. Photochem. Photobiol. B Biol., 41, 83), and Au-decorated Ftn was an effective photocatalyst for reducing Cr(VI, which is the toxic form of chromium. Pd and Au metal nanoparticles encapsulated in Ftn have shown catalytic transformations in several organic redox reactions and C–C bond-formation reactions (Uchida, M. et al., 2007, Adv. Mater., 19, 1025).

Different drug molecules can be encapsulated in the nanocavity or attached to modified amino acid residues on the inner or outer surfaces of Ftn using the 'disassembly-reassembly of Ftn' method (Gu, C. et al., 2020, ACS Nano, 14, 17080; Tu, Z. et al., BMEMat, e12022). In addition, the passive diffusion method of small drug molecules (Ex. Cisplatin) through the hydrophilic and hydrophobic channels under controlled temperature conditions has been investigated (Pontillo, N. et al., 2016, Chem. Commun., 52, 4136).

A primary function of the intracellular Ftn is preventing the oxidation of toxic Fe(II) to Fe(III) by storing Fe(II) inside the cavity as an insoluble form of iron, Fh (Harrison, P. M. et al., 1996, Biochim. Biophys. Acta – Bioenerg., 1275, 161). Hence, it supports inhibiting the Fenton reaction from occurring inside the cells. On the contrary, Ftn can be degraded and release Fe(II) to the cells and can be accumulated. The phenomenon is called Ferritinophagy (Sun, K. et al., 2022, Front. Pharmacol., 13). As the metabolism, DNA synthesis, and proliferation rate of cancer cells are generally elevated compared to healthy cells, cancer cells indicate a higher demand for iron (Guo, Q. et al., 2021, Front. Oncol., 11). As Ftn is a source of mineralized iron, naturally, cancer cells prefer Ftn. The excess Fe(II) accumulated can react with H₂O₂ available in the cancer cells and produce reactive oxygen species (ROS) (Nakamura, H. et al., 2021, Cancer Sci., 112, 3945). Hence, there can be an imbalance between ROS production and the available antioxidant (oxidation stress) (Betteridge, D. J., 2000, Metabolism, 49, 3). Ultimately, it

can provoke lipid peroxidation followed by plasma membrane damage to regular cell death (Ferroptosis). The transferrin receptor 1 (TfR1) is a transmembrane glycoprotein receptor that naturally binds with transferrin (Candelaria, P. V. et al., 2021, Front. Immunol., 12), HFtn (Cheng, X. et al., 2020, Cell Death Dis., 11, 92), and HSFn, regulating cellular iron uptake. Subsequent studies have shown that the TfR1 receptor is significantly up-regulated and over-expressed in various cancers such as glioma, breast cancer, leukemia, and ovarian cancer (Lelievre, P. et al., 2020, Cancers, 12, 12). Hence, by targeting the TFR1 receptor, cancer progression (Proliferation, migration, and invasion) could be suppressed.

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Ftn is an iron-sequestration protein found in archaea, bacteria, algae, higher plants, and animal kingdoms. With a hollow spherical structure, apoFtn consists of 24 protein subunits that, when assembled, form a nanocage architecture (~450 kDa) with an internal and external diameter of 7 and 12 nm, respectively (Andrews, S. C. et al., 1992, J. Inorg. Biochem., 47, 161). While wild-type Ftns are designed by nature to sequester iron as a ferric iron oxyhydroxide inner core, similar in structure to the mineral Fh, laboratory-based research has shown that various non-iron bearing cores can also be mineralized within the hollow cage. Among the inorganic compounds that have been grown in the internal volume of Ftn are Co(O)OH (Douglas, T., 2000, Inorg. Chem., 39, 1828), Mn(O)OH (Mackle, P. et al., 1993, J. Am. Chem. Soc., 115, 8471), magnetite (Meldrum, F. C. et al., 1992, Science, 257, 522), Silver (Kasyutich, O. et al., 2010, J. Am. Chem. Soc., 132, 3621), Nickel and Chromium nanoparticles (Okuda, M. et al., 2003, Biotechnol. Bioeng., 84, 187), CuFe Prussian Blue derivative nanoparticles (Galvez, N. et al., 2005, Dalt. Trans., 15, 2492), CaCO₃ (Wan, X. et al., 2022, Ind. & Eng. Chem. Res., 61, 7842), CeO₂ (Liu, X. et al., 2012, Chem. Commun., 48, 3155), precious metals like Platinum (Fan, J. et al., 2011, Biomaterials, 32, 1611), Palladium (Kanbak-Aksu, S. et al., 2012, Chem. Commun., 48, 5745), Rhodium (Pekarik, V. et al., 2017, Dalt. Trans., 46, 13690), and Iridium (Taher, M. et al., 2022, Angew. Chemie Int. End., 61, e202116623), contrast agents like Gadolinium (III) (Neburkova, J. et al., 2020, Nanoscale Adv., 2, 5567), bio-organic compounds like curcumin (Chen, L. et al., 2014, Food Res. Int., 62, 1147), quantum dots like PbS (Hennequin, B. et al., 2008, Adv. Mater., 20, 3592), CdSe

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(Xing, R. et al., 2009, *Dalt. Trans.*, 10, 1710), CdS (Naito, M. et al., 2010, *Angew. Chemie Int. Ed.*, 49, 7006) and CuS nanoparticles (Wang, Z. Z. Z. et al., 2016, *ACS Nano*, 10, 3453). Overall, Ftn is a versatile nanoreactor with a fixed volume for biomimetic inorganic material synthesis to generate unique structures for applications in
5 medicine (Xue, L. et al., 2019, *Int. J. Mol. Sci.*, 20, 2426; Babincova, M. et al., 2000, *Med. Hypotheses*, 54, 177), environmental remediation (Nikandrov, V. V. et al., 1997, *J. Photochem. Photobiol. B Biol.*, 41, 83; Michel, F. M. et al., 2010, *Biochimica et Biophysica Acta - General Subjects*, 871; Jacobs, J. F. et al., 2010, *Biotechnol. Bioeng.*, 105, 918), water splitting (Abdi, Z. et al., 2019, *Sci. Rep.*, 9, 1), imaging and drug
10 delivery (Deans, A. E. et al., 2006, *Magn. Reson. Med.*, 56, 51; Fan, K. et al., 2012, *Nat. Nanotechnol.*, 7, 459), and nanodevice production (Yamashita, I. et al., 2010, *Biochimica et Biophysica Acta - General Subjects*, 846).

Prior research has shown that Ftn containing the native iron oxyhydroxide, Fh core behaves as a small band gap semiconductor, and in the presence of hole
15 scavengers, aqueous Cr(VI) can be reduced by Ftn to Cr(III) when the system is exposed to wavelengths of light shorter than 475 nm (Cerkez, E. B. et al., 2019, *Environ. Sci. Nano*, 6, 85). A fundamental question arising from this research is whether the mechanism of Cr(VI) reduction proceeds via a homogenous or heterogenous pathway. Specifically, in a homogenous scenario, photo-produced ferrous iron (Fe(II)) would leave
20 the Ftn cage and reduce Cr(VI) in solution, while in a heterogenous pathway, photoexcited conduction band electrons could traverse the protein shell and reduce Cr(VI) on the outer surface of the protein (Kim, I. et al., 2002, *Chem. Mater.*, 14, 4874; Bou-Abdallah, F., 2010, *Biochimica et Biophysica Acta - General Subjects*, 719; Behera, R. K., 2014, *Proc. Natl. Acad. Sci. U. S. A.*, 111, 7925). The possibility of the latter
25 channel, which includes the transfer of electrons through the 2 nm protein cage, is a particularly interesting possibility. Unfortunately, prior research has been somewhat inconclusive on whether such an electron transfer process can occur due to the inability to isolate this process from the homogeneous redox chemistry of aqueous Fe(II). For
30 example, one study concluded that the excited electrons generated by bandgap excitation could not cross the Ftn outer shell by using a large polyoxomolybdate (POM) complex, a moiety that could not enter the protein due to the mismatch of size to the protein pore

(Saenz, N. et al., 2016, Inorg. Chem., 55, 6047). The work observed that the reduction of POM only occurred when Fe(II) ions were available in the solution. However, prior studies show evidence that electron transfer can occur through the Ftn protein shell (Keyes, J. D. et al., 2011, J. Nanoparticle Res., 13, 2563). When Ftn was attached to plasmonic AuNSps, photoreduction of Cr(VI) was observed below the threshold bandgap energy of the Fh core. Control reactions ruled out the possibility of reduction directly on the gold surface, and the observed concentration of aqueous Fe(II) was below the stoichiometric value necessary for the quantity of Cr(VI) reduced. The evidence suggested that Cr(VI) must either be reduced at the protein surface or within the protein cage.

In this contribution, copper hydroxide $\text{Cu}(\text{OH})_2$, a small bandgap semiconductor, was mineralized within the protein cage (i.e., CuFtn). The photochemistry of the system was investigated to isolate and understand the transfer of excited conduction band electrons across the protein cage. The use of Cr(VI) as the probe oxidant allowed a convenient comparison to prior iron-based work. Importantly, the use of CuFtn instead of FeFtn (with a Fh core) eliminated the potential for the photoproduction of an aqueous reductant (i.e., Fe(II) in the Ftn circumstance), as Cu(II) would be the only potential stable aqueous species (Cu(I) would be expected to be less stable) and would not undergo redox chemistry with Cr(VI). Results, however, presented in this contribution will show that $\text{Cu}(\text{OH})_2$ mineralized within the CuFtn does not release Cu(II) into solution when exposed to simulated solar radiation (SSR) and a hole scavenger. In addition, and critical to the focus of this contribution, it is shown that the photoexcitation of the mineralized $\text{Cu}(\text{OH})_2$ in the presence of aqueous Cr(VI) resulted in the reduction of Cr(VI) to Cr(III), suggesting that the transfer of electrons through the 2 nm protein cage is a viable mechanistic pathway.

Chemicals and Mineralization of CuFtn

All chemicals were used as received. Pure analytical grade solid chemicals, solvents, and Deionized (DI) water ($18 \text{ M}\Omega \text{ cm}^{-1}$) were used in all solution preparations. ApoFtn from equine spleen (25 mg mL^{-1} , $443000 \text{ g mol}^{-1}$), sodium hydroxide (NaOH, ACS reagent, Pellets, $\geq 97.0\%$), copper(II) sulfate pentahydrate

($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, ACS reagent, $\geq 98.0\%$), phosphotungstic acid hydrate ($\text{H}_3[\text{P}(\text{W}_3\text{O}_{10})_4] \cdot x\text{H}_2\text{O}$, 99.995%), 4-(2-Pyridylazo)resorcinol monosodium salt hydrate (PAR, powder, 96%), Trisma base (primary standard and buffer, $\geq 99.9\%$), sodium acetate (CH_3COONa , anhydrous, molecular biology grade $\geq 99\%$) were purchased from Sigma-Aldrich. Sodium dichromate ($\text{Na}_2\text{Cr}_2\text{O}_7$, crystalline/certified ACS Plus, inclusive between 99.5%), Sodium potassium tartrate ($\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$, Certified ACS), glacial acetic acid (CH_3COOH , Certified ACS plus, Assay: $\geq 99.7\%$ w/w), hydrochloric acid (HCl , Certified ACS plus, 36.5 to 38.0%), were purchased from Fisher Chemical™. HEPES buffer (ultra-pure) was purchased from American bio.

10 The preparation of CuFtn was adapted from prior methods with slight modifications (Xu, N. et al., 2020, ACS Omega, 5, 10415; Ceolin, M. et al., 2008, Eur. J. Inorg. Chem., 2008, 795). Briefly, 200 μL of HSFtn solution (25 mg mL^{-1}) was added to 10.0 mL of acetate ($\text{CH}_3\text{COOH}/\text{CH}_3\text{COONa}$) buffer (pH 5.5, 0.1 M) and was mixed at room temperature. To purify the HSFtn, 10 mL of HSFtn in acetate (pH 5.5, 0.1 M) was
15 mixed with 10 mM EDTA and dialyzed three times using a molecular porous dialysis membrane (MWCO: 12-14 kDa) with 60 min time slots. Then the protein solution was centrifuged (14,000 rpm, 15 min) using ultra-0.5 mL protein concentrator (Pierce™ Protein Concentrators-Collector kit, PES, 30K MWCO, Thermo-Fisher) and the concentrated protein was resuspended in 10.0mL of acetate buffer (0.1 M, pH 5.5). Then,
20 aliquots of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (12 mM, 2.5 mL) were added to the apoFtn solution over a 50 min period at 10 min intervals. During the initial Cu(II) incubation period, the pH of the system was maintained at pH=5.0 to ensure the maximum Cu(II) binding to the HSFtn (Pead, S. et al., 1995, J. Inorg. Biochem., 59, 15). The solution mixture was slowly stirred throughout the additions. The Cu(II)-apoFtn solution was left standing overnight for
25 incubation after the final $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ addition, which resulted in a final Cu(II) concentration of 2.4 mM. At this point, a pale sky-blue homogenous solution was observed without any visible precipitation. Excess Cu(II) was removed by centrifugation (14,000 rpm, 15 min) using ultra-0.5 mL protein concentrator (Pierce™ Protein Concentrators-Collector kit, PES, 30K MWCO, Thermo-Fisher) and the concentrated
30 protein was resuspended in 12.5 mL of acetate buffer (0.1 M, pH 5.5) by sonication. The resulting solution was dialyzed three times (1.5 h per dialysis) to remove trace Cu(II)

(Molecular porous dialysis membrane, MWCO: 12-14 kDa) in fresh acetate buffer (pH 5.5, 0.1 M, 1000 mL). After the dialysis, the pale blue homogeneous solution became colorless. Next, the pH of the solution was carefully adjusted to 8.2 with 0.01 M NaOH while stirring. The solution acquired a pale blue color consistent with Cu(OH)₂ formed inside the apoFtn cavity (Sokolowska, M. et al., 2005, J. Inorg. Biochem., 99, 1653). The resultant mixture was dialyzed against DI water and heated to 60 °C for 20 min, and then cooled with ice. The CuFtn-containing solution was centrifuged (14,000 rpm, 15 min) by ultra-0.5 mL protein concentrator (Pierce™ Protein Concentrators-Collector kit, PES, 30K MWCO, Thermo-Fisher) and the concentrated protein was resuspended in 10.0 mL of Tris HCl (pH 8.2, 0.1 M). The CuFtn solution was further dialyzed 3 times with Tris HCl (pH 8.2, 0.1 M) for 6 h for remove excess chemicals and then kept at 4 °C (Figure 5)

A reference Cu(OH)₂ solid was also synthesized to augment the CuFtn studies. The reference material was synthesized in the absence of apoFtn (CuCtrl), but the solution conditions (e.g., pH and temperature) were kept similar to the conditions used to synthesize CuFtn.

Characterization

The concentration of CuFtn was quantitatively determined by the Precision Red advanced protein assay (Kikuchi, D. S. et al., 2019, J. Neuroinflammation, 16, 241; Bordeleau, F. et al., 2020, J. Cell Sci., 133) and the A280 method (Desjardins, P. et al., 2010, J. Vis. Exp., 33, 33; Chopra, A. et al., 2019, Sci. Rep., 9, 1) using a 150 W UV-Visible spectrophotometer (Thermo-Fisher Scientific, UL 61010-1). The Precision Red advanced protein assay was performed according to the manufacturer's instructions. For highly concentrated protein samples (> 10 mg mL⁻¹), 200 µL of the assay was added to 1800 µL of protein, thoroughly mixed by inverting, and then the sample was incubated for 1 min at room temperature. The protein concentration was calculated by measuring the absorbance at 600 nm, where an absorbance of 1.00 equals the presence of 100 mg/mL of protein. The A280 method uses the unique molar absorptivity of a protein at 280 nm, assigned to the aromatic amino acids, to determine the concentration of the protein present. For Ftn, the molar absorptivity (Barnes, C. M. et al., 2003, JBIC J. Biol. Inorg. Chem., 8, 195) at 280 nm is $4.8 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

TEM and SEAD analysis were carried out using a JOEL JEM-1400 microscope operating at 120 kV. All Ftn samples were prepared on copper mesh-supported holey carbon TEM grids (Ted Pella) and allowed to dry as a thin film. Some samples were negatively stained with a 2% PTA solution at pH 7. For these samples, a 10 μ L aliquot of protein solution was deposited on the grid and allowed to sit for 10 min. Excess liquid was removed by filter paper, and the grid was then rinsed with 10 μ L of water. Finally, 2 μ L of the PTA solution was deposited and allowed to sit for 2 min, and then the excess PTA solution was removed by filter paper. Individual core particles were measured from TEM images using the freeware, ImageJ (NIH, USA).

PXRD measurements were carried out using a Bruker D8 advance A25 powder diffractometer with CuK α 1 radiation and a Johansson monochromator. Reference samples that were prepared without protein or were bought commercially were finely ground and suspended in isopropyl alcohol. Prior to the analysis, samples were drop cast and concentrated on a Si low background sample holder (BrukerAXS) and dried.

A Malvern Zetasizer particle size analyzer was used to measure the hydrodynamic particle size distribution of CuFtn before and after photochemical reactions. In general, 1 mL of 0.25 μ M solutions were placed inside a disposable plastic cell (DTS0012), for DLS analysis.

ICP-OES (Thermo-Scientific, iCAP 7400-ASX520) was used to quantify copper (324.754 nm, axial mode) and chromium (360.533 nm, axial mode) solution concentrations. Samples for analysis were diluted to 10 mL and acidified by 2% by volume with nitric acid (HNO₃).

Photochemical Batch Reactions

All photochemical experiments were conducted in a 1 cm² quartz cuvette (Thor Labs) using a 200 W Xe lamp (Oriel Instruments, 66002/58) as the SSR radiation source. An absorbance spectrum was acquired for each photochemical reaction using a UV-vis spectrophotometer at t=0 before the sample was exposed to SSR. At each time point of interest (t=5, 10, 15... min), the cuvette, which contained the reaction solution, was removed from the SSR light path, and an absorbance spectrum was obtained.

Samples were individually illuminated for a total of 120 min for all photochemical experiments. All reactions contained 3.2 mL total volume.

Copper release studies were conducted in a solution containing CuFtn (0.8 μ M apoFtn, 45 μ M Cu(II)), 32 mM sodium potassium tartrate, and 80 mM PAR in 0.1 M Tris buffer (pH 8.2). Cr(VI) reduction experiments were conducted under the same conditions but replaced PAR with 0.200 mM potassium dichromate ($K_2Cr_2O_7$). Any volume changes were compensated by adding Tris HCl buffer (pH 8.2). Unless specifically stated, all control studies were conducted under the same conditions for both apoFtn and CuFtn solutions. Quantitation of Cu(II)-PAR and Cr(VI) were determined by UV-Vis spectroscopy at 510 nm (Ramadan, A. A. et al., 2009, Asian J. Chem., 21, 7367) and charge-transfer band at 372 nm (Hoshi, S. et al., 1998, Talanta, 47, 659), respectively.

Results and Discussion

The structure and the morphology of CuFtn was analyzed by TEM obtained from both stained (Figure 6A) and unstained (Figure 6B) samples, which emphasized the structure of the Cu-bearing core and protein cage, respectively. TEM grids containing CuFtn were briefly exposed to 2% PTA to obtain negatively stained images. An image of a negatively stained CuFtn (Figure 5A) displays the protein shells as white hollow circles against a dark field, which were ~ 10 -12 nm in diameter. Inspection of the image also indicates that there was no disruption of the protein cages during copper mineralization. Figure 6B exclusively shows the Cu-bearing cores within Ftn as dark features. The lack of aggregation of these particles is consistent with the inorganic material being confined within the Ftn cages, as protein-free $Cu(OH)_2$ nanoparticles would likely aggregate during drying on the TEM grid (Figure 7). Particle size analysis (TEM images and ImageJ) of the core particles yielded an average diameter of 3.9 ± 0.8 nm (Figure 5C). SAED of the CuFtn particles yielded diffuse rings (Figure 5D), likely indicating that the mineralized $Cu(OH)_2$ core lacked a significant degree of crystallinity.

DLS was used to analyze the hydrodynamic diameter (HD) size distribution of the apoFtn and CuFtn, assuming they are approximately spherical before and after mineralization. Consistent with the microscopy analysis, the DLS of CuFtn

exhibited a primary peak at ~13 nm, slightly larger than the apoFtn cages (Figure 8). This result is consistent with the Cu(OH)₂ nanoparticles being encapsulated within the Ftn since Ftn with a mineralized core would be expected to result in only small changes in HD (Yu, J. et al., 2019, RSC Adv., 9, 39381). Also, the absence of a DLS peak at ~5-10 nm supports the notion that there are no free, unaggregated Cu(OH)₂ particles outside of the Ftn interior volume.

PXRD analysis was performed on samples including CuCtrl, Commercial Cu(OH)₂, and Commercial CuO (Figure 9). Upon examining the PXRD pattern of the commercial Cu(OH)₂ sample using the peak positions from the JCPDS database for Cu(OH)₂ (JCPDS 13-420) (Wang, L. et al., 2014, Nano Res., 7, 199), it was observed that there were additional peak positions present, corresponding to CuO (JCPDS 54-937). On the other hand, the PXRD pattern of the commercial CuO sample exhibited an exact match with the data provided by the JCPDS database (Li, Z. et al., 2015, Sci. Rep., 5, 10617; Vinothkumar, P. et al., 2019, J. Mater. Sci. Mater. Electron., 30, 6249). The PXRD spectrum of CuCtrl exhibits prominent reflections at (020), (021), (111), (022), and (200), which can be attributed to Cu(OH)₂. Additionally, there are noticeable reflections at (-110), (-112), and (-202), which are indicative of CuO. Based on the PXRD analysis, it can be concluded that CuCtrl predominantly consists of Cu(OH)₂, accompanied by a certain presence of CuO, which is in agreement with the prior literature.

The optical bandgap (E_g) of the Cu(OH)₂ core of CuFtn was determined using the Tauc analysis (Nasiri, A. et al., 2017, J. Mater. Sci. Mater. Electron., 28, 9096). Central to the Tauc analysis is that the absorbance, α , of the inorganic core, can be related to the optical band gap by the following equation (1.1):

$$\alpha = \frac{\beta(h\nu - E_g)^n}{h\nu} \quad (1.1)$$

where β is the band tailing parameter, h is Planck's constant, ν is the frequency of light, and E_g is the energy of the average optical bandgap. The final constant, n , can be either 2 or $\frac{1}{2}$ for allowed electronic transitions associated with indirect or direct band gaps, respectively. The energy intercept of the plot of $(ah\nu)^2$ versus $h\nu$ yields E_g for a direct transition (Mokhtar, H. H. et al., 2015, RSC Adv., 5, 93438). Based on the tauc analysis, the average optical direct bandgap of the core in CuFtn was estimated to be 3.65 eV, and

Cu(OH)₂ particles, formed in the absence of Ftn (CuCtrl), exhibited a smaller value of 3.21 eV. Prior research demonstrates that the optical bandgap values are in a comparable range for Cu(OH)₂ bulk nanoparticles (3.08 eV) (Gurav, K. V. et al., 2013, J. Alloys Compd., 573, 27). The increase in the band gap of the mineralized Cu(OH)₂ core (Figure 10) may be attributed to quantum confinement effects as the size of the materials becomes proximate to the de Broglie wavelength of an electron (Jia, W. et al., 2009, J. Appl. Phys., 105, 64917).

Experiments were carried out to determine whether the photoexcitation of the Cu(OH)₂ core of Ftn with SSR in the presence of the hole scavenger, tartrate, led to photocorrosion of the core material and the release of stable Cu species from the Ftn cage. It is mentioned that under similar conditions, Ftn with an Fh core releases Fe(II) into the solution, which can be detected in situ by the Fe(II)-specific chelating agent, Ferrozine (Fz). To assess the possibility of Cu release from the Ftn cage during photoexcitation, a Cu(II) chelator, PAR, was used. In the absence of Cu(II), PAR shows a distinct absorbance at 414 nm but once complexed with Cu(II), PAR exhibits a strong absorbance at 510 nm (Figure 11A and Figure 11B) (Tabata, M. 1976, J. Inorg. Nucl. Chem., 38, 1529). The absorbance spectra of the PAR-Cu(II) complex as a function of Cu(II) concentration (Figure 12A) shows that as the concentration of Cu(II) increased to a max of 100 mM, the absorption feature at 510 nm steadily increases. In contrast, the 414 nm feature, associated with uncomplexed PAR, decreases to zero. The illumination of CuFtn in the presence of the hole scavenger tartrate resulted in an insignificant amount of Cu(II) release during the exposure to SSR for 120 min (Figure 13A). Based on the change in the 510 nm absorbance, the concentration of Cu(II) released from CuFtn after 120 min was <1 μM. This amount of Cu(II) represents <2% of the total Cu within CuFtn. In contrast, previous work under similar conditions demonstrated that >82% of Fe available in the core of Ftn was released as Fe(II).

Exposure of a PAR complex to SSR resulted in an insignificant change in the intensity of the 414 nm feature until 30-40 min. For 120 min longer exposure, the PAR does show slight degradation (~24.3 μM), indicating that the complex is slightly unstable for more prolonged exposure to SSR (t ≥ 60 min). A control reaction carried out with the same conditions, PAR (80 mM) in Tris-HCl (0.1 M, pH 8.2), and sodium

potassium tartrate (32 mM), showed the same PAR degradation ($\sim 23.6 \mu\text{M}$) at 414 nm (Figure 12B). According to the UV-Vis absorbance spectrum of PAR and PAR-Cu(II) complex shown in Figure 12A, if the $24.3 \mu\text{M}$ [PAR] drop was due to Cu(II) chelation, the chelated [Cu(II)] should have been between $10 \mu\text{M}$ - $50 \mu\text{M}$ (Calculated [Cu(II)] in the main reaction was $< 1 \mu\text{M}$). Hence, it confirmed the [PAR] drop during a SSR long exposure was due to a slight degradation of the PAR and not due to the Cu(II) chelation. Thus, by externally replenishing the degraded PAR, any loss or degradation of the chelator during the SSR exposure was effectively compensated, enabling reliable observations and measurements throughout the study. Under dark conditions, a solution of CuFtn ($45 \mu\text{M}$), sodium potassium tartrate (32 mM), PAR (80 mM), and Tris-HCl (0.1 M, pH 8.2) showed no change in absorbance at 414 or 510 nm, the uncomplexed and complexed moieties respectively, after 120 min (Figure 13B). These results indicate that PAR does not induce the release of Cu from the protein core or that PAR can enter into the protein cage.

The exposure of a solution containing CuFtn ($45 \mu\text{M}$) in Tris HCl (pH 8.2) sodium potassium tartrate (32 mM), and Cr(VI) ($200 \mu\text{M}$) to SSR for 120 min resulted in an approximately 80% reduction of the initial concentration of Cr(VI). The change in Cr(VI) concentration over the exposure time is shown in Figure 14A (blue circles), where the Cr(VI) concentration was determined by quantitatively following the absorbance of the chromate charge transfer band at 372 nm (Figure 15A). Control experiments showed that in the absence of light, there was no reduction of Cr(VI) in the presence of CuFtn. Also, in the presence of apoFtn (no mineralized core material) and light there was no Cr(VI) reduction (Figure 14B).

To determine the wavelength dependence of the Cr(VI) reduction in the presence of CuFtn, SSR was passed through various long-pass filters: $\lambda \geq 355 \text{ nm}$, $\lambda \geq 330 \text{ nm}$, $\lambda \geq 300 \text{ nm}$, corresponding to energies $h\nu \leq 3.49 \text{ eV}$, $h\nu \leq 3.75 \text{ eV}$ and $h\nu \leq 4.13 \text{ eV}$, respectively (Figure 14B). Analysis of the data shows that there is no reduction of Cr(VI) when $h\nu \leq 3.49 \text{ eV}$, consistent with prior determination of the bandgap of the mineralized core in CuFtn to be 3.65 eV. In contrast, Cr(VI) reduction was experimentally observed when photons with energies of $h\nu \leq 3.75 \text{ eV}$ or $h\nu \leq 4.13 \text{ eV}$ impinged on the reaction solutions. After individual light exposures for 120 min, using

the $\lambda \geq 330$ nm and $\lambda \geq 300$ nm filters, the final [Cr(VI)] was 150.9 μM (21.5 % of Cr(VI) reduced) and 114.4 μM (41.9% of Cr(VI) reduced), respectively (Figure 14B). The total Cr(VI) reduction study for each cutoff filter throughout 120 min exposure time has been illustrated in Figure 15B. The reduction of Cr(VI) in these circumstances is expected, considering that a fraction of the photons impinging on the reacting solution had energies greater than the bandgap of the semiconductor core of CuFtn.

To investigate the presence of the reduced specie Cr(III), resulting from the Cr(VI) reduction reaction, EDTA was employed as a chelating agent. EDTA forms a stable complex with Cr(III), which can be conveniently detected using UV-Vis spectroscopy (Traboulsi, H. et al., 2020, ACS Omega, 5, 31352). However, the formation of the Cr(III)-EDTA complex is highly dependent on the pH of the reaction medium (Resende, J. E. et al., 2014, J. Am. Chem. Soc., 2014, 754526). The reaction mixture was maintained at a pH of 8.2. According to the speciation diagrams at pH=8.2, the predominant complex expected to form is $[\text{Cr}(\text{EDTA})(\text{OH})]^{2-}$, which exhibits a characteristic absorption peak at 594 nm (Carbonaro, R. F. et al., 2008, Geochim. Cosmochim. Acta, 72, 3241). The gradual increase in absorbance at 594 nm over the time of Cr(VI) reduction indicated the formation of the $[\text{Cr}(\text{EDTA})(\text{OH})]^{2-}$ complex (Figure 16A). Furthermore, to confirm the pH sensitivity of the Cr-EDTA complex, the pH of the reaction medium was modified to 5.5 subsequent to the reduction of Cr(VI) with CuFtn. The observed peak at 594 nm was blueshifted to 541 nm ($\epsilon = 202.5 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$) (Cerar, J., 2015, Acta Chim. Solv., 62, 538), indicating the formation of the $[\text{Cr}(\text{EDTA})]^-$ complex, which is known to be the stable complex under acidic conditions (Figure 16B). Therefore, these results validate the formation of Cr(III) from the Cr(VI) reduction reaction with CuFtn exposed to SSR.

Data for Cr(VI) reduction in the presence of CuFtn, hole scavenger, and PAR is found in Figure 17. According to the results, UV-vis absorbance peaks at 372 and 267 nm gradually decreased over 120 min. An additional broad peak growing around 414 nm indicated that there was the degradation of PAR during the reaction. However, no peak growth at 510 nm confirmed that there was no Cu(II) during the Cr(VI) reduction reaction (Figure 17A). In addition, the experimental results for reaction kinetics of Cr(VI) reduction are similar to if PAR is absent from the solution (Figure 17B). Hence, it could

not be concluded from these experiments that the strong absorbance of the PAR in solution does not affect the photoexcitation of the CuFtn core and the subsequent Cr(VI) reduction chemistry.

There are perhaps two likely potential mechanisms for the catalyzed photochemical reduction of Cr(VI) by the conduction band electrons of photoexcited CuFtn: (1) Cr(VI) enters the protein cavity, where reduction occurs directly on the photoexcited Cu(OH)₂ core, and/or (2) Cr(VI) is reduced on the outer surface of the Ftn cage via electron transfer from the Cu(OH)₂ core (Figure 18A and Figure 18B). In the latter scenario, a mechanistic requirement would presumably be the transfer of reducing electrons through the 2 nm thick protein shell.

To address the first possibility, where Cr(VI) reduction occurs within the Ftn cage, experiments were carried out where the same sample of CuFtn was exposed to three successive Cr(VI) reduction cycles. Each individual cycle consisted of exposing solutions containing CuFtn (45 μ M) in Tris HCl (pH 8.2), Cr(VI) (0.020 mM), to 180 min of SSR. As noted above, this exposure time led to the complete reduction of the Cr(VI) initially present. After each full Cr(VI) reduction cycle of 180 min, a small aliquot was removed from the reaction cuvette for ICP analysis, where the aqueous samples were either analyzed directly or dialyzed (into pH 8.2 Tris-HCl buffer) prior to analysis. The expectation here was that if Cr was deposited within the cage, it would not be removed by dialysis, and the Cr concentrations in both the non-dialyzed and dialyzed samples would be equivalent.

After cycles 1, 2, and 3, the Cr concentrations [Cr(VI)] of non-dialyzed samples were 182.5, 370.4, and 554.7 μ M, representing percent [Cr(VI)] recoveries of 91.3, 92.6, and 92.5 %, respectively. The theoretical [Cr(VI)] for cycles one, two, and three would be 200, 400, and 600 μ M, respectively, a finding which was consistent with ICP-OES data. As these samples were not dialyzed, all Cr, whether aqueous or precipitated, was sampled. After dialysis of each cycle against Tris-HCl buffer, no Cr was detected by ICP-OES in the fractions associated with the CuFtn (Figure 19A). This result shows that after irradiation, any Cr product was present in the solution outside of the protein cage; thus, it was able to pass through the dialysis membrane into the dialysate.

Hence, the ICP-OES results for the cycling experiments show no evidence of Cr residing in the interior of CuFtn after irradiation (Figure 19C and Figure 19D).

The location of the Cu component—inside or outside the Ftn cage—was also investigated by ICP-OES. It was found that the concentration of Cu [Cu(II)] was constant, where the percent recovery of [Cu(II)] was greater than 95% for all samples, whether dialyzed or not (Figure 19B), indicating that the Cu remained inside the Ftn cage. It is also mentioned that this result further supports the notion that the Cu(OH)₂ core of CuFtn does not undergo photocorrosion during exposure to SSR.

Prior work demonstrated that the reduction of Cr(VI) by iron-loaded Ftn is facile and may precede by both a homogeneous and heterogeneous mechanism. The current study of CuFtn suggests that only the heterogeneous mechanism is feasible. To directly compare Ftn and CuFtn, for the reduction of Cr(VI) prior experiments for the reduction of Cr(VI) in the presence of Ftn and SSR were duplicated (Figure 20A).

Examination of these data show that the reduction kinetics of Cr(VI) in the presence of Ftn were faster than CuFtn. Photoreduction of Ftn includes the conversion of Fe(III) to Fe(II), with the latter species being soluble and able to exit the protein shell to reduce Cr(VI) directly. Hence, for Ftn there are at least two mechanistic pathways that can lead to the reduction of Cr(VI) in the presence of SSR. Additionally, the band gap of the Fh core within Ftn is smaller than that in CuFtn: 2.60 vs. 3.65 eV, respectively. The band gap value determines the minimum energy required to excite the valence electrons to the conduction band. The larger the band gap value, the smaller the number of electrons that can be promoted to the conduction band via photoexcitation. FeFtn and CuFtn can utilize ~15 and ~6% of the energy output of the SSR based on the spectral distribution of the Xe arc lamp intensity output. Thus the enhanced rate of Cr(VI) reduction observed with Ftn can be attributed in part to both the multiple mechanistic pathways and the increased flux of photons, which can excite the bandgap, resulting in more Cr(VI) reduction.

Prior studies have shown that the conduction band of copper-oxides is positioned above the conduction band of Fh within Ftn. (Ex. In copper oxides (Jeyalakshmi, V. et al., 2013, Mater. Sci. Forum., 734, 1) (CuO and Cu₂O) ~ -(3.00-4.00) eV, copper sulfides (Li, J. et al., 2020, Catal., 10, 89) (CuS, Cu₂S) ~ -(4.00-4.50) eV, Cu(OH)₂ ~ -(4.00-4.50 eV) (Yu, J. et al., 2011, Energy Environ. Sci., 4, 1364). Compared with the literature values, the

conduction band of the CuFtn (Cu(OH)₂) core (4.00–4.50 eV) is above the level of the Fh conduction band (–5.08 eV). Hence, the excited electron in the copper system has a lower potential (–5.83 eV) to reduce Cr(VI) (Figure 20B).

The experimental data taken as a whole suggest that the photoexcitation of the Cu(OH)₂ core of CuFtn with photons having an energy greater than its band gap leads to the photoreduction of aqueous Cr(VI). Since the copper core of the CuFtn does not undergo photocorrosion and the Cr photoproduct is located in solution (outside the Ftn cage), it was surmised that Cr(VI) reduction occurs via the transfer of electrons across the 2 nm thick protein shell of Ftn. This contention is not inconsistent with prior nonphotochemical studies. For example, while research has shown that small molecules, including dithionite, cysteine, glutathione, and ascorbate, can enter the Ftn cage and reduce the Fe(III) core (Sirivech, S. et al., 1974, *Biochem J.*, 143, 311; Funk, F. et al., 1985, *Eur. J. Biochem.*, 152, 167). Other studies have shown that relatively large molecules, such as phenothiazine and phenoxazine dyes, plastocyanin, stellacyanin, and cytochrome c, can undergo charge transfer with the inorganic core through the protein shell (Koochana, P. K. et al., 2019, *Dalt. Trans.*, 48, 3314; Bou-Abdallah, F. et al., 2018, *Pharmaceuticals*, 11, 120; Carmona, U. et al., 2014, *Chem. Commun.*, 50, 15358). Further, it has been suggested that the entry of oxidants to the interior of the protein cage is not a strict requirement for inorganic core formation in Ftn. Instead it has been postulated that long-range electron transfer processes are operative, allowing Fe(II) oxidation to a Fe(III) bearing core to occur by indirect interaction with external oxidants (Watt, G. D. et al., 1988, *Proc. Natl. Acad. Sci. U. S. A.*, 85, 7457). For example, one study indicated that this long-distance electron transfer can occur by electron tunneling through the 2.0–2.5 nm Ftn protein shell with the assistance of adequately arranged aromatic amino acids. Additional research has suggested that electron transfer occurs through the protein shell during the interaction with *Azotobacter vinelandii* bacteria via redox-active centers (Zhang, B. et al., 2005, *Inorg. Chem.*, 44, 3738). Moreover, a long-distance electron diffusion through the protein shell was observed by Chen et al. from photosensitizers to the iron-sulfur model catalyst mineralized in apoFtn (Chen, W. et al., 2019, *Chem. Sci.*, 10, 2179). Finally, prior research has shown the ability of electrons to tunnel across Ftn-based junctions (Kumar, K. S. et al., 2019, *Adv. Mater.*, 28, 1824).

Based on prior studies, the photochemical heterogeneous reduction pathway seems the most reasonable explanation for Cr(VI) reduction in the CuFtn system. Here photoexcitation would generate a conduction band electron, which would be followed by electron transfer (ET) through the ~2 nm protein cage to initiate Cr(VI) reduction to Cr(III) on the outside of the protein. The possibility that the nature of the Ftn used may influence the photochemistry of the protein is presented herein. Ftn is composed of 24 monomers, which can either be the heavy (H) or light (L) chain subunit. Prior studies have shown that the two different subunits have been demonstrated to have different properties in regard to potential electron transfer, with the L-chain showing a higher conductivity (Carmona, U. et al., 2014, Chem. Commun., 50, 15358). Furthermore, electron transport through L-chain in Ftn is thought to be bi-directional, facilitating both mineralization and demineralization of the inner core. In this study, the protein used to synthesize CuFtn was HSFtn, which consists of 90% L-chain subunit. One might speculate that differences in the photochemistry of Ftn may arise from samples containing different proportions of L and H-chain subunits, and the investigation of this possibility would present an interesting path forward to better understand electron transfer in Ftn.

Summary

The research conducted in this study revealed that HSFtn has the ability to mineralize an insoluble inorganic core predominantly consisting of Cu(OH)₂ (PXRD analysis of the Cu(OH)₂ bulk material (CuCtrl) suggests that there are trace amounts of CuO). TEM images provided visual evidence showing the mineralized core to have a size of approximately 4-5 nm. Analysis of Tauc plots derived from UV-Vis absorption data indicated that the band gap of the semiconductor core was determined to be 3.65 eV. Furthermore, experiments involving the irradiation of CuFtn with steady-state radiation (SSR) in the presence of a hole scavenger and Cu-chelator demonstrated that no copper ions were released from the interior of the Ftn core. This finding suggests that the reducing electrons present in the conduction band of the Cu(OH)₂ core of CuFtn have the ability to traverse the 2 nm thick protein cage of Ftn during exposure to SSR, ultimately facilitating the reduction of Cr(VI) on the exterior surface of the protein. These results

highlight the unique properties and functionality of CuFtn in mediating redox reactions and provide valuable insights into the mechanisms involved in the reduction of Cr(VI) by this protein-based system.

5

Example 2: Synthesis, Characterization, and Photochemistry of Gold Nanorod H-Chain Ferritin Bioconjugates

The present invention further relates to, in part, the bioconjugation of anisotropic AuNPs—gold nanorods (AuNRs) and gold nanostars (AuNSs)—to human H-type ferritin (HFtn). After attaching the AuNRs or AuNSs to HFtn, it was shown that the
10 near-infrared radiation (NIR) excitation of the localized surface plasmon resonance (LSPR) of the AuNR or AuNS conjugated to HFtn led to the activation of the Fh core of the protein. This NIR photochemistry ($\lambda=850$ nm light) resulted in the release of Fe(II) from the Ftn and also led to the reduction of Cr(VI) when it was present in the aqueous
15 phase. The novel synthetic protocols to synthesize the bioconjugates focused on attaching the AuNRs and AuNSs to the solvent-exposed cysteines (Cys) on HFtn. The research also developed techniques for the removal of colloidal stabilizing surfactants, such as CTAB and TritonX-100 (TX-100), from anisotropic AuNPs (AuNR/AuNS) before their attachment to HFtn. The removal of the surfactant was not only important for attachment
20 to the HFtn, but it also removed a cytotoxic species so that the bioconjugates could be used in research that had applications to biomedicine.

Synthetic strategies to form bioconjugates that consisted of spherical gold nanoparticles (AuNSps) attached to HSFtn were also investigated. In contrast to HFtn, HSFtn does not have solvent exposed cys groups. Hence, a challenge that was overcome
25 in this research was to populate the outer surface of HSFtn with thiol groups (-SH) so that AuNSps could be attached. To meet this challenge, the surface primary amine-containing amino acids (lysine) in HSFtn were modified to active cys using N-Succinimidyl S-acetylthioacetate (SATA). After this chemical modification of HSFtn, it was shown that a relatively high density of AuNSps could be attached to HSFtn. This SATA-modified
30 HSFtn bioconjugate system (AuNSp-HSFtn) exhibited the release of Fe(II) at wavelengths of light where $\lambda > 475$ nm. In the absence of AuNSp, HSFtn released Fe(II)

during exposure to light at wavelengths of light where $\lambda < 475$ nm. The activation of the bandgap at longer wavelengths of light ($\lambda > 475$ nm) was due to the excitation of the 532 nm plasmon resonance of AuNSp and the presumed transfer of hot electrons to the inner Fh core of HSFTn.

5

Plasmonic Gold Nanomaterial Synthesis and Properties

A major focus of the present work is to form bioconjugates containing AuNPs and Ftn. This section briefly reviews information about AuNP synthesis. By varying the size, shape, and dimension of gold nanostructures, unique optical properties can be obtained. Brust and coworkers prepared simple single-step gold quantum dots (<5 nm) using tetrachloroaurate (AuCl_4^-) freshly prepared sodium borohydride, alkyl thioether end-functionalized poly(methacrylic acid) (Hussain, I. et al., 2005, J. Am. Chem. Soc., 127, 16398). Changing polymer concentration could change the particle sizes from 2.2-4.6 nm. Sardar and Parry synthesized gold quantum dots (<5 nm) using 9-borabicyclo[3.3.1]nonane (9-BBN) as the reducing agent. 9-BBN concentration and reaction temperature was found to affect the nucleation rate and growth of the particles (Sardar, R. et al., 2011, J. Am. Chem. Soc., 133, 8179). Jana et al. fabricated uniform AuNPs between 5–40 nm by 3.5 nm gold particles as seeds prepared by borohydride reduction of AuCl_4^- in citrate (Jana, N. R. et al., 2001, Langmuir, 17, 6782). Various morphologies were obtained by further modifying the spherical gold seed particles with more AuCl_4^- , reductants (NaBH_4 , ascorbic acid, hydrazine, and dimethylformamide (DMF)), other cations (Ag^+), and shape-templating surfactants Cetyltrimethyl ammonium bromide (CTAB), Cetyltrimethyl ammonium chloride (CTAC), sodium dodecyl sulfate (SDS), Polyvinylpyrrolidone (PVP), TX-100, DMF and hydroxylamine sulfate). A popular approach for AuNR synthesis is seed-mediated growth by either citrate or CTAB-capped AuNPs. Cationic surfactant micelles function as a soft template for AuNR growth, Ag^+ ions control the shape and crystalline structure, and ascorbic acid is a mild reducing agent during the reaction. A few research groups (Mulvaney and coworkers (Perez-Juste, J. et al., 2004, Adv. Funct. Mater., 14, 571), El-Sayed and coworkers (Nikoobakht, B. et al., 2003, Chem. Mater., 15, 1957), Murphy and coworkers (Gao, J. et al., 2003, Langmuir, 19, 9065) and Guyot-Sionnest and coworkers (Liu, M. et al., 2005,

J. Phys. Chem. B, 109, 22192)) have improved seed-mediated AuNR synthesis methods to strengthen their chemical stability and monodispersity and change the aspect ratio. Chang et al. developed an electrochemical method to generate suspended AuNRs with controlled aspect ratios and high yields (Yu et al., 1997, J. Phys. Chem. B., 101, 6661).

5 Figure 21 shows how the color and longitudinal UV-Visible absorbance of the AuNRs change when the aspect ratio is altered. Other anisotropic nanostructures such as triangular, hexagonal, and polygonal gold plates exhibit inherent sharp edges that result a high local electric field in applications like optical biosensing and surface-enhanced Raman spectroscopy (SERS). Chen et al. developed a method to get
10 monodispersed triangular gold nanoprisms (morphological yield >90%) through a seedless growth process with edge lengths tuned between 40 and 120 nm (Chen, L. et al., 2014, Nano Lett., 14, 7201), whereas Porel et al. developed polygonal gold nanoplates using a heat-treated poly(vinyl alcohol) film. The synthesis span over a large variety of triangular, square, hexagonal, and rare pentagonal plates by tuning the Au/PVA ratio
15 (Porel, S. et al., 2005, Chem. Commun., 18, 2387).

 Another anisotropic gold nanostructure, AuNS, exhibits complex structures and morphology. They can be synthesized using either the seeded-growth method or the one-pot synthesis method. A pH-controlled one-pot AuNS synthesis method was used by Das et al. using gelatin to enhance the radio-sensitization of cancer
20 therapeutics (Das, R. P. et al., 2021, New J. Chem., 45, 13271). Srivastava and coworkers performed a protein-Mediated one-pot Green Synthesis of AuNS for Imaging and photothermal cancer therapy (Sasidharan, S. et al., 2017, ACS Sustain. Chem. & Eng., 5, 10163). According to Vorster and coworkers, by adding AgNO₃, HEPES-mediated one-pot AuNS synthesis was improved (Mulder, D. W. et al., 2019, R. Soc. Open Sci., 6,
25 190160). A surfactant-free one-pot AuNS synthesis conducted by Huynh et al. showed its antibacterial properties (Huynh, P. T. et al., 2021, J. Nanomater., 2021, 6650661). In 2009, the first synthesis of penta-branched AuNS by a seeding growth approach was reported by Huang and coworkers (Wu, H.-L. et al., 2009, Chem. Mater., 21, 110). Fabris and coworkers produced six-branched AuNS using a high-yield, seed-mediated method
30 (Atta, S. et al., 2019, Nanoscale, 11, 2946). They have further shown how the morphology of the AuNS can be tuned by varying TX-100, ascorbic acid, and AgNO₃ as

precursors. Moreover, Kereselidze et al. used a silver seed-mediated AuNS synthesis method for biomedical applications (Kereselidze, Z. et al., 2012, JoVE, 59, e3570).

Excitation of the LSPR of a gold nanostructure occurs when the oscillation frequency of free electrons at the surface resonates with the frequency of the impinging electromagnetic radiation (Figure 22). This process results in interesting surface-enhanced optical properties (Li, X. et al., 2009, Colloids Surfaces A Physicochem. Eng. Asp., 332, 172). As an example, for AuNSps (<60 nm), the LSPR absorbance peak appears around 500–550 nm (resulting in a red color) (Li, W. et al., 2015, Nanomedicine, 10, 299). The optical properties due to LSPR may depend on the size and shape, type of solvent, surface ligand, charge density, pH, and temperature (Gezgin, S. Y. et al., 2020, Nanomaterials, 10). Unlike isotropic AuNPs such as AuNSps, anisotropic gold nanostructures possess distinct shapes, such as AuNRs, AuNSs, nanocubes, or nanowires, which give rise to unique plasmonic properties. Anisotropic nanoparticles that include AuNRs (Harris, N. et al., 2008, Gold Bull., 41, 5) and AuNSs (Atta, S. et al., 2019, Nanoscale, 11, 2946) are associated with two distinct plasmon bands (i.e., transverse and longitudinal bands). The transverse band occurs around ~520 nm, whereas the longitudinal band position may vary depending on the length/width ratio (ranging from 500 to 1600 nm). By altering the shape of AuNPs, the optical properties can be extended across a broad spectrum (visible to NIR).

LSPR-induced hot electrons generated by plasmonic gold nanostructures attached to semiconductors have been widely investigated in photovoltaics and photocatalysis (Subramanian, V. et al., 2004, J. Am. Chem. Soc., 126, 4943; Li, P. et al., 2011, J. Am. Chem. Soc., 133, 5660; Sahu, K. et al., 2020, Mater. Res. Bull., 123, 110707). The LSPR decay results in photothermal effects and plasmonic heating. It triggers with Landau damping, generating hot electron-hole pairs (Furube, A. et al., 2017, NPG Asia Mater., 9, e454). Plasmonic heating is induced by electron–electron scattering (<100 fs), electron–phonon scattering (1–10 ps), and ‘phonon–phonon (ph–ph) scattering’ (~100 ps) and this heat is dissipated to the surroundings. Fe and Cu-based metal oxides (within Ftn) investigated herein have a high density of states in the conduction band due to d-orbitals. Hence, they are better electron acceptors which can efficiently couple with hot electrons.

There is an ongoing discussion on the mechanisms of plasmon-driven chemistry. One commonly accepted mechanistic pathway during excitation of the LSPR involves hot carrier injection into the conduction band of the semiconductor and tunneling across the Schottky barrier (S.B) (Sundararaman, R. et al., 2014, Nat. Commun., 5, 5788). A Schottky junction is created when metal and semiconductors come in contact. Hot carriers can be created by both intraband (from occupied s-band to empty s-band within the conduction band) and interband (from other bands such as d band to the unoccupied s-band states in the conduction band) transitions, with the type of transition being influenced by the energy of excitation (Yamada, K. et al., 2007, J. Phys. Chem. C, 111, 11246). Notably, these two processes result in distinct hot carrier distributions. The hot carriers generated from $d \rightarrow sp$ interband transitions of gold nanostructures have much lower kinetic energies but higher populations. In addition, the process requires an excitation energy greater than 2.38 eV (<539 nm) (Hoven, C. V. et al., 2008, Proc. Natl. Acad. Sci., 105, 12730). These highly energetic hot electrons can be extracted by n-type acceptor semiconductors with a high Fermi level and high density of state (DOS) in the conduction band (Liu, Y. et al., 2020, Nano Lett., 20, 4322). The S.B height is crucial for injecting hot electrons into semiconductors through inter or intra-band transition (Lee, C. et al., 2019, RSC Adv., 9, 18371). When the energy of the radiation is low, the intraband transition is a common occurrence in gold. The schematic in Figure 23 illustrates how hot electrons are produced within a metal nanorod that has been optically stimulated and then transferred over the S.B at the metal-semiconductor interface and injected into the semiconductor.

The development of heterostructures composed of proteins chemically attached to plasmonic particles has been of significant interest to image biomolecules. The intense plasmonic absorption of the nanoparticle (e.g., gold) makes spectroscopic detection schemes feasible even at relatively low concentrations. In the context of the environment, research also has been interested in understanding how nanorods interact with proteins (Liu, H. et al., 2012, Gold Bull., 45, 187; Scaletti, F. et al., 2015, J. Inorg. Biochem., 150, 120; Moghadam, T. T. et al., 2011, Int. J. Biol. Macromol., 49, 629; Thioune, N. et al., 2013, Gold Bull., 46, 275). The attachment of the nanoparticles to the

biomaterial often results in a protein corona (Lynch, I. et al., 2008, *Nano Today*, 3, 40). There is a dynamic equilibrium between the protein corona on a nanomaterial. The adsorption-desorption rates and the equilibrium binding constants depend on the affinity and curvature of the nanomaterial surface, the presence of binding ligands, and the surrounding medium. In these types of prior studies, the energy transfer between the plasmonic particle and bound protein has generally not been a direct motivation.

One common AuNP morphology that has been intensely investigated is the AuNR, which features distinctive optical properties depending on its size, aspect ratio, and the refractive index of the medium in which they reside (Polito, A. B. et al., 2015, *J. Nanoparticle Res.*, 17, 485; Liu, S.-Y. et al., 2011, *Opt. Lett.*, 36, 1296; Yang, D.-P., 2008, *Chem – An Asian J.*, 3, 2010; Burrows, N. D. et al., 2016, *Langmuir*, 32, 9905). AuNRs, depending on their size and aspect ratio, can absorb both in the visible and NIR region, generating two unique LSPR bands. The transverse plasmon excitation band is positioned around 530-540 nm in the visible region, and the longitudinal excitation band can be in the visible or NIR region (Varkavski, O. P. et al., 2003, *J. Phys. Chem. B*, 107, 3101). One area of intense interest that exploits the NIR absorbance and likely the interaction of plasmonic AuNPs and biomaterial are photothermal therapies that rely on the local heating effect of AuNRs upon NIR excitation. Here AuNRs (typically capped by PEG) localized during in-vitro studies local to tumor cells have been shown to undergo induced NIR localized heating that kills the tumor cells.

Research has also recently shown that the chemical attachment of AuNPs to protein architectures allows for the repurposing of the protein function via the light-induced excitation of the nanoparticle (Cerkez, E. B. et al., 2019, *Environ. Sci. Nano*, 6, 85; Huttmann, G. et al., 2003, *Therapeutic Laser Applications and Laser-Tissue Interactions*, 5142; Wang, X. et al., 2009, *J. Mater. Chem.*, 19, 8944; Zhou, J. C. et al., 2010, *Mater. Sci. Eng. C*, 30, 20). One such example involves the chemical attachment of AuNSps (plasmon resonance at 532 nm) to the globular protein Ftn. Ftn is a 24-mer protein that nature has designed to sequester, store and release iron in living organisms. It primarily consists of an iron mineralized ~ 7-8 nm inner core surrounded with an 11-12 nm thickness protein shell (Lv, C. et al., 2014, *Biochemistry*, 53, 2232; Uchida, M. et al., 2010, *Biochim. Biophys. Acta – Gen. Subj.*, 1800, 834; Huard, D. J. E. et al., 2013, *Nat.*

Chem. Biol., 9, 169). In Ftn, up to ~4000 (Yang, J. et al., 1992, Ultramicroscopy, 45, 199) iron atoms can be stored as superparamagnetic crystalline ferric oxyhydroxide, known as Fh (Chua-anusorn, W., Iron Oxide Deposits in Iron Overload Diseases, 1997). Fh is a semiconductor with a tunable bandgap between 1.3 and 2.6 eV (Rakshit, T. et al., 2020, Nano-Structures & Nano-Objects, 24, 100582).

The present research investigates the synthesis and photochemical properties of AuNR-HFtn bioconjugates. Results detailed herein demonstrate that AuNRs can be attached to the outer surface of HFtn, presumably due to the formation of Au-S bonds via the interaction of the AuNR and solvent exposed thiol groups on HFtn. To make this possible a protocol to remove the surfactant on the AuNRs, CTAB, prior to attachment to the HFtn is presented. Novel photochemistry is presented where the NIR excitation of the LSPR of the AuNR attached to HFtn results in electron transfer between the AuNR and Fh core of HFtn. This electron transfer across the Ftn shell results in the formation of soluble Fe(II) and if Cr(VI) is present in solution, the formation of Cr(III).

Experimental

All chemicals were used as received. Pure analytical grade solid chemicals, solvents, and Deionized (DI) water ($18 \text{ M}\Omega \text{ cm}^{-1}$) were used in all preparations. Sodium borohydride (NaBH_4 , 98+%), Sodium hydroxide (NaOH , ACS reagent, Pellets, $\geq 97.0\%$), Sodium tetrachloroaurate(III) dihydrate ($\text{NaAuCl}_4 \cdot 2\text{H}_2\text{O}$, >99%), Hexadecyltrimethylammonium bromide (CTAB, For synthesis), Phosphotungstic acid hydrate ($\text{H}_3[\text{P}(\text{W}_3\text{O}_{10})_4] \cdot x\text{H}_2\text{O}$, 99.995%), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid monosodium salt hydrate (Ferrozine, 98+% pure), Tris base (primary standard and buffer, $\geq 99.9\%$), L-(+)-Ascorbic acid (AA, 98+%), were purchased from Sigma-Aldrich. Silver nitrate (AgNO_3 , 99%), Hydrochloric Acid (HCl , Certified ACS plus, 36.5 to 38.0%), Hydrogen Peroxide (H_2O_2 , 30%, Certified ACS, Assay: 29.0 to 32.0%), Ferrous Ammonium Sulfate Hexahydrate ($\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, Crystalline/Certified ACS, Assay: ≥ 98.5 to 101.5%), Sodium potassium tartrate ($\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$, Certified ACS), were purchased from Fisher Chemical™ and HEPES buffer (ultra-pure) from American bio.

Expression of HFtn (homopolymer consists of 100% H chain) was performed in *E.coli* BL21 cells containing the plasmid with the gene for HFtn (Amos, F. F. et al., 2013, JBIC J. Biol. Inorg. Chem., 18, 145). 1000 mL Lysogeny broth (LB) culture media mixed with 30 $\mu\text{g/mL}$ kanamycin was inoculated with 5 mL of *E.coli* BL21 cell culture grown at 37 °C overnight with 225 rpm shaking. (OD=0.6) Then 1 mM Isopropyl β - d-1-thiogalactopyranoside (IPTG) was added to the medium and grown the culture for an additional 5 hours. Next, the cells were centrifuged (3000 xg, 30 min), and the cell pellet was resuspended in 20 mL lysis buffer (100 mM HEPES, 50 mM NaCl, pH 8.0), and cooled for 30 min in an ice bath. Finally, the cells were sonicated and centrifuged (10000xg, 10 min) to remove any remaining cell debris.

The supernatant was separated and heated at 65 °C for 10 min to precipitate out the denatured proteins. Then, they were centrifuged at (10000 xg, 10 min), and the supernatant containing the HFtn was dialyzed overnight in 1000 mL buffer (100 mM Tris, 200 mM NaCl, pH 7.4). The protein sample was concentrated (Millipore Centriprep centrifugal concentrators, MWCO 30,000) and filtered (0.2 μM syringe filter) prior to loading on a size-exclusion chromatography column (HiPrep 16/60 Sephacryl S-300). The column was pre-equilibrated with buffer. (100 mM Tris, 200 mM NaCl, pH 7.4). The HFtn (the 24-mers) was eluted using the same buffer from other oligomers. Protein purity was evaluated by SDS PAGE, and the concentration of the apoferritin ($\epsilon = 23,000 \text{ M}^{-1}\text{cm}^{-1}$) was measured by a UV-vis spectrophotometer at 280 nm for each subunit of the 24mer (Bou-Abdallah, F. et al., 2002, Biochemistry, 41, 11184).

The ToxinSensor™ Endotoxin Detection System Kit from GenScript was used according to the manufacturer's instructions. The endotoxin was removed using 0.2 μM Mustang E membrane filters. A working concentration of 0.30 mg/mL (0.6 μM) Ftn was used for the studies with an endotoxin concentration of 0.084 EU mL^{-1} ApoFtn samples were stored at 4 °C prior to use.

Briefly, 2.0 mL of endotoxin-free purified apoFtn (0.631 mg mL^{-1}) was added to 20 mL of HEPES buffer (pH=7.4, 0.1 M), and prepared by bubbling $\text{N}_2(\text{g})$ for 1.5 h. Then aliquots of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (4.06 μM , 500 μL) were added to the apoFtn solution over a 150 min period at 30 min intervals targeting 2000 iron loading. (HFtn). In each Fe(II) addition, the solution was shaken slowly and kept air-tight for 30

min. The solution slowly turned to a yellowish-orange color from colorless, and a homogeneous solution was observed without any visible precipitation. Excess Fe(II) and other excess ions were removed by dialysis (Molecular porous dialysis membrane, MWCO: 12-14 kDa) in fresh Tris buffer (pH 7.4, 0.1 M, 1000 mL) for 180 min intervals three times and stored in 4 °C.

CTAB stabilized AuNRs (AuNR-CTAB) were synthesized using a method by Nikoobakht and El-Sayed mentioned elsewhere (Nikoobakht, B. et al., 2003, Chem. Mater., 15, 1957). Briefly, CTAB solution (5 mL, 2.0×10^{-1} M) and 5.0 mL of 5.0×10^{-4} M HAuCl₄ solution were mixed while stirring, and 0.60 mL of the ice-cold 1.0×10^{-2} M NaBH₄ was added, which suddenly turned the solution into a brownish yellow solution. Then the solution was shaken vigorously by hand for 2 min and kept in a water bath at 25 °C for 5 min.

AuNR growth solution was prepared by the following method. CTAB (100.0 mL, 2.0×10^{-1} M) was added to 5.0 mL of 4.0×10^{-3} M AgNO₃ solution kept at 25 °C. Then, HAuCl₄ (100.0 mL, 1.0×10^{-3} M) was added, and the solution was gently mixed, followed by 1.4 mL of 7.88×10^{-2} M ascorbic acid. Ascorbic acid changed the solution from dark yellow to colorless, yielding the growth solution. Finally, 240 µL of the seed solution (kept at 27–30 °C) was added to the growth solution to form AuNR with the plasmonic band at 800 nm. The color of the growth solution gradually changed from colorless to pale pink within 10–15 min. Then the solution was purified by centrifugation at 6k rpm for 30 min and redispersed in 100.0 mL of 10 mM CTAB.

To attach the CTAB-AuNRs to the HFtn, the CTAB needed to be removed as the capping agent. The following procedure replaces the function of the CTAB with HFtn that stabilizes the AuNRs in solution. 2.0 mL of AuNR-CTAB were centrifuged at 12000 rpm for 15 min, and the supernatant was discarded carefully. Then 2.0 mL of distilled water was added to the first AuNR pellet, mixed by vortexing, and centrifuged at 12000 rpm for 15 min. Next, 2.0 mL of distilled water was added to the remaining AuNR pellet, mixed by vortexing, and centrifuged at 10000 rpm for 10 min. In order to adjust the dispersion to CMC of CTAB (1.0 mM), 1.0 mM CTAB 1.0 mL was added to the AuNR pellet, mixed by vortexing, and HFtn (0.068 µM) protein solution 1.0 mL was added slowly under ultrasonication for 2 min. (HFtn/AuNR dispersions, 1:1 v/v). Then

the AuNR-HFtn conjugate solution was further sonicated for 30 min, centrifuged at 5000 rpm for 2 min, decanted excess protein/ CTAB, and dispersed in 2.0 mL of HFtn in Tris-HCl buffer. (pH =7.4, 0.1 M), and stirred for at least 24 h. Next, the AuNR-HFtn solution was centrifuged at 10000 rpm for 2 min, decanted excess protein/ CTAB, and dispersed
5 in a 2.0 mL Tris-HCl buffer. (pH =7.4, 0.1 M), and concentrated as desired.

Characterization

The Precision Red advanced protein assay was used to determine the concentration of the HFtn quantitatively. The assay was performed according to the
10 manufacturer's instructions. Briefly, 200 μ L of the assay was added to 1800 μ L of HFtn protein, thoroughly mixed by inverting, and then the sample was incubated for 1 min at 25 °C. The protein concentration was calculated by UV-Vis spectroscopy ((Thermo-Fisher Scientific, UL 61010-1.) at 600 nm. A single absorbance unit equals 100 mg mL⁻¹ of HFtn protein.

15 TEM was used to image pure AuNR, AuNR-HFtn bioconjugates using a JOEL JEM-1400 microscope operating at 120 kV (under 50,000-100,000 magnifications and 20 nm/ 50 nm resolution). All the samples were prepared on copper mesh-supported holey carbon TEM grids (Ted Pella) and allowed to dry as a thin film. The AuNR-HFtn sample was centrifuged at 5000 rpm for 5 min thrice and diluted each time with Tris-HCl
20 buffer (0.1 M, pH =7.4) to remove excess HFtn in the soft corona and used for the TEM imaging. TEM Images were taken with and without staining. To visualize the outer shell of the cheerio-like HFtn protein in the AuNR-HFtn bioconjugates, a 2% PTA negative staining solution at pH 7 was used. When the TEM grids were prepared, 10 μ L aliquots of the sample were deposited on the grid and air-dried for 10 min. The excess sample was
25 removed with filter paper and then rinsed with 10 μ L of water. Next, 3 μ L of the PTA solution was deposited, allowed to sit for 2 min, and the excess was removed by filter paper to prepare stained grids.

The Malvern Zetasizer particle size analyzer was used to measure the hydrodynamic particle size distribution of AuNR-CTAB and dispersed in HFtn(Tris-HCl
30 buffer (0.1 M, pH =7.4)) 1 mL solutions was used in a disposable plastic cell (DTS0012) for the particle size analysis and Z.P measurements.

To determine the iron content mineralized inside the HFtn and the gold concentration([Au]) in the AuNR, ICP-OES (Thermo-Scientific, iCAP 7400-ASX520) was utilized. HFtn protein samples for analysis were diluted to 10 mL with DI water and acidified by 2% by volume with nitric acid (HNO₃). To analyze the [Au] in the AuNR, a
5 AuNR sample was dissolved in an aqua regia solution. Briefly, the AuNR and AuNR-HFtn conjugates were treated with 5 mL concentrated aqua regia (25% HCl, 75% HNO₃) and digested for at least 24 h, and the final samples were diluted to 10 mL DI water prior to metal analysis with ICP-OES.

ATR-FTIR analysis was performed to confirm the CTAB removal from
10 the AuNR-HFtn bioconjugates. All experiments were conducted in Nicolet Magna 750 FTIR spectrometer with a single bounce diamond crystal ATR cell (SpecacTM) and mercury cadmium telluride A (MCTA) detector cooled by liquid N₂.

Results and Discussion

15 TEM images and NIR plasmon spectra of the AuNR-CTAB are shown in Figure 24. Figure 24A shows the UV-Vis spectrum of the AuNRs and they exhibit two different modes, a weak transverse band (perpendicular to the long axis of the rod) at 520 nm and a sharp and intense longitudinal band (parallel to the long rod axis) at 800 nm. TEM images (Figure 24B) showed that the average width and length of the AuNRs are
20 ~12 and ~50 nm, respectively (an aspect ratio of ~1:4). The AuNR-CTAB was stable in 10 mM CTAB solution. However, the AuNRs became unstable and started to aggregate if CTAB was removed from the solution (via centrifugation).

TEM images of HFtn stained with PTA shows the protein shell (white features, Figure 25A). Unstained TEM images show the black spherical inner core with
25 an ~ 8 nm diameter, consistent with the stained TEM images. (Figure 25B). The unstained and stained TEM images of the AuNR-HFtn conjugates have been illustrated in Figure 24C and Figure 24D, respectively. Both unstained and stained TEM images support the contention that the AuNRs are attached HFtn. Black dense spheres represent the inner core of HFtn consisting of Fh core, and the white color coating surrounding the
30 core in the stained TEM image represents the HFtn shell which has a thickness of ~2 nm.

The interactions of AuNRs with HFtn were further analyzed using UV-Vis spectrophotometry. Figure 26A and Figure 26B, the unstained TEM images of AuNR illustrate the AuNR-CTAB and AuNR-HFtn conjugates dispersed in Tris HCl (0.1 M, pH=7.4). The LSPR longitudinal band position of AuNR-CTAB is at 800 nm. The unstained TEM image of the AuNR-CTAB has a distinct rod feature resulting from seed-mediated AuNR synthesis. Figure 26B, it is shown that the transverse absorbance intensity and full-width half maxima (FWHM) of the LSPR band of AuNR increased after HFtn conjugation. The longitudinal band of the nanorods is more susceptible to chemical changes and the dielectric constant around the vicinity of the AuNR (Jacobs, J. F. et al., 2010, *Biotechnol. Bioeng.*, 105, 918). The longitudinal LSPR absorbance of AuNR-HFtn shows a protein-induced redshift (~50 nm) to 850 nm compared with AuNR-CTAB's absorbance at 800 nm. The peak broadening likely indicates coupling interactions between plasmonic AuNRs with HFtn (Abdi, Z. et al., 2019, *Sci. Rep.*, 9, 1). AuNR-HFtn conjugates were totally dispersed in the Tris buffer with no aggregation, whereas AuNR showed a rapid, irreversible aggregation when dispersed alone in Tris (Figure 26B). Typically, the longitudinal peak position depends on the aspect ratio (A.R) of the AuNR. According to TEM images shown in Figure 27A and Figure 27B, the average width ranges from 10-15 nm whereas the length varies from 45 to 55 nm, resulting in an A.R of ~4. The A.Rs calculated from the TEM images using ImageJ, for the AuNR and the AuNR-HFtn were 4.00±0.07 and 4.02±0.03, respectively (Figure 27C and Figure 27D). This result signifies that the A.R remains almost the same even after the bioconjugation and longitudinal band redshift. According to a study carried out by El-Sayed and coworkers, there was a linear relationship between the A.R and the LSPR wavelength maximum (λ_{max}) (Deans, A. E. et al., 2006, *Magn. Reson. Med.*, 56, 51).

$$\lambda_{max} = (445.4 + 90.6 \times (\text{A.R})) \quad (2.1)$$

Calculations based on this equation yields λ_{max} values for AuNR-CTAB and AuNR-HFtn of 807.8 nm and 809.6 nm, respectively. These values agree with the LSPR of the AuNRs observed from the TEM images.

During the seed-mediated synthesis of AuNRs, quaternary ammonium surfactant CTAB stabilizes the AuNRs as a colloidal suspension. The chemical structure of CTAB surfactant can be found in Figure 28A. Although, AuNR-CTAB cannot be used

for biological and/or therapeutic applications due to the proven cytotoxicity of CTAB. The present work developed a protocol to remove the CTAB from the AuNR surface prior to attachment to HFtn. The procedure was a modified version of a protocol developed for the attachment of bovine serum albumin (BSA) protein and AuNR (Fan, K. et al., 2012, *Nat. Nanotechnol.*, 7, 459). The CTAB concentration of AuNR stock solution (10 mM) was lowered by a factor of ten below its critical micelle concentration (CMC=1 mM) before HFtn addition. Furthermore, the higher protein concentration (~ 10 mg mL⁻¹) and its higher binding ability with the AuNR produced an additional reason for protein to replace CTAB ligands.

ATR-FTIR analysis (Figure 28B) provided evidence for the removal of the positively charged CTAB bilayer from AuNR-HFtn. An intense asymmetric stretching at 2915 cm⁻¹, symmetric C-H stretching at 2850 cm⁻¹, C-H scissoring at 1430 cm⁻¹ and 1462 cm⁻¹, C-H bending at 720 cm⁻¹ and 730 cm⁻¹ can be observed for both pure CTAB and AuNR-CTAB corresponding to the (CH₂)_n chain of the CTAB. The distinctive C-N stretching frequency of the quaternary ammonium nitrogen bound to the carbon chain appears at 935 cm⁻¹. HFtn and AuNR-HFtn conjugates do not show any of these peaks corresponding to CTAB, indicating that the CTAB was wholly removed from the AuNR surfaces (Yamashita, I. et al., 2010, *Biochim. Biophys. Acta – Gen. Subj.*, 846). Furthermore, a surface Z.P analysis study carried out on the CTAB-AuNR and AuNR-HFtn confirmed the CTAB removal from the AuNR surface. At first, the Z.P recorded for CTAB-AuNR was +64.6 mV. However, as the synthesis of AuNR-HFtn conjugates progressed, this potential gradually became negative, ultimately reaching a value of -13.9 mV. This significant shift in value indicated the removal of CTAB from the AuNR-HFtn bioconjugates.

When HFtn is attached to an AuNR, the size of the nanoparticles will increase due to specific chemical binding and non-specific adsorption and interactions (Cerkez, E. B. et al., 2019, *Environ. Sci. Nano*, 6, 85). DLS studies were carried out to analyze AuNR-CTAB and AuNR-HFtn conjugates. The results show two distinguishable DLS peaks for AuNRs. Prior studies have shown that the DLS size distribution curves for non-spherical AuNRs show two distinctive peak sizes. The small size peak, around 2.3 nm, represents the rotational diffusion of the AuNRs, and the large size peak is the

hydrodynamic diameter positioned at 59.3 nm. HFtn shows a hydrodynamic diameter of 13.4 nm. When AuNR was conjugated with HFtn, the small size peak disappeared, and two new broad DLS bands emerged at 42.8 nm and 301.1 nm. (Figure 29).

In Z.P studies, the surface charge is typically used to determine the particle stability in a dispersed medium, surface adsorption, and surface chemistry due to the net charge of the different particles (Kim, I. et al., 2002, Chem. Mater., 14, 4874). When AuNRs are dispersed in CTAB, an electric double layer (EDL) is developed. The immobile layer close to the AuNR surface (the stern layer) neutralizes the surface charge of the AuNR, and an outer layer (the diffuse layer) is in equilibrium with diffusive ions with the bulk solution due to random thermal motion (Bou-Abdallah, F., 2010, Biochim. Biophys. Acta – Gen. Subj., 719). The boundary between the edge of this solvated diffuse layer and the bulk solvent is called the slipping plane. Hence, the potential of the slipping plane referenced to the bulk would provide the Z.P measurement (Behera, R. K. et al., 2014, Proc. Natl. Acad. Sci. U. S. A., 111, 7925). The Z.P study and ATR-FTIR characterization were performed to investigate the removal of CTAB from AuNR surfaces. The initial positive Z.P of AuNR-CTAB (+64.6 mV) decreased to -13.9 mV after the successful synthesis of AuNR-HFtn conjugates, as depicted in Figure 29B. This change in Z.P closely matched the Z.P value of HFtn (-14.5 mV), indicating the attachment of HFtn to the AuNRs, leading to the colloidal stability of the AuNR-HFtn conjugate system. Normally, AuNRs have a small Z.P ranging from +10 mV to -10 mV. It was hypothesized that an electrostatically induced positive charge on the surface of AuNRs contributes to the stabilization of the bioconjugate.

Conclusion

The study investigated the interaction synthesis and properties of AuNRs conjugated with HFtn. The UV-Vis spectrum of AuNR-CTAB exhibited distinct transverse and longitudinal bands at 520 nm and 800 nm, respectively. TEM images showed support for the attachment of HFtn to AuNRs. The UV-Vis spectrophotometry analysis revealed a ~50 nm redshift and broadened longitudinal plasmon resonance absorbance in AuNR-HFtn conjugates compared to AuNR-CTAB. The A.R of AuNR-CTAB and AuNR-HFtn were calculated to be ~4. The study focused on the removal of

CTAB from the surface of AuNRs during their synthesis and the subsequent functionalization of the AuNRs with HFtn. ATR-FTIR analysis confirmed the complete removal of CTAB from the AuNR surface in the presence of HFtn. DLS studies revealed that AuNR-CTAB exhibited two distinct hydrodynamic diameter peaks. This
5 experimental observation suggested that the presence of large HFtn assemblies on AuNRs facilitated the formation of spherical-shaped AuNR-HFtn conjugates. The initial positive Z.P of AuNR-CTAB was measured at +64.6 mV. However, after the complete synthesis of AuNR-HFtn bioconjugates, the Z.P shifted to -13.9 mV. This value was close to the Z.P of HFtn (-14.5 mV), indicating that the HFtn molecules became attached
10 to the surface of the AuNRs. Overall, the findings highlight the successful conjugation of HFtn to AuNRs and the modulation of their plasmonic properties.

Photochemistry of Gold Nanorod H-Chain Ferritin Bioconjugates

The prior research shows that the excitation of the Au nanosphere at 532
15 nm results in the (1) release of Fe(II) from the protein cage structure into solution and (2) the transfer of conduction band electrons from Fh core through the 2 nm protein shell, to reduce an exogenous oxidizing agent (e.g., Chromate) in solution. The primary motivation tested in the current chapter is to determine whether AuNRs chemically attached to HFtn can result in similar chemistry to when AuNSps are attached to Ftn. In
20 particular, will the NIR excitation of AuNRs attached to the exterior of Ftn lead to activation of the Fh core and result in Fe(II) release and electron transfer between the core and exogenous redox active species in the solution? AuNRs (adsorbs at 800 nm) were chemically attached to HFtn (AuNR-HFtn). HFtn has solvent-exposed cysteine groups on its 24 subunits, allowing Au-S bond formation to secure the AuNRs to the Ftn.
25 Firstly, the experimental procedure involved subjecting the AuNR-HFtn hybrid heterostructure to the illumination of an 850 nm NIR lamp. Through this process, a detailed examination and precise quantification of the release of Fe(II) into the surrounding solution was conducted. To accomplish this, a sensitive analytical method utilizing Fz was employed, which enabled us to accurately measure and quantify the
30 concentration of Fe(II) ions that were liberated during the experimental procedure. In a subsequent experiment, chromate (Cr(VI)) was introduced into the solution containing

the AuNR-HFtn and proceeded to expose it to NIR light. This step aimed to investigate the reduction of Cr(VI) to Cr(III) that occurred during the NIR exposure. By monitoring the amount of Cr(VI) reduction, insights were gained into the capability of the hybrid bioconjugate system to facilitate this specific redox chemical transformation under NIR illumination. Additionally, a pulsed laser source characterized by a higher energy density compared to the 810 nm NIR lamp was employed. By subjecting the heterostructure to this laser exposure, the dynamics of Fe(II) release and Cr(VI) reduction were analyzed.

Experimental

All chemicals were used as received. Pure analytical grade solid chemicals, solvents, and Deionized (DI) water ($18 \text{ M}\Omega \text{ cm}^{-1}$) were used in all preparations. TEOS (99.9%), Sodium hydroxide (NaOH, ACS reagent, Pellets, $\geq 97.0\%$), 5,5'-Dithiobis(2-nitrobenzoic acid (DTNB, $\geq 98\%$), Tetraethyl orthosilicate (TEOS, analytical standard), Ethanol (200 proof, ACS reagent, $\geq 99.5\%$), Phosphotungstic acid hydrate ($\text{H}_3[\text{P}(\text{W}_3\text{O}_{10})_4] \cdot x\text{H}_2\text{O}$, 99.995%), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate (Ferrozine, 98+% pure), Trisma base (primary standard and buffer, $\geq 99.9\%$), were purchased from Sigma-Aldrich. Sodium potassium tartrate ($\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$, Certified ACS), Hydrochloric Acid (HCl, Certified ACS plus, 36.5 to 38.0%), Hydrogen Peroxide (H_2O_2 , 30%, Certified ACS, Assay: 29.0 to 32.0%), were purchased from Fisher Scientific and HEPES buffer (ultra-pure) from American bio.

In a typical synthetic process, the initial synthesis and purification of a 10 mM AuNR-CTAB solution were followed by centrifugation at 14000 rpm for 15 min. The resulting supernatant was carefully removed, and the precipitate was subsequently dispersed into a 1 mM CTAB solution (5.0 mL). While gently stirring the mixture, the pH was adjusted to 10.4 by adding 0.1 M NaOH. Over the course of 30 min, three 5 μL injections of 20% (v/v) Tetraethyl orthosilicate (TEOS) in ethanol (TEOS: ethanol ratio of 1:9, v/v) were introduced at 30 min intervals. The solution was then left under stirring conditions for a minimum of 12 h at room temperature. Following this, the solution underwent centrifugation at 12000 rpm for 10 min, followed by three washes with

ethanol and subsequently with DI water. Then 50 μL of HFtn was added to the AuNR pellet and mixed in 2 mL of Tris-HCl (0.1 M, pH=7.4) to prepare the AuNR(SiO_2)-HFtn.

Ellman's reagent (4.0 mg L^{-1}) in sodium phosphate buffer (0.1 M, pH=8, 1mM EDTA) was prepared, and 20 μL was added to HFtn, mixed, and incubated for 15 min at room temperature. Afterward, UV-Vis absorbance was measured at 412 nm. The total solvent-exposed free thiol residues in the HSFn molecules were determined using the calibration curve established with L-cysteine (Figure 30).

Photochemical batch reactions

All photochemical experiments were conducted in a 1 cm^2 quartz cuvette (Thor Labs) with a 3.2 mL total sample volume using an 850 nm NIR illuminator (Univivi IR illuminator with 12 V/ 1A adaptor) as the light source. AuNR-HFtn samples and controls were individually exposed for 120 min, and absorbance spectra (1100–200 nm) were acquired for each photochemical reaction from the UV-vis spectrophotometer. Iron release studies were conducted with AuNR-HFtn, 32 mM sodium potassium tartrate, and 80 mM Fz in 0.1 M Tris buffer (pH=7.4). Quantitation of Fe(II) release was determined by UV-Vis spectroscopy at 562 nm due to Fe(II)-Fz complex formation. The Laser used in the experiments is produced from a Titanium Sapphire regenerative amplifier system. The linearly polarized laser pulse wavelength is in the 750-900 nm range with an adjusted center wavelength of $\sim 830 \text{ nm}$. The pulse width (FWHM) is $<100 \text{ fs}$ at a repetition rate of 1 kHz, with a maximum energy per pulse of 2.5 mJ.

Results and Discussion

Releasing bioavailable Iron for metabolic processes is one of the functions of Ftn (Koorts, A. M., 2007, Arch. Physiol. Biochem., 113, 30). According to Carmona et al., two well-known mechanisms for the iron release as Fe(II) cationic form are proposed (Carmona, F. et al., 2013, Coord. Chem. Rev., 257, 2752). Various reductants and iron chelators can be transported through the C3, and C4 channels, corresponding to hydrophilic and hydrophobic molecular pathways in Ftn, respectively (Plays, M. et al., 2021, Metallomics, 13; Sala, D. et al., 2017, J. Chem. Inf. Model., 57, 2112). These small molecules can reduce the mineralized iron oxyhydroxide core of Ftn to generate Fe(II)

and chelate them out of the Ftn (Watt, R. K. et al., 2013, Catal. Sci. Technol., 3, 3103; Cerkez, E. B. et al., 2019, Environ. Sci. Nano, 6, 85).

Prior studies have shown that the Ftn protein can be repurposed to release Fe(II) during exposure to light having an energy greater than the bandgap of the Fh bandgap: ~ 2.6 eV ($\lambda \leq 475$ nm), at least for laboratory mineralized Ftn. The chemical attachment of gold nanospheres on Ftn with a plasmonic resonance at 532 nm allows Fe(II) release to occur at much longer wavelengths of visible light. Data in Figure 31A and Figure 31B was acquired from a solution consisting of AuNR-HFtn in Tris HCl buffer (pH=7.4), Fz and, sodium potassium tartrate. Fz was used as a Fe(II) chelator that gives a sharp absorbance peak at 532 nm, which can be used to calculate the [Fe(II)] quantitatively. Analysis of data in Figure 31A shows that the AuNR-HFtn heterostructure allows Fe(II) release to occur during exposure to NIR radiation (well below the bandgap of the Fh core of Ftn). The Fz was used to chelate any Fe(II) leading to the AuNR-HFtn heterostructure during exposure to 850 NIR for 2 h.

When the reaction mixture was exposed to 850 nm NIR, 12.8 nM of Fe(II) was released from the heterostructure within 2 h. The Fe(II) release increased gradually with exposure times. According to Figure 31B, the Fe(II) release was 30.7 nM and 61.5 nM after 3 h and 5 h of exposure, respectively. Furthermore, a significant release of Fe(II) did not occur when the system was kept in the dark or when either HFtn or apoHFtn were utilized without AuNR under 850 nm NIR irradiation. These control experiments indicate that AuNRs must be attached to HFtn to facilitate Fe(II) during exposure to 850 nm NIR. Tartrate was added as a hole scavenger to provide sufficient electrons to the valence band of the AuNR to enhance hole-electron separation. The Fe(II) release was close to zero when the system did not contain tartrate (Figure 32A).

To further understand the mechanism involved with the iron release from the conjugated system, AuNR was synthesized incorporated with HSFtn (AuNR-HSFtn), (AuNR(SiO₂)-HFtn), and AuNR-HFtn(DTNB). A study was conducted to determine the release of Fe(II) in these three systems and compared it with (AuNR-HFtn) (Figure 32B). In addition, TEM images were taken for the diluted AuNR-HFtn, AuNR-HSFtn, AuNR(SiO₂)-HFtn, and AuNR-HFtn(DTNB) solutions after removing excess proteins. (Figure 32C-Figure 32F). AuNR-HSFtn bioconjugates were prepared by the same

method as AuNR-HFtn preparation except for incorporating HSFn instead of HFtn. HSFn does not contain surface-exposed cysteine residues for AuNR to attach to, which means that there is no strong binding between AuNR to HSFn (Figure 32C). TEM images showed no attachments between AuNR and HSFn except for a few proteins
5 closely arranged significantly far from the AuNR as protein islands (Figure 32D). In ongoing research, insulated silica shells have been utilized to create a thick barrier that prevents the formation of the Schottky barrier (S.B), to enhances the solubility and dispersion in water, and chemically protects the material. The presence of a S.B can impede the injection of hot electrons. SiO₂ insulating barrier (thickness >10 nm) could
10 interfere with the hot electron transfer from the metal to the semiconductor unless spectrally overlapped (Cushing, S. K. et al., 2015, J. Phys. Chem. C, 119, 16239). The control reaction done with the AuNR(SiO₂)-HFtn shows barely any release of Fe(II). Ellman's reagent (DTNB) is a water-soluble reagent used to quantify thiols. This rapid and stoichiometric reaction with thiol adds one mole of thiol to DTNB. The TNB²
15 released from DTNB can be analyzed spectroscopically by measuring the absorbance at 412 nm ($\epsilon = 14,150 \text{ M}^{-1} \text{ cm}^{-1}$). Every solvent-exposed cys residue in HFtn protein is capped and modified by DTNB. According to the results shown in Figure 33B, there is no Fe(II) release with AuNR-HSFn, AuNR(SiO₂)-HFtn, and AuNR-HFtn(DTNB) under 850 nm NIR exposure. The TEM images indicate that there is no strong interaction
20 between AuNR and HFtn in both AuNR(SiO₂)-HFtn and AuNR-HFtn(DTNB) systems. Hence, it suggests that without a firm attachment between the HFtn and AuNR, no photochemistry occurs in the bioconjugate system (Figure 32E and Figure 32F).

Experiments that investigated Fe(II) release from AuNR-HFtn when exposed to a pulsed laser source operating at 810 nm were conducted. After 1 h to the
25 laser, approximately 25 μM of Fe(II) was released into the solution (Figure 34), and after a 2 h exposure, the solution concentration of Fe(II) increased to 31.2 μM . Based on the ICP results, the maximum Fe(II) concentration that could be produced, assuming all the Fe in the HFtn was released, would be ~47 mM. After the same time, using the LED at 810 nm led to the release of ~9 nM. This difference in Fe(II) yield was attributed to the
30 laser source having a much higher power density (320 mW cm⁻²) compared to the NIR lamp (4 mW cm⁻²). Data in the Figure 34A also shows that the amount of iron released in

the absence of aqueous tartrate is lower ~15 mM than if the tartrate is present. It may be that hole-scavenging species are formed during water ionization that occurs with the high-intensity pulsed laser (Xu, J. et al., 2021, J. Am. Chem. Soc., 143, 10382). Without AuNR attached to the HFtn, there is no release of Fe(II), which implies that HFtn alone cannot release iron under NIR laser exposure under present reaction conditions. Compared to a continuous laser output, femtosecond pulsed laser light is beneficial due to its ability to introduce excessive local heat for only a short time around the AuNRs (Guan, G. et al., 2021, Adv. Healthc. Mater., 10, 2001158). Without AuNR in the system, there is no release of Fe(II), which implies that HFtn alone cannot release iron in these reaction conditions. Even when AuNR and tartrate are both absent in the system, it still doesn't release Fe(II). Hence, it clearly indicates that the AuNR is the critical component of the system that can facilitate the band gap excitation of the Fh core. Based on Figure 34B, it was observed that the release of Fe(II) from the AuNR-HFtn bioconjugate system increased as the exposure time to the laser was prolonged. Within the first 30 min, 13.9 mM of iron was released. Over the next 30 min, it increased to 25.6 mM and then to 31.2 mM in the following 60 min. However, as shown in Figure 35A and Figure 35B, after exposure to the femtosecond pulsed laser for 30 min, the AuNR did undergo reshaping (Gonzalez-Rubio, G. et al., 2017, Science, 358, 640) and the welding of particles together (Albrecht, W. et al., 2020, ACS Nano, 14, 12558). Figure 35A shows that the UV-Vis broad absorbance of the modified AuNRs showed new absorbance modes: one blue-shifted to 920 nm and another red-shifted to 750 nm.

To further understand the photochemistry of AuNR-HFtn, experiments were performed where solutions containing the present plasmonic heterostructure and Cr(VI) were exposed to 800 nm light. Prior research showed that the photoexcitation of Ftn with photons with energies ≥ 2.61 eV ($\lambda \leq 475$ nm) results in the bandgap of the Fh core being excited, and if Cr(VI) is present in solution it is reduced to Cr(III). Solutions investigated in these experiments consisted of AuNR-HFtn, 200 μ M Cr(VI), and 32 mM tartrate, stabilized in Tris HCl (pH= 7.4). The Cr(VI) concentration was determined by measuring the UV-vis absorbance of chromate at 372 nm. The reaction mixture containing AuNR-HFtn in Tris HCl, tartrate, and Cr(VI) received light exposure from the NIR illuminator for 120 min. This exposure time resulted in a reduction of the Cr(VI)

concentration by 11.7%. Interestingly, a reduction of Cr(VI) by 5.6% occurred even in the absence of HFtn. It is important to mention at this point that the reduction of Cr(VI) was insignificant in the control reactions that were carried out with apoFtn but in the absence of AuNR and Tartrate (Figure 36A). Hence, the excitation of AuNRs with the NIR is likely driving some Cr(VI) reduction. Prior studies have shown that gold nanomaterials can produce energetic hot electrons that are beneficial in redox reactions (Willner, B. et al., 2006, Curr. Opin. Biotechnol., 17, 589). Therefore, it is speculated that the AuNR has the ability, under NIR exposure, to reduce Cr(VI) by producing hot electrons through the LSPR effect.

Figure 36B exhibits Cr(VI) reduction studies in the presence of AuNR-HFtn when exposed to laser light. After 1h of laser exposure, 76.3% of Cr(VI) was reduced to Cr(III), whereas without tartrate in the reaction medium, the percentage conversion of Cr(VI) was 36.4%. This result suggests that tartrate can enhance the reaction progression rate, but without tartrate, the reaction still proceeds to an appreciable extent. If AuNR and tartrate are both removed from the system, no Cr(VI) reduction is observed. When there is no HFtn in the system, a 17.8% Cr(VI) reduction can be observed. Femtosecond laser pulses can ionize water molecules through multiphoton absorption. The possibility of hydrated electron generation may account for the reduction of Cr(VI) (Putri, K. Y. et al., 2022, Radiat. Phys. Chem., 199, 110269). When AuNR is absent from the reaction solution, there is hardly any conversion of Cr(VI) to Cr(III).

The Fh core in HFtn needs a minimum of 2.6 eV for the direct band gap. Therefore, it is impossible to achieve Fh direct band gap excitation with an incident energy of 1.45 eV (Figure 37A). Prior research has shown that metal-semiconductor heterojunctions can induce charge separation in the semiconductor via photonic enhancement (PE), plasmon-induced resonance energy transfer (PIRET), direct electron transfer (DET) or plasmonic hot electron transfer (PHETr), and plasmonic hot electron tunneling (PHETu) (Figure 38A and Figure 38B). A requirement for PE is that the energy of the incident photon is greater than the bandgap of the semiconductor. In the PIRET mechanism, plasmonic energy is transmitted and induces charge separation in a semiconductor through a dipole-dipole interaction. This mechanism involves the efficient collection of visible and NIR light with energies below the semiconductor band edge and

uses an insulating spacer layer to transfer energy non-radiatively, preventing interfacial charge recombination losses. The UV-vis absorbance band positions of AuNR-HFtn, HFtn, and Fh are shown in Figure 37B. Based on the band positions, the transverse band at 520 nm falls within the range of the Fh broad absorbance peak (300-600 nm.), while the longitudinal band has an absorbance too low of a wavelength range. The PIRET mechanism, however, is unlikely to occur if the localized surface plasmon resonance of the metal does not overlap with the absorption bands of the semiconductor. Therefore, the PIRET mechanism is not expected to result in a significant energy transfer within the AuNR-HFtn system.

Most LSPR effects for metal photocatalysis mechanisms, for (Au, Ag, Cu, etc.)/semiconductor (TiO₂, ZnO, CuO, etc.) heterojunctions, are commonly explained by the PHETr or PHETu phenomenon (Mubeen, S. et al., 2011, Nano Lett., 11, 5548; Shiraishi, Y. et al., 2017, Nanoscale, 9, 8349). Gold nanostructures are appropriate as metals due to their significant and tunable extinction coefficients in the visible and NIR regions (Jain, P. K. et al., 2006, J. Phys. Chem. B, 110, 7238; Ni, W. et al., 2008, ACS Nano, 2, 677). Semiconductors are selected based on their electron-accepting ability and density of state (DOS) in the conduction band. Energetic hot electrons can overcome the metal–semiconductor S.B if the semiconductor is located spatially within the plasmon's near field or in direct contact. Mechanistically, excited localized surface plasmons would decay non-radiatively due to ultrafast scattering processes (few tens of femtoseconds to picoseconds) such as electron–electron scattering and electron–phonon scattering followed by phonon–phonon relaxation with the surrounding medium (Qi, Z. et al., 2017, Nanotechnology, 28, 275202). These relaxation processes can generate high-energy hot electrons with energies between the vacuum energy and the Fermi level or closer to the Fermi level of the gold nanostructure on average. The hot electron injections should occur prior to loss of energy due to relaxation processes, or the energy of the hot carriers needs to be maintained far from the Fermi level to overcome the S.B for efficient hot electron-based applications (Kumarasinghe, C. S. et al., 2015, Sci. Rep., 5, 12140).

The hot-electron generation and injection efficiency in metal nanostructures depends on various factors such as the S.B height, surface roughness of the materials, morphology, the electric field enhancement inside the metal, and direction

of the electric field direction to maintain the correct orientation of the hot electrons. The electric field enhancement has been proven to be the highest inside nanorods compared with other traditional nano-shapes. Prior research has explained in detail the theoretical explanation and mathematical derivation of the hot electron generation in AuNRs and injection rates of hot electrons in contact with a semiconductor. For gold, the excited "hot holes" occur when energy levels reach 2.6 eV (476 nm) to 2.8 eV (442 nm), which is slightly above the interband transition (Wei, H. et al., 2020, Proc. Natl. Acad. Sci., 117, 15473). However, AuNR-HFtn conjugates absorb photons mainly around 1.46 eV (850 nm), which would suggest only hot electrons generation.

Even though the hot electrons are very energetic, they cannot transfer distances > 10 nm without losing the energy due to scattering (Scales, C., 2010, IEEE J. Quantum Electron., 46, 633). Intrinsic to the AuNR-HFtn bioconjugate is a ~ 2 nm protein shell barrier, which could be the rate-determining step for PHETr from AuNR to the conduction band of Fh. Previous research also suggests that hot electrons can transfer to the semiconductor conduction band more efficiently by tunneling through the S.B with lower energy requirements (Xue, J. et al., 2018, Chem. Commun., 54, 6052; Lee, Y. K. et al., J. Phys. Condes. Matter, 28, 254006). Hot electron-related ROS generation is a common phenomenon that explains the photochemical photocatalytic reactions that occur via PHETr mechanism (Gao, L. et al., 2014, ACS Nano, 8, 7260; Liu, Y. et al., 2022, J. Mater. Chem. B, 10, 7760). Prior research has reported various reactions involving hot carriers, including dissociation of hydrogen (Mukherjee, S. et al., 2014, J. Am. Chem. Soc., 136, 64), CO oxidation (Park, J. Y. et al., 2008, Nano Lett., 8, 2388), and NH_3 oxidation (Oshikiri, T. et al, 2016, Angew. Chemie Int. Ed., 55, 3942). Therefore, drawing upon previous scientific literature, a hypothesis involves the PHETr or PHETu process from the plasmonic AuNR to the conduction band of Fh via HFtn protein shell. This mechanism pathway is speculated to be the most plausible explanation for the excitation of the Fh bandgap under NIR exposure, even when the energy of the NIR radiation falls significantly below the band gap energy of the Fh material. However, to fully understand how the AuNR-HFtn bioconjugate facilitates electron/energy transfer for photocatalytic reactions, additional theoretical, computational, and experimental research is required.

In conclusion, the findings of this study highlight the remarkable capability of the AuNR-HFtn heterostructure to facilitate Fe(II) release under NIR radiation, even at energy levels significantly below the bandgap energy of the Fh core material in HFtn (>2.6 eV). The exposure of the reaction mixture to 850 nm NIR resulted in the release of 12.8 nM of Fe(II) within 2 h, with a gradual increase observed over time, reaching 61.5 nM after 5 h. In contrast, the control study involving AuNR-HSFtn, AuNR(SiO₂)-HFtn and AuNR-HFtn(DTNB) demonstrated minimal Fe(II) release compared to the AuNR-HFtn bioconjugate system. It signifies the proper attachment between AuNR and HFtn for effective Fe(II) release. Notably, femtosecond pulsed laser exposure led to a substantial release of Fe(II), with approximately 25 μ M released after 1 h compared to 9 nM under NIR lamp exposure for 2 h, owing to the significantly higher power density of the laser source. However, the exposure to a femtosecond pulsed laser caused the reshaping of AuNR and the welding of particles with a shift in longitudinal peak position. Furthermore, the reduction of Cr(VI) demonstrated a 12% reduction under 2 h of NIR light exposure, while laser exposure resulted in a remarkable 76% reduction. These results suggest that the Fe(II) release from the AuNR-HFtn bioconjugate system has the potential to induce redox reactions in the surrounding medium. The proposed mechanistic explanation suggests that PIRET is unlikely to occur. Instead, the postulated mechanism involves PHETr/PHETu through HFtn shell from AuNR to the Fh core material, thereby facilitating Fe(II) release under NIR exposure, even below the direct band gap excitation energy of Fh. These findings contribute towards the understanding of the underlying processes and potential applications of the AuNR-HFtn heterostructure in controlled release and redox reactions.

Example 3: Gold Nanorod H-Chain Ferritin Bioconjugates to Invoke Cellular Ferroptosis and Destroy Cancer Cells

The present work is further drawn to, in part, the use of the AuNR-HFtn bioconjugates as a photodynamic strategy utilizing NIR to suppress the growth of cancer cells through the process of ferroptosis. Experiments were carried out to expose prostate cancer cells (PC3) to AuNR-HFtn, and during NIR irradiation, they showed the ability to limit the growth of the cells compared to experiments where the cells were exposed to

just HFtn or AuNRs. The results suggested that Fe(II) released from the HFtn led to cancer cell death through a process that might be ferroptosis. Future studies will need to investigate this possibility and whether the bioconjugates developed herein will offer a novel therapeutic strategy for cancer tumor suppression.

5 The primary goal of the ideal cancer therapeutic is to eliminate cancerous cells in their entirety while leaving healthy cells to function normally. The benefits of traditional chemotherapies are severely limited by the overall toxicity of the active component, thereby limiting the drug dose and length of treatments. Recently, the use of gold nanoparticles (AuNPs)—typically nanorods (AuNRs) —under near infrared
10 radiation (NIR) exposure for plasmonic photothermal therapy (PPTT) of tumors has received significant interest in the medical community and entered clinical trials in 2016. However, scientific challenges that exist and need to be overcome to realize the full potential of PPTT include (1) a low specificity of AuNPs for cancerous cells, (2) a need for high fluxes of NIR to reach deep tissue tumors to induce thermal heating of AuNPs,
15 and (3) a low biocompatibility of surface ligands attached to AuNPs that are necessary to facilitate their transport in living tissue. The present work tests whether these challenges can be overcome by using a novel bioconjugate platform that consists of AuNPs chemically complexed to the iron storage metalloprotein, ferritin (Ftn), where the Ftn acts as both a biocompatible stabilizing surface “ligand” and photoactive center. It is
20 hypothesized that this bioconjugate (Au/Ftn) will overcome the low specificity of AuNPs for cancer cells, since cancerous cells exhibit high concentrations of transferrin receptors (TfR1) that will bind the Au/Ftn bioconjugate. Furthermore, the Au/Ftn will exhibit unique properties in the presence of NIR to overcome low NIR fluences in deep tissue tumors. In particular, exposure of Au/Ftn to NIR not only produces heat for PPTT, but
25 also results in Fe²⁺ release from Ftn at low NIR fluence that may kill cancer cells through ferroptosis. This unique photochemistry afforded by Au/Ftn makes these bioconjugates potential agents for photodynamic therapy (PDT) that relies on the generation of reactive oxygen species at cancerous sites.

 There is an extensive literature detailing the development of plasmonic
30 gold nanoparticles (AuNPs) for plasmonic photothermal therapy (PPTT) treatment of tumors by exploiting the local hyperthermia that results when AuNPs within the tumor

are exposed to light aligned with the surface plasmon resonance (SPR). A recent clinical trial using PPTT showed promising results for the ablation of prostate tumors and does bode well for the future investigation and application of PPTT as a tumor suppression therapy. Prior research has shown that the size and shape of the AuNP affect the energy position of the SPR, but these physical characteristics of the particles also affect the uptake of the AuNP by cellular components that make up healthy and/or cancerous tissue. Significant challenges still exist in this area since relying on the porous vascular system of cancerous tissue, as hypothesized by the enhanced permeability and retention effect (EPR), does not lead to enough selectivity for effective localization of the AuNPs at the tumor site. A well employed method is to use polyethylene glycol (PEG) as the stabilizing ligand on AuNPs to increase the residence time of the particles so that their delivery to tumor cells can be optimized, yet even with extended time this type of passive diffusion based delivery is hardly tumor-specific. Further, the extension of these systems to clinical trials have suffered in part due to the scattering of NIR light as it passes through tissue resulting in the loss of intensity and ultimately the loss of hyperthermal effects at the AuNP site.

In contrast to PPTT, the use of photodynamic therapy (PDT) has had a significant presence in the area of cancer treatment in the clinic for over 40 years. In general, the PDT photosensitizer agent when irradiated with specific light wavelengths produces reactive oxygen species (ROS). Traditional PDT therapies generally have used porphyrins that absorb visible light to produce singlet oxygen that can kill cancerous tumors (when localized in the tumor). Prior research has investigated AuNP-based systems that can exhibit both plasmonic heating (i.e., hyperthermia) and photodynamic properties, but each property can only be activated by a different region of the electromagnetic spectrum. For example, a Au/Ag nanocage with a photodynamic sensitizer showed plasmonic heating near 800 nm and ROS generation during 630 nm irradiation that could be used to kill cancerous cells. The largest barrier facing PPTT therapies is that human tissue is not transparent to visible light, thus treatments typically occur via surgical means.

The present synthetic strategy uses ferritin (Ftn) to template the chemical attachment of plasmonic gold nanorods (AuNRs) and gold nanostars (AuNSs). The

heterostructures that are developed and investigated have photothermal properties conducive to PPTT, but more importantly they will exhibit distinct and novel properties that will overcome the challenges that limit the more conventional AuNP systems that solely rely on PPTT for tumor suppression. The use of Ftn as the present scaffold will
5 allow the docking of the moiety at cellular receptors such as transferrin receptor (TfR1), which is overexpressed by various types of cancer cells. Hence, the presence of the Ftn allows the selective localization of the heterostructured therapeutic particles at the tumor site. Furthermore, the present heterostructures provide a photodynamic therapy (PDT), where the light-induced release of Fe^{2+} from AuNP/Ftn will drive ferroptosis for tumor
10 suppression (i.e., PDT) at NIR intensities that would not support hyperthermia effects central to PPTT.

Currently, the potential application of AuNPs for PPTT is limited due their low specificity for cancerous cells and the need for high intensity NIR to reach deep tissue tumors to induce thermal heating of the particles. The present research is
15 innovative in that it will generate Trojan horse heterostructures-composed of AuNPs and Ftn-that inherently target cancer cells that will be operative in deep tissue therapies, since lower NIR exposures can be used. The novel property of the bioconjugate is that excitation of the AuNR (or AuNS) complexed to the metalloprotein, ferritin, will lead to ferrous release, leading to ferroptosis and the killing of the host cancer cells. This ability
20 to control iron release from Ftn through the low NIR intensity excitation of the plasmonic particle-by imparting new function to a supramolecular protein assembly-is innovative. A short-term objective is to demonstrate proof of concept for the selective and effective treatment of high priority cancer targets, including prostate cancer (CRPC) and triple negative breast cancer (TNBC) lines that present unmet biomedical needs, by varying the
25 building blocks of the Au/Ftn heterostructures. The proposed studies will develop synthetic methods to create functional hybrid plasmonic bioinorganic materials that can ultimately be used in biomedical therapeutic applications.

Synthetic routes are developed herein to functionalize Ftn protein cages (~12 nm diameter) with AuNRs, schematically shown in Figure 39, or gold nanostars (AuNSs) that exhibit photodynamic and photothermal properties within the 600 to 1000
30 nm NIR window and investigate the NIR-induced photochemical and photothermal

properties exhibited by the heterostructures. The aim will include quantifying the release of ferrous iron (Fe^{2+}) and formation of oxidizing species under NIR irradiation from the heterostructures. Further, the potential of the present bioconjugates for inducing ferroptosis ex-situ by using phospholipid vesicles is probed—a proxy for cell membranes—to study PDT induced lipid peroxidation.

Further, the effect of the novel heterostructures under NIR irradiation on the viability of cancerous cell lines was investigated. NIR power densities were varied to understand how ferrous release from Ftn affects cell viability and whether the combination of iron release for ferroptosis and plasmonic induced heating effects show synergy for enhanced suppression of cancer cell growth, compared to either strategy alone. The work was benchmarked against current clinical methods and identify the specificity of the treatment by also treating healthy cells.

An objective of the present work is the development of a Au/Ftn framework for novel therapeutics which can overcome the current limitations identified for gold nanoparticle PPTT. Through the fundamentally linked aims strategies for synthesis of Au/Ftn heterostructures are honed to determine whether the novel bioconjugates have long-term potential as transformative materials for the selective elimination of cancerous cells via PPTT and PDT.

An objective of the present work was to understand mechanistic aspects of the photochemistry of Ferritin (Ftn) and repurpose this protein—designed by nature to sequester iron in organisms—to drive chemistry relevant to environmental remediation and biomedicine. Toward this end, the first goal was to understand how the visible light-induced photochemistry of Ftn with solution phase redox active species depends on the nature of the mineralized metal hydroxide-oxide (hydr(oxide)) core. The second goal was to utilize this understanding to develop photoactive bioconjugates consisting of Ftn functionalized with plasmonic gold nanospheres (AuNSps), nanorods (AuNRs), and nanostars (AuNSs) and to understand their photochemistry in the presence of visible and near-infrared light. A final objective was to investigate the application of these bioconjugates for diverse applications such as environmental chemistry and as cancer tumor suppression strategies.

Prior research has shown that Ftn containing an iron-bearing oxyhydroxide core material can, if exposed to the light of particular wavelengths, drive redox reactions in an aqueous solution (Shin, K. M. et al., 2010, *Electrochim. Acta*, 55, 3486; Kim, I. et al., 2002, *Chem. Mater.*, 14, 4874; Watt, R. K. et al., 1992, *Biochemistry*, 31, 9673; Chen, P. et al., 2020, *Sci. Rep.*, 10, 4033). There are still outstanding issues that prevent a more complete understanding of the photochemistry of Ftn with a Fh core. The first issue that needs to be resolved is whether the photochemistry exhibited by the protein is due to light-induced Fe(II) release into the solution. Prior studies have shown this to be a photochemical pathway if Ftn is exposed to photon energies equal to or greater than the bandgap of the inorganic core material. The aqueous Fe(II) can then presumably undergo reactions with redox-active reactants in the solution. A second issue is to determine whether the photochemistry exhibited by Ftn is due to the excitation of the small bandgap semiconductor core material, and the subsequent production of conduction band electrons that transfer across the Ftn protein shell to reduce redox active species in solution. Prior research has not been able to distinguish between these two possibilities in a conclusive manner. This issue was addressed by investigating the photocatalytic behavior of Ftn after assembling a small bandgap copper-based core material within the globular protein that was insoluble under visible light irradiation. The photochemistry of this copper hydr(oxide)-Ftn (CuFtn) was then investigated in the presence of the oxidizing agent chromate (Cr(VI)). CuFtn photochemistry was shown to include the reduction of hexavalent chromium (Cr(VI)) to trivalent chromium (Cr(III)), showing for the first time that the transfer of reducing electrons from the semiconductor core of Ftn to an exogenous redox active species was a viable mechanistic pathway. The research also showed the potential application of CuFtn for the removal of hazardous environmental pollutants (i.e., Chromate).

Surface Functionalization/Modification of Ftn

As stated previously, bioconjugates investigated herein consist of AuNPs chemically linked to HFtn or HSFtn. In the HFtn circumstance, solvent-exposed cysteine groups are present on the 24 self-assembling H-subunits allowing Au-S bonding to link the AuNPs and protein. In the HSFtn circumstance, there are no solvent-exposed cysteine

groups, so HSFtn with -SH groups were functionalized by reacting Ftn with N-succinimidyl S-acetylthioacetate (SATA).

Cys has a significantly high affinity to gold due to the interaction between its sulfhydryl/thiol (-SH) side chain and gold surface (Nie, H.-Y. et al., 2021, Biointerphases, 16, 21005). Exploiting the affinity of the Au to S has been central to prior studies that have been interested in the bonding of Cys-containing peptides and proteins to gold nanostructures (Shao, Q. et al., 2016, Langmuir, 32, 7888). In Ftn, zwitterionic Cys residues have been utilized as a cross-linker to attach proteins to gold nanostructures. The formed “Au-S” bond (thiolate–Au⁺) is a coordinative interaction with a homolytic strength of ~40 kcal/mol (Burgi, T., 2015, Nanoscale, 7, 15553). The HFtn carries 100% H subunits and three cys residues per subunit, namely Cys90, Cys102 located on the shell surface, and Cys130 inside the pore (Lucignano, R. et al., 2022, Int. J. Mol. Sci., 23, 22). HSFtn contains about 90% L-chain and 10% H-chain. The HSFtn L-chain subunits contain two Cys, (Cys52, Cys130), but they are not solvent exposed and available for linking to AuNRs and AuNSs (Takeda, S. et al., 1995, J. Biochem., 117, 267).

To overcome the lack of solvent-exposed cys groups on the surface of HSFtn, SATA, a well-known thiolation reagent was used. SATA reacts with primary amines via its N-hydroxysuccinimide (NHS) ester, forming a stable covalent amide linkage (Figure 40) (Duncan, R. J. S. et al., 1983, Anal. Biochem., 132, 68). Various antibodies, antigens, proteins, and viruses modified by SATA to introduce thiol groups for different applications have been widely investigated in the literature. Rösch and coworkers found a novel method to synthesize stable radioarsenic labeled proteins by modifying the two globulins, the mabs ch3G4 and Rituximab antibody, with SATA. After deprotection of the thiol by deacetylation of the SATA-modified antibody, radioactive Arsenic iodide [⁷⁵As]AsI₃ was conjugated via As-S bond formation (Jennewein, M. et al., 2006, Nucl. Instruments Methods Phys. Res. Sect. A Accel. Spectrometers, Detect. Assoc. Equip., 569, 512). Similarly, an anti-TAR DNA-binding protein 43 (anti-TDP-43) was modified by Dai et al. using SATA-mediated thiolation as a sensing scaffold for identifying various biomarkers (Dai, Y. et al., 2018, Biosens. Bioelectron., 117, 60). Lin et al. developed a ruthenium-based fluorescent complex for biosensing by crosslinking with SATA-modified protein G. The complex has been

proven to be a universal fluorescent probe for immunoassays (Lin, J.-T. et al., 2012, PloS One, 7, 1). Furthermore, Greener bioreduction of AuCl_4^- at neutral pH on the SATA-modified M13 bacteriophage (M13-SH) was introduced by Zhang and coworkers. They showed residual hydroxylamine converted AuCl_4^- to fused AuNPs, which linearly deposited on the virus. Pfs230 antigen is a candidate for a potential Malaria transmission-blocking vaccine (Wei, Z. et al., 2022, ACS Omega, 7, 9951). SATA-modified Pfs230 antigen has been tested for improving the functional activity of the vaccine (Scaria, P. V. et al., 2019, npj Vaccines, 4, 24).

In the present work, SATA was employed to conjugate the amino acid Lys present in HFtn. Modification of Lys using SATA resulted in the formation of stable and protected -SH groups. These protected -SH groups could be subsequently deprotected using hydroxylamine-HCl. Furthermore, this work provides comprehensive details regarding the utilization of SATA-modified HFtn for achieving highly efficient attachment of AuNSps via Au-S interactions.

To quantify the amount of thiolation, 5,5'-dithiobis(2-nitrobenzoic acid (DTNB) was used herein. DTNB reacts with the free sulfhydryl side chain of cys, forming a S-S bond between the Ftn protein and a thionitrobenzoic acid (TNB) residue (Figure 41). The modification of Cys with DTNB is usually performed at pH 7.0-8.0 and can be quantitatively and qualitatively analyzed by Ultraviolet-Visible (UV-Vis) spectroscopy. The TNB^{2-} anion released from the reaction shows a peak at 412 nm, which corresponds to its yellow color.

Synthesis and Photochemistry of Au/Ftn Heterostructures

Ftn, which sequesters and stores iron in biological systems, is an excellent choice as the biological surface stabilizing ligand for AuNP for potential therapeutic applications. Ftn is a well-studied protein, though controversies remain. Ftns are comprised of 24 subunits that self-assemble to form a protein cage structure. The outside diameter of the cage is 12 nm, surrounding a cavity of roughly 8 nm in diameter (Figure 39). Many ferritins feature variable ratios of heavy (H-chain) subunits, which have a diiron ferroxidase center, and light (L-chain) subunits, which lack this center. The protein is remarkably stable; its isolation includes heating to $\geq 65^\circ\text{C}$ for ≥ 10 min. The protein

cage is also electrically conductive. Prior studies have generally concluded that electron transport across the shell of Ftn is rather facile, and that the shell conductivity depends on the relative proportion of H- and L-chain subunits, the suggestion being that the L-chain is more conductive than the H-chain subunit. Within the interior of the 24mer, up to 4500 Fe atoms are stored as a NP of iron oxide. The resulting mineralized iron oxyhydroxide has been shown using X-ray scattering techniques to be the ferrihydrite phase (nominally, Fe(O)OH).

The interaction (via energy and/or electron transfer) between plasmonic Au nanospheres and the inorganic Fe(O)OH core of Ftn can be achieved and exploited for facilitating photoredox chemistry of metals during visible light exposure. In particular it has been shown that the excitation of the SPR of plasmonic gold spheres (7 nm) chemically attached to Ftn with visible light (532 nm) resulted in the liberation of Fe²⁺ in the presence of a hole scavenger. Importantly, and most relevant to the present studies are preliminary results shown below that show that the same release of Fe²⁺ can be achieved by exposing AuNR/Ftn to NIR, according to the schematic in Figure 42. It was believed that the mechanistic pathway for Fe²⁺ release from Au/Ftn involves the transfer of hot electrons from the plasmonic Au to the conduction band (CB) of the semiconductor core of Ftn where they can participate in redox chemistry (i.e., as a reducing electron).

20 *Synthesis of Heterostructure Components*

Purified H-chain and L-chain human Ftn are in hand (~50 mg/L of *E. coli* culture). Various mixed compositions from 100% H-chain to 100% L-chain will be prepared by denaturing mixtures of the two proteins and renaturing the heteropolymer 24mers. Horse spleen ferritin (HSFtn) is readily commercially available and is 90-95% L-chain and 5-10% H-chain. H-chain Ftn has ferroxidase sites allowing the efficient mineralization of the ferrihydrite core by catalyzing the oxidation of Fe(II) to insoluble Fe(III). The iron oxyhydroxide core in the L-chain protein were mineralized per established procedures in which Fe(II) can be mineralized in the presence of H₂O₂ and methanol. In this case the mineralization process is not catalyzed by a ferroxidase site, but instead is a Fe(III) hydrolysis reaction. Necessary additional mutants can be produced by using standard mutagenesis techniques.

The synthesis of AuNRs and AuNSs will follow protocols described in the literature. In the case of AuNRs, an established protocol was used to synthesize the plasmonic materials with aspect ratios (AR, length divided by width) ranging between ~2.2 and 3.8. AuNRs with an AR of 3.8 (22 nm length and 6 nm width) absorb at $\lambda_{\text{max}} =$
5 ~790 nm (position of the longitudinal SPR). These synthetic protocols will yield AuNRs capped with cetyltrimethylammonium bromide (CTAB). This capping agent is displaced by chemical moieties having thiol functionality to form a strong S-Au interaction. The thiol groups (i.e., cysteine) on Ftn provide this affinity for the AuNRs. Following established protocols, AuNS morphologies will be generated via an established seeded
10 method using Triton-X, ascorbic acid, and AgNO₃. Nominal sizes of ~50 nm will be targeted although the number and lengths of branches will be determined with transmission electron microscopy (TEM).

Conjugation to Form Heterostructures

15 Purified H-chain and L-chain human Ftn are in hand (~50 mg/L of *E. coli* culture). Various mixed compositions from 100% H-chain to 100% L-chain will be prepared by denaturing mixtures of the two proteins and renaturing the heteropolymer 24mers. Horse spleen ferritin (HSFtn) is readily commercially available and is 90-95% L-chain and 5-10% H-chain. H-chain Ftn has ferroxidase sites allowing the efficient
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Studies of Au/Ftn for PDT

The rate of Fe²⁺ release from AuNP/Ftn heterostructures where the Ftn component has different, but well-defined relative proportions of H- and L-chain subunits was investigated. Ferrozine was used as the chelating agent of Fe²⁺ to quantify the concentration of Fe²⁺ by monitoring the Fe²⁺-ferrozine complex at 562 nm with UV-Vis. Prior research has suggested that the L-chain protein subunit allows more facile electron transport across the Ftn cage than does the H-chain subunit. It is pointed out that horse-spleen Ftn, which is predominately composed of L-chain subunits has already exhibited conductivity through the cage as evidenced by its successful imaging by scanning tunneling spectroscopy (STM), and conductive AFM.

The rate of Fe²⁺ release from the AuNR/ and AuNS/Ftn end members (i.e., all H-chain or L-chain Ftn) was compared to determine which AuNP/Ftn heterostructures are most amenable for inducing ferroptosis. Furthermore, on a fundamental level, comparing the kinetics of the photochemistry of AuNP/Ftn (L-chain) and AuNP/Ftn (H-chain) allows us to discern the contribution of the H- and L- chain to the charge transfer process. A hypothesis to be tested here is that AuNP/Ftn (H-chain) will show a reduced rate of Fe²⁺ release in the presence of NIR relative to AuNP/Ftn (L-chain). If the hypothesis is experimentally confirmed it will further support the belief that electron transfer from the plasmonic AuNP (as a hot-electron) to the inorganic metal oxide is a critical mechanistic step.

The AuNP morphology that leads to the most efficient AuNP/Ftn architecture for NIR induced iron release was determined. The best performing Ftn composition with regard to Fe²⁺ release in the presence of NIR will be used as the scaffold to individually bind AuNRs and AuNSs of different sizes. The different sizes

will be associated with a range of SPRs throughout the NIR region (800-1000 nm). The effect of the SPR energy position on the rate of iron release was investigated. LEDs emitting in the NIR will be used to access the different wavelengths for these experiments.

5 The photochemistry of AuNP/Ftn under conditions relevant to ferroptosis mimicking *in-vivo* conditions was investigated. Experiments will be carried out using AuNP/Ftn architectures shown to be effective in Fe^{2+} liberation under NIR. Solutions containing AuNP/Ftn were prepared with and without bilayer-forming polyunsaturated phosphocholine lipids (PL) that will serve as proxies for the bilayer forming cellular
10 membranes. Work was initiated with L- α -phosphatidylcholine (Soy PC) that contains two double bonds in a fatty acid chain bridged by a methylene group. Polyunsaturated lipids with similar structures are most susceptible to ferroptosis. Reactions will be carried out in solutions that are anoxic, oxic, or ones that contain H_2O_2 . In the absence of PL, for example, but in the presence of AuNP/Ftn and H_2O_2 , hydroxyl radicals are produced due
15 to Fenton chemistry initiated by Fe^{2+} liberation under NIR. The production of any short-lived $\text{OH}^\bullet$ will be trapped by coumarin to form the fluorescent 7-hydroxycoumarin adduct. A key product resulting from the peroxidation of the unsaturated lipid is malondialdehyde (MDA) and this species can be quantified via the thiobarbituric (TBA) acid assay where a the reaction of TBA and MDA forms a red-adduct with a λ_{max} of 532
20 nm. Research suggests that TBA can yield false positives in the presence of aqueous iron, thus high pressure liquid chromatography will be used to quantitate MDA in the present reactions.

Studies of Au/FTN for PPTT

25 Here, the stability and solution thermal effects (i.e., temperature rise) of the plasmonic particles during the excitation of the SPR of the AuNP with laser light is investigated. The photothermal conversion efficiency (η) of the different Au morphologies attached to Ftn will be investigated using a method described in the literature. In particular NIR lasers (including 532 and 808 nm) with power densities in the
30 range of 1.3, 2.6, and 4.2 W/cm^2 were used. At a given excitation wavelength, the photothermal properties of the AuNR/Ftn and AuNS/Ftn will be compared at a similar

optical density (achieved by varying the concentration of a particular AuNP/Ftn system).⁵¹ The time dependence of the temperature change of the solutions (in a cuvette) will be measured with a thermocouple up to the steady state maximum temperature.

Along with this temperature, η can be calculated utilizing the optical density and laser power. The photothermal properties of the AuNP/Ftn will be compared to the protein free systems (and compared to the literature).

Preliminary Results

AuNRs were complexed with Ftn by the co-mixing of AuNRs with H-Ftn. Each of the 24 protein subunits that make up H-Ftn have two solvent exposed cysteines that allow the linkage through the creation of Au-S bonds. AuNRs were prepared in solutions containing the surfactant cetyl trimethyl ammonium bromide (CTAB). Removal of the CTAB was accomplished by centrifugation of the AuNRs. The supernatant, which contained CTAB was decanted off and the AuNRs were redispersed into a buffered solution containing H-Ftn. This procedure led to the capping of the AuNRs with H-Ftn. Samples analyzed by TEM were prepared by pipetting an aliquot of AuNR/Ftn solution and then dispersing it on a TEM grid. The grid was then washed with DI water and exposed to 2% phosphotungstic acid, which negatively stained the sample. Shown in Figure 43A, a gold nanorod (dark rod) is surrounded by ferritin protein, visualized by white hollow circles. Figure 43B shows the UV-vis spectrum of the AuNRs (black) prior to exposure to H-Ftn. These data exhibit characteristic transverse and longitudinal surface plasmon resonances associated with AuNRs at 530 and 800 nm, respectively. After conjugation to H-Ftn, a decrease in intensity due to dilution is observed but additionally, a shift to 850 nm is observed for the longitudinal SPR. This shift in the SPR was due to changes in the local environment around the nanorod.

NIR light emitting diodes ($\lambda=850$ nm) were used to photoexcite AuNR/Ftn, showing a release of 60 nM ferrous iron from AuNR/Ftn after 300 minutes of NIR irradiation. In these experiments ferrozine was used as a chelator to complex Fe^{2+} released from the Ftn. Tartrate was used as a hole scavenger in solution. No Fe^{2+} was released from the Ftn in the absence of light. Due to the relatively low flux of NIR photons, minimal heating of the solution was experimentally observed. This result

supports the contention that iron release to produce ferroptosis in cells can occur at radiation fluxes lower than those needed for photothermal heating of the plasmonic AuNPs. AuNR/Ftn were irradiated in solutions containing H₂O₂, which has relevance to the increase presence of this molecule in tumor cells relative to healthy cell. To determine whether the release of Fe²⁺ led to Fenton chemistry and the production of OH• coumarin was introduced into the solution. The reaction of OH• with coumarin leads to the fluorescent product, 7-hydroxycoumarin. The results indicate that upon irradiation with NIR, fluorescence at 455 nm is observed, associated with the 7-hydroxycoumarin adduct. Hence, ROS species were formed via NIR excitation of the heterostructures.

The first two conjugation protocols will work well for attachment of Ftn to AuNPs, but a third protocol is available if needed. A linker could be attached with amine and thiol functionality to the protein via reaction between a surface carboxylate and the linker amine. One study, for example, used a thiol-PEG (polyethylene glycol)-amine linker to anchor HSFtn to a gold surface. This protocol, however, is the least preferred because the linker will lead to an increase in the distance between the protein cage (and inorganic core) and the AuNP, which would presumably decrease any electronic interaction between the AuNP and inorganic Fe(O)OH core of the Ftn. This strategy, however, could be used to determine the distance dependence of the AuNP-inorganic core interaction by using linkers of different lengths.

Cancer Killing Effectiveness of Heterostructures

Relevant to the goals of the present work is prior research that has shown that specific sites on the Ftn protein cage show selective binding to receptors on cancer cells. In particular, Ftn has a high affinity for binding to the transferrin receptor 1 (TfR1), which is over expressed in many primary and metastatic human cancers. In normal tissues TfR1 is actually downregulated, making Ftn selective for cancerous cells. Prior studies have shown that Ftn binding to TfR1 results in its incorporation into cellular endosomes and lysosomes. This affinity of Ftn for cancer cells has been exploited in prior research where Ftn was used as a delivery agent of drugs to cancer cells for tumor suppression. Furthermore, research on the immunoreactivity of HFtn based treatments indicates low proinflammatory cytokine release when fully purified, demonstrating the

safety of HFtn for medical treatments. This affinity of Ftn for cancer cells can be exploited to localize the AuNP/Ftn particles at tumor sites. This expectation is supported by prior research that showed that Au nanospheres with bound transferrin were incorporated into human nasopharyngeal carcinoma cells. A later study also showed that the increased incorporation of the Au nanospheres coated with transferrin markedly reduced the power needed for photothermal treatment with ~530 nm (i.e., visible) light relative to uncoated Au nanospheres.

The combination of photothermal and photodynamic strategies into one system has been investigated. In at least two cases ferroptosis was induced by the inactivation of Fe regulating biochemical channels in the cell. Recent research, for example, has investigated the use of gold AuNS with alloyed Pt to allow the concurrent activation of localized hyperthermia and ferroptosis. Irradiation of this Au/PtNP with 808 nm NIR resulted in hyperthermia and the release of aqueous Au and Pt species that the authors argue induced ferroptosis, either via the inactivation of cellular glutathione peroxidase 4 (GPX4) and/or the depletion of glutathione (GSH). In another study the photothermal properties of Au nanocages (during irradiation with 808 nm light) were used along with the release of RSL3 (an inducer of ferroptosis) which was loaded within the nanocage. By using ferritin as the stabilizing ligand both photothermal and photodynamic strategies can be harnessed while also targeting the cancerous cells.

Design of Cell Line Studies

As a guide prior investigations of Yang et al. where the effect of ferroptosis on different types of cancer cell lines were studied. The representative CRPC cell line, PC-3 used in preliminary work described herein, is used as well as the typical TNBC cell line, MDA-MB-231, that has been shown to be growth-suppressed under ferroptosis conditions. Furthermore, this same cell line has been shown to be growth-suppressed under photothermal conditions using AuNRs exposed to a NIR laser. Hence, it will be determined whether ferroptosis can be initiated at low NIR intensities where photothermal effects are minimal and whether ferroptosis can enhance cell death at higher NIR fluxes where photothermal effects are maximized. The identification of ferroptosis as the specific mechanism for cell killing will be achieved by using standard

western blot assay to confirm the downregulation of glutathione peroxidase (GPX4) protein and upregulation of acyl-CoA synthetase (ACSL4); followed by qPCR-based validation of the upregulation of PTGS2 and CHAC1 genes.

The cell line studies will feature a series of variables including incubation
5 time, length of light exposure, and intensity of light exposure. By changing incubation time, differences in the heterostructures presence in the extracellular fluid versus associated with the cell can be isolated. By changing the length of light exposure, it can identify at what point cell death occurs. And finally, by changing the intensity of exposure, PDT occurring at low fluxes versus combined PDT and PPTT which will occur
10 as the flux increases can be isolated. Additionally, heterostructures which contain no iron are used within the ferritin cage to determine the extent of iron released ferroptosis. Finally, the work is baselined by comparing to the current state of the art, i.e. using PEGylated gold nanorods under the same conditions in cancer lines and the effect that heterostructures have on healthy cell lines, such as prostate tissue cells, RWPE-1, and
15 breast epithelial cells, MCF10A, to ascertain the selectivity of the heterostructures to cells with over-expressed TfR1 receptors.

Preliminary Results

The present work used a prostate cancer cell line (PC-3) to test initial
20 heterostructures for their ability to suppress the growth of cancer cells. Cells were incubated with AuNR/Ftn for 2 or 24 hours and then some wells were exposed to NIR light emitting diodes ($\lambda=850$ nm) for 24 hours at a flux of 4 mW/cm² (Figure 44A). For a 2-hour incubation, control and treatment wells all showed greater than 95% cell viability. In contrast, tests with a 24-hour incubation showed marked differences, where the control
25 group and dark treatment wells continued to exhibit >95% cell viability. However, the treatment group exposed to low flux NIR radiation resulted in <70% cell viability. Bright field microscopy demonstrated that after 24 hours incubation, dark aggregates were within the confines of the cell walls without loss of shape or volume (Figure 44C) compared to untreated cells (Figure 44B). After exposure to NIR radiation, however, the
30 cells appeared shriveled and blurry, indicating cellular stress (Figure 44D).

The present strategy provides clear results and the ability to distinguish which variables affect cell killing. If more detailed microscopy is necessary, beyond brightfield, confocal microscopy can be used with fluorescently tagged Ftn to track not just the uptake of gold AuNPs but also the Ftn into the cells.

5

Cancer is a major deadly disease encountered worldwide for many years. Analyzing the global data and research, conventional treatments, such as radiotherapy (RT) and chemotherapy (CT), have various challenges and side effects due to the utilization of high doses of harmful radiation and toxic drugs (Payne, H. et al., 2013, *BJU Int.*, 112, 885; Mathan, S. V. et al., 2022, *Understanding Cancer*, pp 217-236; Wang, K. et al., 2021, *CA Cancer J. Clin.*, 71, 437). Recently, photothermal therapy (PTT) and photodynamic therapy (PDT) have attracted considerable attention as potential cancer therapies. In PTT, a photothermal agent (PTA) is utilized to generate heat and destroy cancer cells upon irradiation, typically with NIR light that has a significant transmission distance through human tissue (Hu, J.-J. et al., 2018, *Nanoscale*, 10, 22657). The working hypothesis is that a moderate elevation of the temperature (Hyperthermia) inside the body tissues ($> 42^{\circ}\text{C}$) where the PTA is localized will damage cancer cells with minimal damage to surrounding healthy tissues (Bettaieb, A. et al., 2013, *Cancer Treat.- Conv. Innov. Approaches*). To date, NIR-triggered inorganic nanomaterials, such as transition metal nanoparticles (Szewczyk, O. K. et al., 2022, *Int. J. Mol. Sci.*, 23), metal sulfides (Sheng, J. et al., 2018, *Small*, 14, 1702529), platinum nanoparticles (Zhu, X.-M. et al., 2016, *Adv. Healthc. Mater.*, 5, 3165), and gold nanomaterials (Chuang, Y.-C. et al., 2022, *J. Nanotheranostics*, 3, 117), have been used PTA. In the PDT process, ROS are generated by exposing a photosensitizer (PS) to light of a particular wavelength. However, a challenge in PDT is that photobleaching may occur and the PSs show an insignificant absorption of NIR (650– 900 nm) (Vankayala, R. et al., 2018, *Adv. Mater.*, 30, 1706320).

In this study, a novel bioconjugate consisting of AuNRs chemically linked to the protein HFtn is introduced. Inside HFtn, there is a small-band semiconductor known as Fh. The purpose of designing this bioconjugate is to address two key challenges in the advancement of PPT and PDT for addressing health concerns such as

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cancer. The first challenge involves developing strategies that enable the concentration of nanoparticles (in PPT) or photosensitizers (in PDT) specifically at the targeted region of interest, such as a tumor in the case of cancer. By achieving this localization, the potential damage to healthy surrounding tissues can be minimized, leading to more effective and targeted treatments. The second challenge is to explore therapeutic approaches that utilize light wavelengths capable of effectively transmitting through human tissue. This is crucial for practical implementation since utilizing wavelengths with good tissue penetration enables the treatment to reach deep-seated tumors or affected areas within the body. By constructing the bioconjugate with AuNR and HFtn, this study aims to address these challenges and provide a promising and synergistic approach for enhancing PPT and PDT strategies. The goal is to develop therapies that concentrate the therapeutic agents at the target site while utilizing light wavelengths that can efficiently penetrate human tissue, thus advancing the field of cancer treatment and potentially addressing other health issues as well.

The bioconjugate—HFtn chemically linked to AuNRs (AuNR-HFtn)—investigated in this contribution has chemical and physical properties that address these challenges. Ftn is a globular protein constructed from 24 self-assembling polypeptide subunits to form a protein shell with a ~12 nm outer diameter and an internal cavity with an ~8 nm diameter (Moglia, I. et al., 2020, *J. Inorg. Biochem.*, 206, 111016). Typically, in mammalian Ftms, the 24 subunits consist of two different subunit types: H-chain and L-chain subunits. HFtn contains 100% H subunits (Xu, X. et al., 2022, *Molecules*, 27). The primary function of Ftn is to sequester and store cellular iron and protect the cellular lipids, DNA from ferrous iron-induced redox chemistry (Plays, M. et al., 2021, *Metallomics*, 13). Importantly, for the potential use of the bioconjugate, HFtn has an affinity for cancer cells (Dong, Y. et al., 2021, *Mol. Pharm.*, 18, 3365). Specifically, the transferrin receptor 1 (TfR1), is a transmembrane glycoprotein that is overexpressed in tumor cells and readily binds HFtn due to the requirements for cancer cell growth. Hence, the HFtn potentially provides a strategy to selectively target cancer cells for the delivery and localization of AuNRs at cancer tumor sites.

Cancer cells contain up to 100 mM of elevated H_2O_2 , while normal cells have less than 20 nM (Wu, Y. et al., 2019, *Chem. Sci.*, 10, 7068). The high levels of

H₂O₂ in cancer cells are due to the disproportionation of superoxide dismutase in mitochondria (Lin, B. et al., 2019, ACS Appl. Mater. & Interfaces, 11, 11157). Therefore, cancer cells are susceptible to H₂O₂. Prior research has shown that the exposure of the AuNR-HFtn bioconjugate to NIR that can excite the plasmonic mode of the AuNR
5 results in the release of ferrous iron (i.e., Fe(II)). It triggers the Fenton reaction, which leads to the accumulation of ROS, lipid peroxidation, and iron-dependent cell death, also known as Ferroptosis (Shintoku, R. et al., 2017, Cancer Sci., 108, 2187). In cancer cells with high H₂O₂ levels, the release of Fe(II) will inevitably trigger the Fenton reaction, which generates radicals that cause Ferroptosis. Hence, not only is PPT a viable means to
10 kill cancer cells, the release of Fe(II) into the cellular media can potentially induce ferroptosis and cell death. Ferroptosis is a well-studied process in the context of human physiology where the programmed release of Fe(II) in cells leads to ROS and the destruction of the cell membranes via the oxidation of bilayer lipids (Stockwell, B. R., 2022, Cell, 185, 2401).

15 The present work is further drawn to the effect of NIR-irradiated AuNR-HFtn bioconjugates on the viability of PC3 human prostate cancer cells. This cell line was selected since TfR1 receptors are significantly more abundant in all prostate cancer cell lines than in normal prostate epithelial cells (Deng, Z. et al., 2017, Oncotarget, 8, 82231). Present studies utilized AuNR-HFtn bioconjugates that were free of the
20 surfactant CTAB, which was used to prepare the AuNRs (Yu, C. et al., 2007, Langmuir, 23, 9114). The removal of the CTAB was necessary due to its cytotoxic properties. It is shown that if the AuNR-HFtn bioconjugates ([AuNR]=174.5 μ M) are incubated with the prostate cancer cells for 24 h and then irradiated with NIR, there is a 32% loss in cell viability relative to experiments that were conducted in the dark. Importantly, it is shown
25 that the AuNR-HFtn bioconjugate is more effective in decreasing the cell viability compared to AuNRs alone in the presence of NIR.

Experimental

Human prostate cancer cell line (PC3) was seeded in two 96-well plates
30 (10 000 cells per well) in DMEM/F-12 culture media supplemented with 10% FBS, 100 IU mL⁻¹ penicillin, and 100 μ g mL⁻¹ streptomycin, maintained at 37 °C. The PC3

cancer cell line is cultured using Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM F12). The UV-visible spectrum of this culture medium is shown in Figure 45A. Prior to studying the cancer cell line, the stability of AuNR-HFtn with DMEM medium was tested. The effect of the AuNR-HFtn bioconjugate on the viability of PC3 human prostate cancer cells with and without NIR exposure was investigated.

The UV-vis spectra of AuNR-HFtn in DMEM F12 with different AuNR concentrations ($[AuNR]=49.1, 24.5, 12.3, \text{ and } 4.9 \mu\text{M}$) are illustrated in Figure 45B. AuNR has a plasmonic absorbance peak at 850 nm with varying intensities depending on its concentration. HFtn protein has a primary absorbance in the UV range at 280 nm due to the presence of tryptophan, tyrosine, and phenylalanine amino acid residues. The AuNR-HFtn bioconjugates ($[AuNR]=49.1 \text{ and } 4.9 \mu\text{M}$) in Tris HCl (pH=7.4) and DMEM F12 equal volumes were mixed and kept for 30, 120, 360, and 720 min. The absorbance at 850 nm and 280 nm were measured, and it was observed that the absorbance remained almost constant for each time interval up to 720 min (Figure 45C and Figure 45D). Therefore, the DMEM F12 culture medium has provided stable chemical conditions to avoid the AuNR and HFtn aggregation in the medium.

About 24 h after seeding, each well was treated with AuNR-HFtn conjugates diluted AuNR concentrations ranging from $1.39 \mu\text{M}$ to $174.5 \mu\text{M}$. Then the cells were incubated for 2 h and 24 h at 37°C with 5% CO_2 . Subsequently, cells in the plate were exposed to NIR lamp ($\lambda = 850 \text{ nm}$), setting the power to 4 mW/cm^2 in each well for 2 and 24 h. On completion, the number of metabolically active/viable cells was determined by measuring the concentration of ATP using the CellTiterGlo® Luminescent cell viability assay (Promega). The same procedure was applied to the dark control. Percent cell viability was calculated as a percentage with respect to the untreated cells used as control.

For studies involving PC3 cancer cell viability% studies with the AuNR-HFtn bioconjugates vs. control system (AuNR-apoHFtn), Dark and light conditions of each system and untreated cancer cell control, the Student's t-test was performed to determine statistical significance and a $P < 0.05$ was utilized as the threshold limit for a significant difference.

Results and Discussion

The novel bioconjugation system AuNR-HFtn has undergone comprehensive characterization using various analytical techniques. In summary, the initial step involved synthesizing AuNR-CTAB with an absorbance band centered at 800 nm. These AuNRs were then conjugated with HFtn, as depicted in Figure 46A – Figure 46C. To make the system suitable for biological applications, the cytotoxic CTAB was removed from the synthesis process. This modification was confirmed through analyses such as DLS, Z.P, and ATR-FTIR spectroscopy. Subsequently, the photochemistry of the AuNR-HFtn bioconjugate system was investigated, specifically exploring its response to NIR light. The research conducted revealed that the AuNR-HFtn bioconjugate system exhibited the capability to release Fe(II) when exposed to NIR light. This finding indicates the potential of the system for applications involving NIR-triggered responses in cancer therapeutics.

Prior studies have shown that the exposure of AuNR-HFtn bioconjugate to NIR (840 nm, 1.46 eV) leads to the release of Fe(II) from the HFtn. The mechanism that facilitates this process is PHETr/PHETu from the AuNR to the inorganic Fh core of HFtn. According to the prior work, 120 min of exposure to 850 nm NIR results in the release of 12.8 nM Fe(II). The amount of Fe(II) released increases up to 61.5 nM after 300 min. Experiments carried out without the NIR resulted in no Fe(II) release. As mentioned above, one of the processes by which the NIR photochemistry of AuNR-HFtn could affect cancer cell viability is through the release of Fe(II) and subsequent formation of ROS that could lead to the disruption of the bilayer membrane of cells. Hence, experiments were carried out to investigate whether the NIR-irradiated bioconjugate could indeed lead to ROS formation. It was investigated whether hydroxyl radical was formed by using the knowledge that the reaction of hydroxyl radical with coumarin produces hydroxycoumarins which are detectable via fluorescence spectroscopy.

The most common hydroxycoumarin is 7-hydroxycoumarin, also known as umbelliferone (Figure 47A). The fluorescence of 7-hydroxycoumarin can be probed in the visible region, with the highest intensity at approximately 455 nm (Yang, S. et al., 2014, Tetrahedron, 70, 8914). The results in Figure 47B clearly indicate that when

exposed to NIR irradiation at 850 nm, AuNR-HFtn gradually generates OH radicals over time, as opposed to the dark control (Table 1).

5 Table 1. Absorbance values of the HFtn absorbance of the AuNR-HFtn ([AuNR]=49.1 and 4.9 μ M) at 850 nm and 280 nm in the DMEM F12 medium with varying time intervals (t=30, 120, 360, 720 min).

	30 min	120 min	360 min	720 min
850 nm absorbance/ A.U				
AuNR-HFtn ([AuNR]=49.1 μ M)	0.15339	0.1537	0.15145	0.14928
AuNR -HFtn ([AuNR]=4.9 μ M)	0.06623	0.06681	0.06644	0.06614
280 nm absorbance/ A.U				
AuNR-HFtn ([AuNR]=49.1 μ M)	3.14251	3.12158	3.13054	3.13144
AuNR -HFtn ([AuNR]=4.9 μ M)	3.12748	3.15359	3.14227	3.13273

This result suggests that the NIR-induced Fe(II) release from AuNR-HFtn, in the presence of biological cells (e.g., cancer cells), could potentially lead to the killing of the cells via a ferroptosis-like cell death mechanism.

Table 2 shows the cancer cell viabilities% for AuNR-HFtn and AuNR-apoHFtn (AuNR attached to HFtn without a Fh core) treated PC3 cancer cell lines under dark and light conditions.

15 Table 2. Absorbance values of the HFtn absorbance of the AuNR-HFtn ([AuNR]=49.1 and 4.9 μ M) at 850 nm and 280 nm in the DMEM F12 medium with varying time intervals (t=30, 120, 360, 720 min).

	30 min	120 min	360 min	720 min
850 nm absorbance/ A.U				
AuNR-HFtn ([AuNR]=49.1 μ M)	0.15339	0.1537	0.15145	0.14928
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280 nm absorbance/ A.U				
AuNR-HFtn ([AuNR]=49.1 μ M)	3.14251	3.12158	3.13054	3.13144
AuNR-HFtn ([AuNR]=4.9 μ M)	3.12748	3.15359	3.14227	3.13273

Two different AuNR concentrations ([AuNR]= 1.39 μ M and 174.5 μ M) and incubation times (2 h and 24 h) were studied. Data in Figure 48A shows that there is no significant cell viability% reduction ($P>0.05$) when after the AuNR-apoHFtn and AuNR-HFtn bioconjugates are individually incubated with the cell line for 2 h either under dark or light conditions. In contrast, data in Figure 48B shows that if the bioconjugate and cells are incubated for 24 h prior to NIR exposure for both AuNR-HFtn ([AuNR]= 1.39 μ M) and AuNR-HFtn ([AuNR]= 174.5 μ M) there is a significant reduction in cell viability% ($P < 0.05$) under light conditions (compared to dark conditions). AuNR-apoHFtn did not result in a significant decrease in cell viability ($P>0.05$) when exposed to light relative to dark conditions. The small decrease in cell viability for the AuNR-apoHFtn (24 h incubation) system in the presence of NIR was attributed to be due to the photothermal effect of AuNRs. The larger effect of the AuNR-HFtn NIR irradiated system on decreasing cell viability may be due to the release of Fe(II) and the subsequent reaction of ROS with the cellular membrane material.

Besides, small amounts of iron might be mineralized in the HFtn, contributing to photodynamic cancer cell destruction. Thus, to avoid the possible minute iron contamination in the process, Ftn protein-free AuNR capped with methoxy poly(ethylene glycol) (AuNR-mPEG-SH) can be utilized as an additional control system for the comparison with the AuNR-HFtn bioconjugate system. Moreover, to evaluate the synergistic (PTT+PDT) effect and solitary PTT effect on eradicating PC3 cancer cells, it is reasonable to compare the AuNR-HFtn bioconjugate system with the AuNR-mPEG-SH control system. However, the AuNR-apoFtn and AuNR-mPEG-SH control systems are expected to behave differently in the vicinity of cancer cells. Although AuNR-apoFtn can be targeted to cancer cells via TfR1 receptors, AuNR-mPEG-SH, being a pure AuNR without a protein component, most likely enter the cancer cells through the endocytosis

pathway. Therefore, allowing adequate incubation time for both systems to travel to the cancer cells is essential for a reasonable comparison.

When the incubation time is increased from 2 h to 24 h, the cell viability% for the system with AuNR-HFtn bioconjugates in the dark condition was insignificant ($P > 0.05$). In the light conditions for AuNR-HFtn ($[AuNR] = 1.39 \mu M$) the cell viability% was reduced by almost 24%, whereas for the AuNR-HFtn ($[AuNR] = 174.5 \mu M$) the reduction ($P < 0.05$) was 32% (Figure 48C). Control investigations were carried out using untreated PC3 cells under both dark and light conditions. It was found that there was no significant difference ($P < 0.05$) in AuNR-apoHFtn ($[AuNR] = 174.5 \mu M$) and AuNR-HFtn ($[AuNR] = 174.5 \mu M$) when compared to untreated PC3 cancer cells under dark conditions. Under light conditions, there was no significant reduction in cell viability% ($P < 0.05$) for AuNR-apoHFtn ($[AuNR] = 174.5 \mu M$) in untreated PC3 cancer cells. However, a substantial decrease in cell viability% ($P < 0.05$) for AuNR-HFtn ($[AuNR] = 174.5 \mu M$) bioconjugates under the same conditions was observed (Figure 48D).

Figure 49 exhibits optical microscopic images of PC3 cancer cells, PC3 cancer cells incubated with AuNR-HFtn for 24 h before NIR irradiation and PC3 cancer cells incubated with AuNR-HFtn for 24 h after NIR irradiation. The microscopic image in Figure 49A shows that the PC3 cancer cells appear as elongated features with a maximum length of approximately $100 \mu m$. They have various sizes, most of which have a polygonal shape with a spherical nucleus in the middle, surrounded by the cytoplasm. After a 24 h incubation with AuNR-HFtn, the cancer cells appear to have internalized AuNR-HFtn clusters (Figure 49B). When these cells were exposed to NIR light for another 24 h, a significant morphological change was observed by microscopy. In particular, there was a decrease in the number of cancer cells compared to the control uninfected PC3 cells. The cancer cells became rounded and shrunken, while the untreated cells remained polygonal. Clusters of AuNR or AuNR-HFtn were observed outside the cancer cells after NIR irradiation, which was not visible before. This observation is supported by prior research which showed a similar behavior for PC3 cells when their proliferation was suppressed with anticancer agents (Rasul, A. et al., 2013, Molecules, 18, 9372) (Figure 49C). Hence, the cell viability results, and microscopic images lend support to the notion that a relatively long incubation time is needed, likely allowing

AuNR-HFtn bioconjugates to enter (by endocytosis) the targeted cell's internal volume prior to NIR exposure.

A system with anticancer properties should possess specific qualities, including cancer-targeting ability, nontoxicity, biocompatibility, biodegradability, and efficient pharmacological properties, such as effective loading controlled prolonged therapeutic effect in vivo and in vitro (Wong, K. H. et al., 2020, *Molecules*, 25; Di, X. et al., 2022, *Pharmaceutics*, 14). In cancer therapeutics, the primary focus is delivering anticancer agents to cancer tissues selectively, maintaining the appropriate concentration level for a particular time to suppress anticancer activities (Wang, X. et al., 2008, *CA. Cancer J. Clin.*, 58, 97). The enhanced permeability and retention effect (EPR) is a pathophysiological passive targeting phenomenon that targets specific molecules like liposomes, nanoparticles, and macromolecular compounds (>40 kDa) in solid tumor tissue (Wu, J., 2021, *J. Pers. Med.*, 11). However, targeting anticancer therapy via the EPR effect in clinical practice is not always successful since it depends on various chemical and physical conditions. Therefore, active targeting is necessary. To make anticancer agents more effective, they are often chemically or genetically modified to target cancer cells (Suggitt, M., 2005, *Clin. Cancer Res.*, 11, 971). The AuNR-HFtn nanocarrier is unique because it potentially will target cancer cells through its specific affinity to TfR1 without additional modifications.

Iron is a crucial element for the growth of mammalian cells, but it can also contribute to the formation of oxygen-free radicals through Fenton chemistry. Ftn is a cytoprotective protein responsible for maintaining the balance of intracellular iron (Liu, M.-Z. et al., 2022, *Front. Pharmacol.*, 13). It can minimize the formation of oxygen-free radicals by sequestering excess free iron. Various studies have suggested that Ftn can protect against oxidative stress-related damage caused by an imbalance between ROS production and insufficient antioxidants to eliminate them within cells. Inside cancer cells, the iron content becomes low due to rapid proliferation and metabolic reactions (DNA synthesis, cellular respiration). Hence the transferrin receptors (TfR), which regulate iron metabolism, are overexpressed in cancer cells. Consequently, there is a notable increase in Ftn, heme production, and the binding of Ftn to TfR receptors within the cancer cells. Prior research has revealed that HFtn binds to the receptor TfR1 and

enters both endosomes and lysosomes (Rafik, S. T. et al., 2023, J. Clin. Med., 12).

Endosomes are a diverse group of organelles within cells that sort and transport materials taken in from the cellular surfaces. Lysosomes are eukaryotic organelles that contain digestion enzymes, which are vital for autophagy. Other studies have found that Ftn from the cytoplasm also reaches lysosomes and that lysosomes may play a role in releasing iron from Ftn (Kidane, T. Z. et al., 2006, J. Physiol. Physiol., 291, C445).

Iron metabolism is crucial in cancer development, survival, proliferation, and metastasis. Two therapeutic approaches have been developed based on intracellular iron metabolism in cancer cells (Chen, X. et al., 2020, Front. Cell Dev. Biol., 8; Guo, Q. et al., 2021, Front. Oncol., 11). The first approach involves depleting iron from cancer cells via iron chelators. The expectation is that the removal of iron will eventually starve the cancer cell metabolism and terminate its growth. The second approach consists of generating cytotoxic levels of ROS by triggering the Fenton reaction with excess iron, leading to Ferroptosis in cancer cells. Present research focused on the latter approach, NIR irradiation of HFtn attached to AuNR to provide cancer cells with excess Fe(II) where the this redox active species can trigger the Fenton reaction (production of hydroxyl radicals) via reaction with the comparatively high H₂O₂ levels (~100 mM) in cancer cells. The elevated oxidative stress can initiate ferroptosis and eliminate cancer cells through plasma membrane damage.

Conclusion

In this chapter, the primary objective was to exploit the potential of the AuNR-HFtn bioconjugate system for cancer therapeutics. The central hypothesis proposed that NIR light-induced Fe(II) release from the system could trigger the Fenton reaction within cancer cells, thereby initiating Ferroptosis. Preliminary fluorescence studies provided confirmation of hydroxyl radical formation during NIR exposure. To investigate the anticancer properties of the system, the PC3 prostate cancer cell line was utilized. The results demonstrated a significant decrease in cancer cell viability% ($P < 0.05$) as the incubation time increased from 2 h to 24 h under NIR exposure for 2 h. The drop in cancer cell viability% was dependent on the concentration of AuNR ([AuNR]) with an increase from 24% to 32% observed when the [AuNR] was raised from 1.39 μ M

to 174.5 μM . Additionally, a significant decrease in cancer cell viability percentage ($P < 0.05$) was observed in the AuNR-HFtn bioconjugate system ($[\text{AuNR}] = 174.5 \mu\text{M}$) compared to the AuNR-apoHFtn control system ($[\text{AuNR}] = 174.5 \mu\text{M}$), as well as the untreated PC3 cells. Furthermore, the potential mechanisms underlying the anticancer properties of the AuNR-HFtn bioconjugate system were discussed. However, further investigations, including control cell line studies, optimization, and system enhancements, are necessary to improve the system's productivity for effective cancer therapy.

10 Example 4: Synthesis, Characterization, and Photochemistry of Gold Nanostar H-Chain Ferritin Bioconjugates

Plasmonic AuNRs and AuNSs were attached to human H-chain Ferritin (HFtn). Present work showed that the photochemistry of HFtn could be sensitized to longer wavelengths by attaching the anisotropic plasmonic nanoparticles that have plasmonic resonances in the near-infrared (NIR) region of the electromagnetic spectrum. In addition, the results showed that the bioconjugate releases Fe(II) during exposure to light wavelengths in the NIR (e.g., $\lambda = 850 \text{ nm}$). The development of these conjugates is not only novel in structure but also in potential application. In the context of a potential application, bioconjugates composed of AuNRs or AuNSs attached to HFtn (AuNR- and AuNS-HFtn) were also investigated as a tumor suppression cancer treatment to complement the ongoing interest in using NIR-activated AuNRs for photothermal tumor suppression strategies (Wu, Y. et al., 2018, ACS Nano, 12, 9279; Ali, M. R. K. et al., 2017, Proc. Natl. Acad. Sci., 114, e5655; Taylor, M. L. et al., 2022, Bioengineering, 9). The advantages of this novel system will be discussed later.

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Ftn is a multifunctional protein that plays a crucial role in iron metabolism and storage (Kuberl, A. et al., 2016, Proc. Natl. Acad. Sci., 113, 4806). It acts as a protective cage, encapsulating iron in a mineral form called Fh (Nihei, M. et al., 2018, J. Am. Chem. Soc., 140, 17753). This mineral form allows for efficient iron storage within cells while preventing the iron from causing oxidative damage (Arosio, P. et al., 2009, Biochem. Biophys. Acta – Gen. Subj., 1790, 589). In mammals, including humans, Ftn is

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composed of two subunits: the heavy chain (H) and the light chain (L). The HFtn is a variant of Ftn that consists solely of H subunits (Park, J.-S. et al., 2007, *Appl. Microbiol., Biotechnol.*, 75, 347). Each H subunit has 1-2 exposed cys ligands, which are sulfur-containing molecules. The formation of -SH bonds due to these cys ligands plays a crucial role in establishing a stable interaction between HFtn and AuNPs (Poole, L. B., 2015, *Free Radic. Biol. Med.*, 80, 148). Besides, Ftn is a promising tool in cancer therapeutics due to its unique properties, such as selective uptake by cancer cells via TfR1 receptors, nanocage structure, biocompatibility, low immunogenicity, and potential for multimodal therapy (Zhang, B. et al., 2021, *Adv. Drug Deliv. Rev.*, 176, 113892).

In prior research, significant efforts have been devoted to exploring the attachment of gold nanostructures to proteins, aiming to harness the unique properties of both materials for various applications (Wu, R. et al., 2020, *Front. Chem.*, 8; Wang, P. et al., 2015, *Sci. Technol. Adv. Mater.*, 16, 34610; Liu, F. et al., 2015, *ACS Appl. Mater. & Interfaces*, 7, 3717). AuNPs offer remarkable optical, electronic, and catalytic properties, while proteins exhibit diverse functionalities and high specificity. Several strategies have been employed to facilitate the attachment process, including direct adsorption (Sotnikov, D. V. et al., 2019, *Colloids Surfaces B Biointerfaces*, 173, 557), covalent conjugation (Singh, V., 2013, *J. Nanobiotechnology*, 11, 7), and functionalization of protein surfaces (Dutta, S. et al., 2022, *Front. Mol. Biosci.*, 9). These approaches often involve the modification of surface amino acids, such as cysteine or lysine, to introduce reactive groups for interaction with gold surfaces (Van der Meer, S. B. et al., 2021, *Chem – A. Eur. J.*, 27, 1451). Herein, a strategy to modify the HFtn with chemically synthesized AuNS through Au-S covalent conjugation is designed. The HFtn bioconjugate system (AuNS-HFtn) is analyzed using various analytical techniques. TEM was used to examine the shape of the AuNS and its attachment to the HFtn outer shell. Furthermore, UV-Vis spectroscopy was utilized to analyze the changes in the LSPR effect of both AuNS and AuNS-HFtn. Additionally, ATR-FTIR was used to study the total surfactant removal from the bioconjugates for non-toxic biomedical utilization. The research has shown that when AuNS is irradiated at 850 nm, it can release Fe(II) from the HFtn inner core consisting of Fh semiconducting material.

It was proposed that the release of Fe(II) can trigger a Fenton reaction in cells (Ex. cancer cells, bacterial cells), leading to the photodynamic pathway in addition to its photothermal effect. Furthermore, the LSPR of AuNSs allows for enhanced antibacterial effects through the generation of ROS upon exposure to light. The dual
5 action of the material can provide a synergistic effect in combating infections and treating cancer simultaneously. This can be particularly beneficial in situations where bacterial infections often occur in cancer patients, leading to complications and compromised treatment outcomes. Overall, the development of materials with both antibacterial and anticancer properties holds great promise for advancing healthcare by addressing the
10 challenges of drug resistance, improving infection control, and enhancing cancer treatment strategies. The present work is also relevant for the study of the simultaneous antibacterial and anticancer properties of the AuNS-HFtn bioconjugate system.

Experimental

15 All chemicals were used as received. Pure analytical grade solid chemicals, solvents, and Deionized (DI) water ($18\text{ M}\Omega\text{ cm}^{-1}$) were used in all preparations. Sodium borohydride (NaBH_4 , 98+%), Sodium hydroxide (NaOH , ACS reagent, Pellets, $\geq 97.0\%$), Sodium tetrachloroaurate(III) dihydrate ($\text{NaAuCl}_4 \cdot 2\text{H}_2\text{O}$, >99%), Phosphotungstic acid hydrate ($\text{H}_3[\text{P}(\text{W}_3\text{O}_{10})_4] \cdot x\text{H}_2\text{O}$, 99.995%), TritonX-100 (2-
20 [4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol, Laboratory grade), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate (Ferrozine, 98+% pure), Trisma base(primary standard and buffer, $\geq 99.9\%$), L-(+)-Ascorbic acid (AA, 98+%), Silver nitrate (AgNO_3 , 99%), Hydrochloric Acid (HCl , Certified ACS plus, 36.5 to 38.0%), Ferrous Ammonium Sulfate Hexahydrate ($\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$,
25 Crystalline/Certified ACS, Assay: ≥ 98.5 to 101.5%), from Fisher Chemical™ and HEPES buffer (ultra-pure, American bio).

Briefly, 2.0 mL of purified apoFtn (0.631 mg mL^{-1}) was added to 20 mL of HEPES buffer ($\text{pH}=7.4$, 0.1 M) and prepared by bubbling $\text{N}_2(\text{g})$ for 1.5 h. Then aliquots of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ ($4.06\text{ }\mu\text{M}$, 500 μL) were added to the apoFtn solution
30 over a 150 min period at 30 min intervals targeting 2000 iron loading. (HFtn). In each Fe(II) addition, the solution was shaken slowly and kept air-tight for 30 min. The solution

slowly turned to a yellowish-orange color from colorless and a homogeneous solution was observed without any visible precipitation. Excess Fe(II) and other excess ions were removed by dialysis(Molecular porous dialysis membrane, MWCO: 12-14 kDa) in fresh Tris buffer (pH 7.4, 0.1 M, 1000 mL) for 1.5 h intervals three times and stored in 4 °C.

5 AuNS stabilized with TX-100 was synthesized using a method developed by Atta et al. (Atta et al., 2019, Nanoscale, 11, 2946). To start, all the necessary solutions were prepared separately before being combined. A 10 mL and 20 mL 0.15 M TX-100 solution were made by adding 0.89 mL to 9.11 mL and 1.78 mL to 18.22 mL of DI water, respectively, from a 1.69 M stock concentration. Next, 500 μ L of 10 mg mL⁻¹ AuCl₄⁻ the
10 solution was prepared by mixing 50 μ L of 100 mg mL⁻¹ stock AuCl₄⁻ solution with 450 μ L of DI water. Finally, 3.9 mM AgNO₃ solution (0.0067 g, 10 mL), 0.788 M Ascorbic acid solution (0.1388 g, 1 mL), and 0.01M NaBH₄ (0.0038 g, 10 mL) were also prepared.

Seed solution was prepared by adding 600 μ L of NaBH₄ solution to a 10 mL mixture of Triton X-100 and 100 μ L of 10 mg mL⁻¹ AuCl₄⁻ solution. The pale
15 yellowish color solution turns to dark orange color. The solution was stirred for 2 min and further aged for 10 min at 2-4 °C before using for the next process.

AuNS growth solution was prepared by the following method. 20 mL of TX-100 solution was mixed with 400 μ L of 10 mg mL⁻¹ AuCl₄⁻ solution and stirred for 2 min. Then a rapid sequential addition of 500 μ L of 3.9 mM AgNO₃, 40 μ L of 0.788 M
20 ascorbic acid and 14 μ L of the seed solution were carried out within 1-2s time interval. Finally, the solution mixture was stirred further for 30 min at room temperature. To achieve various AuNS aspect ratios, the amount of TX-100 added to the initial 10 mL of TX-100 solution can be adjusted. Based on UV-vis spectroscopy results, adding volumes of 1775, 890, 750, and 500 μ L resulted in longitudinal NIR absorbance bands of 1050,
25 900, 850, and 700 nm, respectively.

12.0 mL of TX-100 stabilized AuNSs were centrifuged at 14000 rpm for 15 min, and the supernatant was discarded carefully. Then 2.0 mL of distilled water was added to the first AuNS pellet, mixed by vortexing, and centrifuged at 12000 rpm for 15 min. Next, the remaining AuNS pellet was mixed with 1.0 mL of HEPES (0.1 M,
30 pH=7.4). (If the AuNS in TX-100 needs to be transferred and stabilized in mPEG-SH, 100 μ L of 100 μ M mPEG-SH can be added to the final ~25 mL AuNS solution, kept

overnight, and then centrifuged at 12000, 10 min for purification) Then HFtn protein solution 1.0 mL was added slowly to 1.0 mL of AuNS in HEPES under ultrasonication for 2 min. (HFtn/AuNS dispersions, 1:1 v/ v) Then the AuNS-HFtn conjugate solution was further sonicated for 30 min, centrifuged at 10000 rpm for 5 min, decanted excess HFtn/ CTAB, and dispersed in 2.0 mL of HFtn in HEPES buffer. (pH =7.4, 0.1 M), and stirred for at least 24 h. Next, the AuNS-HFtn solution was centrifuged at 10000 rpm for 2 min, decanted excess HFtn/ CTAB, and dispersed in a 2.0 mL HEPES. (pH =7.4, 0.1 M), and concentrated as desired.

10 *Characterization*

TEM was used to image pure AuNS and AuNS-HFtn bioconjugates using a JOEL JEM-1400 microscope operating at 120 kV (under 50k-100k magnifications and 20 nm/ 50 nm resolution). All the samples were prepared on copper mesh-supported holey carbon TEM grids (Ted Pella) and allowed to dry as a thin film. The AuNS-HFtn sample was centrifuged at 5000 rpm for 5min thrice and diluted each time with HEPES buffer (0.1 M, pH =7.4) to remove excess HFtn in the soft corona and used for the TEM imaging. TEM Images were taken with and without staining. To visualize the outer shell of the cheerio-like HFtn protein in the AuNS-HFtn bioconjugates, a 2% PTA negative staining solution at pH 7 was used. When the TEM grids were prepared, 10 μ L aliquots of the sample were deposited on the grid and air-dried for 10 min. The excess sample was removed with filter paper and then rinsed with 10 μ L of water. Next, 3 μ L of the PTA solution was deposited, allowed to sit for 2 min, and the excess was removed by filter paper to prepare stained grids.

ATR-FTIR analysis was performed to confirm the TX-100 removal from the AuNS-HFtn bioconjugates. All experiments were conducted in Nicolet Magna 750 FTIR spectrometer with a single bounce diamond crystal ATR cell (SpecacTM) and mercury cadmium telluride A (MCTA) detector cooled by liquid N₂.

All photochemical experiments were conducted in a 1 cm² quartz cuvette (Thor Labs) with a 3.2 mL total sample volume using an 850 nm NIR illuminator (Univivi IR illuminator with 12 V/ 1A adaptor) as the light source. AuNR-HFtn samples and controls were individually exposed for 120 min, and absorbance spectra (1100–200

nm) were acquired for each photochemical reaction from the UV-vis spectrophotometer. Iron release studies were conducted with AuNR-HFtn, 32 mM sodium potassium tartrate, and 80 mM Fz in 0.1 M Tris buffer (pH 7.4). Quantitation of Fe(II) release was determined by UV-Vis spectroscopy at 562 nm due to Fe(II)-Fz complex formation.

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Results and Discussion

In Figure 50, the UV-Vis spectrum exhibits two different modes similar to the AuNR, a weak transverse band at 505 nm and a broad longitudinal band at 800, 900 or 1050 nm. It was discovered that by adjusting the TX-100 concentration ([TX-100]) in the seed solution, the longitudinal absorbance band position could be tuned in the NIR window. During the reaction, TX-100 micelles form and can capture free Au(III) ions, which can limit the nucleation and growth steps of the mechanism. When [TX-100] surpasses the critical micelle concentration (CMC) of TX-100 (0.3 mM), the free Au(III) ions available in the solution are significantly reduced. As a result, the nucleation and growth kinetics are anticipated to be more controllable and vice versa. Atta et al. reported that various factors, including the chemical characteristics of seeds, the ascorbic acid, and the AgNO₃ concentration, affect the anisotropic growth of AuNS and its longitudinal NIR absorbance. The same publication thoroughly explored how each reactant influences the morphology and the NIR absorbance. However, the mechanism involved in the seed-mediated TX-100 capped AuNS synthesis remains unresolved.

TEM images and NIR plasmon spectra of the AuNS in TX-100 are shown in Figure 50. According to Figure 50B, the TEM image indicates that most AuNSs have spikes of different lengths and numbers. Figure 51A - Figure 51E shows the diversity of spike sizes among the dispersed AuNSs in TX-100. Based on the TEM images, each AuNS can have between 3 and 7 spikes, either sharp or blunt, and of different sizes. The length of the spikes ranges from 50-100 nm (Figure 50C). Additionally, some shorter spikes have developed from the main branches of the AuNSs, as shown in Figure 52A and Figure 52B.

In Figure 53A, the stained TEM image of HFtn reveals a distinct cheerio-like structure comprising an inner core of Fh and an outer white protein shell. In Figure 53B, the stained AuNS-HFtn clearly demonstrates the conjugation of HFtn with the

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AuNSs at the spikes of the AuNSs. The dark spherical spheres in the unstained image represent the Fh core. Additionally, the unstained TEM image shows that the HFtn can be trapped within the cage-like inner cavities created due to aggregation of the AuNS (Figure 53C). More unstained TEM images of AuNS-HFtn have been provided in Figure 53A-Figure 53F. Area A, shown in Figure 53C, clearly indicates the alignment of the HFtn inner core on the AuNS spikes. The tiny gap between the inner core and the AuNS spike surface denotes the HFtn protein shell thickness of ~ 2 nm.

According to the UV-Vis spectrum shown in Figure 53D, the longitudinal peak was redshifted to 850 nm from its initial absorbance at ~810 nm after the HFtn bioconjugation. This redshift is due to the change in the refractive index of the medium from TX-100 to Tris HCl. The data presented in the text provides detailed insights into the structure of HFtn and offers valuable information on its interactions with AuNS, including the formation of AuNS nanocages and AuNS-HFtn bioconjugation. More unstained TEM images of AuNS-HFtn have been provided in Figure 55A – Figure 55F.

TX-100 is a modified poly(ethylene glycol) derivative and a nonionic surfactant. Its terminal hydroxy group has been converted into the corresponding p-(2,4,4-trimethylpentan-3-yl)phenyl ether (Homma, J. et al., 2008, J. Wood Chem. Technol., 28, 270). It has been shown to cause cytotoxicity due to its prolonged exposure and high concentrations (Koley, D. et al., 2010, Proc. Natl. Acad. Sci., 107, 16783). This is mainly because it disrupts the compactness and integrity of the cellular lipid membrane and organelle membranes. To avoid any adverse effects, removing TX-100 from the AuNS-HFtn bioconjugates is crucial before using them in biological applications. An ATR-FTIR study was conducted to ensure that the TX-100 removal had been successfully accomplished. The ATR-FTIR spectrum of AuNSs stabilized in TX-100 is represented by the dark green spectrum (A), while the AuNS-HFtn bioconjugates in Tris-HCl (0.1 M, pH=7.4) solution corresponds to the light green spectra, respectively (Figure 56A and Figure 56B). The two spectra exhibit noticeable differences. In the dark green spectrum, the OH stretching vibration of the terminal OH group in TX100 is responsible for a broad and weak band with a center at 3480 cm^{-1} . Additionally, the weak and sharp bands observed at 1610 , 1581 , and 1512 cm^{-1} are attributed to C-C in-plane stretching vibrations of benzene (Kimura, N. et al., 1996, J. Colloid Interface Sci., 182, 356). The

aromatic in-plane bending mode is detected at 1186 cm^{-1} , while the C-O-C stretching frequency generated due to oxyethylene units attached to the benzene ring is observed at 1092 cm^{-1} . It is vital to note that this unique feature is a distinguishing characteristic of TX-100 compared to Tris-HCl. According to the pale green spectrum, the infrared spectroscopy analysis of Tris-HCl reveals that the 1272 and 763 cm^{-1} peak represents the C-N stretching and N-H wagging frequency, respectively. The C-O stretching vibration corresponds to Tris-HCl is located at 1041 cm^{-1} . The broad band at $3000\text{--}3400\text{ cm}^{-1}$ is attributed to the N-H and O-H stretching vibrations of primary amines of Tris and the HFtn. In addition, the peak at 1625 cm^{-1} represents the N-H bending frequency of primary amines of Tris and the HFtn (Chen, X. et al., 2018, *Polymers*, 10). The CH_2 bending modes of TX-100 are located at 1458 , 1350 , and 949 cm^{-1} , corresponding to the scissoring, wagging, and rocking, respectively. In Tris-HCl, those modes are slightly shifted and relocated at 1460 , 1390 , and 912 cm^{-1} . The CH_2 twisting modes can be seen at 1295 and 1247 cm^{-1} . In Tris 1296 cm^{-1} , CH_2 twisting mode can be observed. Additionally, the O-H bending mode can be observed at 927 and 882 cm^{-1} for TX-100 and Tris-HCl, respectively. The infrared spectral analysis of the AuNS in TX-100 and AuNS-HFtn in Tris-HCl indicates that the TX-100 surfactant has been successfully removed from the bioconjugates, and AuNS-HFtn has been transferred to Tris-HCl.

The reaction mixture consisting of AuNS-HFtn bioconjugates, Tris HCl buffer (pH=7.4), Sodium potassium tartrate, and Fz was exposed to an 850 nm NIR illuminator for various time intervals, and the amount of Fe(II) released from the system was determined. Fz is a chelator specific to Fe(II) ions, and it exhibits a sharp absorbance peak at 532 nm when it forms a complex with Fe(II) (Capitan-Vallvey, 2001, *Fresenius. J. Anal. Chem.*, 369, 139). This absorbance peak can be used to measure the concentration of Fe(II) ions in the solution quantitatively. According to the results presented in Figure 57A, as the reaction mixture is exposed to the 850 nm NIR illuminator, the absorbance peak at 532 nm gradually increases with the duration of exposure. This indicates that the concentration of released Fe(II) ions in the solution is increasing over time. To quantitatively determine the concentration of released Fe(II), Beer's Lambert law was employed. By applying this law, the concentration of Fe(II) ions released into the solution during NIR radiation exposure is calculated. The results show

that within 2, 5, and 24 h of exposure, the released Fe(II) concentrations are 34, 131, and 447 nM Fe(II), respectively. Control reactions performed without the presence of HFtn and AuNS did not exhibit a significant release of Fe(II) (Figure 57B). This suggests that the observed photochemical effect, resulting in the release of Fe(II) ions, only occurs when AuNS and HFtn are conjugated together. In other words, the specific interaction between AuNS and HFtn is necessary for the photochemical reaction to take place and release Fe(II) ions.

The mechanism behind the Fe(II) release can be attributed to the same mechanism described previously for Fe(II) release from the AuNRs attached to HFtn (AuNR-HFtn) system under 850 nm NIR exposure. The Fh in HFtn requires a minimum energy of 2.6 eV to achieve a direct band gap excitation. Hence, the incident energy of 1.45 eV, corresponding to 850 nm NIR light exposure, is insufficient to excite the Fh core directly. Previous studies have explored metal-semiconductor heterojunctions to induce charge separation in semiconductors using various mechanisms (Dutta, S. K. et al., 2015, J. Phys. Chem. Lett., 6, 936). These mechanisms include plasmon-induced resonance energy transfer (PIRET), plasmonic hot electron transfer (PHETr), and plasmonic hot electron tunneling (PHETu) (Jia, C. et al., 2016, Adv. Energy Mater., 6, 1600431). However, the PIRET mechanism is unlikely to occur if the localized surface plasmon resonance of the metal does not overlap with the absorption bands of the semiconductor (Subramanyam, P. et al., 2022, J. Photochem. Photobiol. C Photochem. Rev., 51, 100472). In the case of AuNS and Fh core materials, their UV-vis absorbance bands do not overlap significantly with each other, suggesting that the PIRET mechanism is not expected to result in significant energy transfer within the AuNS-HFtn conjugate system. Instead, it was postulated that the most common electron transfer mechanisms, PHETr and PHETu involve in photocatalytic reactions via AuNS-HFtn heterojunction (Manuel, A. P., 2021, Nanomaterials, 11). These mechanisms rely on the transfer of high-energy electrons from the metal to the semiconductor to drive desired chemical reactions.

Future Work with the AuNS-HFtn System

Based on preliminary studies, it was observed that the AuNS-HFtn bioconjugate system has the ability to release Fe(II) when exposed to 850 nm NIR light.

The Fe(II) release can be further enhanced by increasing the duration of exposure and the power of the NIR illuminator. It was hypothesized that the AuNS-HFtn system may generate hydroxyl radicals through the Fenton reaction when subjected to elevated levels of H₂O₂ in cancer cells, similar to what was observed in previous studies with the AuNR-HFtn system. Consequently, these photodynamic properties of the AuNS-HFtn bioconjugate system can be harnessed to induce ferroptosis, leading to damage in cancer cell membranes. This suggests the potential utility of the AuNS-HFtn system in targeted cancer therapy by selectively inducing cell death through ferroptosis.

Bacterial infections have become a severe issue for public health (Smith, K. A. et al., 2011, J. Exot. Pet Med., 20, 32). The conventional antibiotics used to treat bacterial infections have proven to be less effective due to the development of antibiotic-resistant bacteria, structural changes, and gene mutations in bacterial strains (Hancock, R. E. W., 2000, Proc. Natl. Acad. Sci., 97, 8856). Interestingly, pathogenic bacteria have evolved sophisticated strategies to acquire iron, as iron is an essential nutrient for their survival and growth (Tanaka, K. J. et al., 2018, Biochim. Biophys. Acta – Biomembr., 1860, 868). These bacteria can obtain iron from various host sources, including host proteins like Ftn (Lewis, J. P., 2010, Periodontol., 52, 94). Hence, Ftn is an attractive target for these bacteria. Recently, nanoparticle (NP)-based antibiotics have been thoroughly researched to combat microbial drug resistance (Gomez-Nunez, M. F. et al., 2020, Front. Microbiol., 11; Makabenta, J. M. V. et al., 2021, Nat. Rev. Microbiol., 19, 23). AuNPs have superior photostability, modifiable surfaces, lower toxicity, and higher bioavailability compared to other metal nanomaterials (Zaidi, S. et al., 2017, Nanomedicine Nanotechnology, Biol. Med., 13, 2281). The presence of sharp tips with high surface-to-volume ratios and the ability to produce heat by absorbing NIR makes branched AuNS a more compelling candidate for attacking bacterial cells (Feng, Y. et al., 2019, ACS Appl. Bio Mater., 2, 747). Therefore, the unique characteristics of AuNS-HFtn bioconjugates, having stable interactions and potentially targeting bacterial cells, make it a promising candidate for various selective antibacterial biomedical applications.

Conclusion

In this study, TX-100 stabilized AuNSs were synthesized chemically, exhibiting a longitudinal band positioned at 810 nm. TEM images revealed that each AuNSs could possess 3 to 7 spikes of varying sizes, either sharp or blunt, with lengths ranging from 50-100 nm. A novel approach was employed to eliminate surfactants from the AuNS and conjugate them with HFtn protein through Au-S interactions. Following the bioconjugation, the UV-Vis spectrum exhibited a red shift in the longitudinal band position, shifting to 850 nm. ATR-FTIR spectroscopy was employed to validate the removal of the toxic TX-100 surfactant from the AuNS-HFtn bioconjugate system. Upon exposure to an 850 nm NIR light source, the system demonstrated Fe(II) release. The results indicated that within 2, 5, and 24 h of exposure, the concentrations of released Fe(II) were 34 nM, 131 nM, and 447 nM, respectively. Additionally, the mechanism responsible for generating ROS through the Fenton reaction can be utilized to harness the dual anticancer and antibacterial properties of the AuNS-HFtn bioconjugate system. This unique hybrid material possesses the capability to inhibit cancer growth while also combating potential bacterial infections, presenting a promising avenue for multifunctional therapeutic applications.

Example 5: Gold Nanosphere Attachment to Thiol-Functionalized Horse Spleen Ferritin

A novel attachment route to link AuNSps to horse spleen ferritin (HSFtn) was also presently investigated. The interaction between chemically synthesized gold nanostructures and HSFtn is negligible compared to their interaction with HFtn due to insufficient surface-exposed, active cys groups (Uchida, M. et al., 2006, J. Am. Chem. Soc., 128, 16626). By modifying the surface primary amine-containing amino acids (Lysine) via N-Succinimidyl S-acetylthioacetate (SATA) to introduce -SH groups in HSFtn, it was found that the surface reactivity and connectivity of HSFtn to AuNSps was enhanced. The -SH are known to form strong covalent bonds with Au, leading to gold-thiol (Au-S) bond formation (Chen, H. et al., 2013, Chem. Soc. Rev., 42, 2679). Hence, by introducing excess active -SH groups through SATA modification, the ability of HSFtn to form stable Au-S bonds with AuNSps has improved. In addition, the bioconjugate system composed of AuNSps attached to SATA-modified HSFtn (AuNSp-

HSFtn) released Fe(II) during exposure to longer wavelength light ($\lambda > 475$ nm) than did HSFtn alone ($\lambda < 475$ nm).

Ftn is an iron-storage globular protein that can sequester free Fe(II) as a Fe(III) bearing iron oxyhydroxide solid via enzymatic oxidation pathways within its cavity. HSFtn having an outer diameter of ~ 12 nm and an inner diameter of ~ 8 nm, is a well-characterized Ftn protein, and its 3D structure and amino acid sequence are well known.¹ HSFtn is defined as a protein shell that is composed of 24 protein subunits with various proportions of H and L chains. HSFtn comprises mainly stable L-chains and only 10–15% H-chains. X-ray crystallographic studies have shown $476000 \text{ g mol}^{-1}$ of the molar mass of oligomers containing 24 subunits (Heusterspreute, M. et al., 1981, FEBS Lett., 129, 322). HSFtn shows promise as a candidate for targeting cancer cells due to its ability to bind with TfR1 receptors which are overexpressed in cancer cells (Li, L. et al., 2010, Proc. Natl. Acad. Sci., 107, 3505). Cancer cells have high metabolic activity, DNA synthesis, and cellular growth, which leads to the elevated enzymatic activity of ribonucleotide reductase. This requires cellular iron as a cofactor, resulting in the over-expression of TfR1 receptors in cancer cells. Due to this affinity of HSFtn for TfR1 receptors, investigators continue to investigate the use of HSFtn as a container for the delivery vehicle for the transport of drugs to cancer cells.

Prior research has shown that AuNSps can be grown on the exterior surface of HSFtn via a photochemical process where HSFtn in the presence of AuCl_4^- in the presence of citrate is exposed to light (Keyes, J. D. et al., 2011, J. Nanoparticle Res., 13, 2563). The plasmon resonance of the 7-8 nm AuNSps grown in this circumstance resides at ~ 520 nm. While the mechanism for the growth of the AuNSps on HSFtn has not been well established, particle formation may occur in the 3-fold pore of HSFtn where cysteine groups are available to form Au-S bonds during exposure to light. A complication related to the potential attachment of AuNPs to HSFtn is the lack of solvent-exposed cysteine on the outer surface of the protein cage.

In the current study, the development of AuNSp-HSFtn bioconjugates composed of HSFtn attached to chemically synthesized AuNSps is studied. As a well-known AuNP, AuNSps play a significant role in the field of biological sciences, mainly

due to their tunable optical, physical, and chemical properties and biocompatibility. They exhibit non-cytotoxicity, making them safe for use in various biological applications. It is shown in the present contribution that the installation of solvent-exposed S-H functionality on the surface of HSFtn allows the attachment of AuNSps. In particular, it is shown first that adding thiol functionality to HSFtn can be accomplished by reacting N-succinimidyl S-acetylthioacetate (SATA) (Reddy, N. C. et al., 2020, Org. Biomol. Chem., 18, 4669) with primary amine groups (Lys) on the exterior protein surface of HSFtn. It is mentioned that SATA-functionalized proteins and antibodies have been widely utilized in potential applications for biosensor fabrication (Dai, Y. et al., 2018, Biosens. Bioelectron., 117, 60), drug targeting (Talelli, M. et al., 2011, J. Control. Release, 153, 93), immunoassays (Chen, C.-S. et al., 2005, Talanta, 67, 205), immunosensors (Genc, R. et al., 2011, Anal. Chem., 83, 563), antibacterial applications (Robinson, A. M. et al., 1998, Biochim. Biophys. Acta – Biomembr., 1369, 278), and liposome coupling (Derksen, J. T. P., 1985, Biochim. Biophys. Acta – Biomembr., 814, 151). Besides, AuNSps are a well-known contrasting agent used for various imaging techniques, including dark-field light scattering, optical coherence tomography (OCT), Photothermal imaging (PTI), Surface-enhanced Raman Spectroscopy (SERS), and Magnetic resonance imaging (MRI) (Wu, Y. et al., 2019, Nano Today, 24, 120). Hence, the AuNSp-HSFtn bioconjugate system holds great potential for cancer cell imaging in addition to targeted cancer therapeutics. With regard to a targeted cancer photothermal therapeutic, the ability of the bioconjugate to release Fe(II) ions opens up the possibility of suppressing tumor growth via ferroptosis.

Experimental

All chemicals used in this study were used as received without any further purification. Analytical grade solid chemicals, solvents, and deionized (DI) water (18 MΩ cm⁻¹) were used in all preparations. Apoferritin from equine spleen (apoFtn), sodium hydroxide (NaOH, ACS reagent, Pellets, ≥ 97.0%), Sodium tetrachloroaurate(III) dihydrate (NaAuCl₄·2H₂O, >99%), SATA, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP powder), 5,5'-Dithiobis(2-nitrobenzoic acid (DTNB, ≥98%), Dimethyl sulfoxide (DMSO, ACS reagent, ≥99.9%), (R)-2-Amino-3-mercaptopropionic acid (L-Cysteine,

Assay: > 99%), Hydroxylamine hydrochloride ($\text{NH}_2\text{OH} \cdot \text{HCl}$, reagent plus, 99%), Phosphotungstic acid hydrate ($\text{H}_3[\text{P}(\text{W}_3\text{O}_{10})_4] \cdot x\text{H}_2\text{O}$, 99.995%), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid monosodium salt hydrate (Ferrozine, 98+% pure), and Trisma base (primary standard and buffer, $\geq 99.9\%$) were purchased from Sigma-Aldrich. Hydrochloric acid (HCl , Certified ACS plus, 36.5 to 38.0%), Sodium phosphate dibasic heptahydrate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, crystalline/certified ACS, Assay: ≥ 98 to 102%), sodium phosphate monobasic monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, crystalline/certified ACS, Assay: ≥ 98 to 102%), sodium citrate dihydrate (Granular/Certified), ferrous ammonium sulfate hexahydrate ($\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, crystalline/certified ACS, Assay: ≥ 98.5 to 101.5%), Sodium potassium tartrate ($\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$, Certified ACS), ethylenediaminetetraacetic acid (EDTA, Electrophoresis grade, Assay: $\geq 99\%$) were purchased from Fisher Chemical™. Finally, HEPES buffer (ultra-pure) was obtained from American bio.

Briefly, 10.0 mL of an apoFtn solution (25 mg mL^{-1} , $443000 \text{ g mol}^{-1}$) in HEPES buffer (pH= 7.4, 0.1 M) containing NaCl (100 mM) was prepared at room temperature. Aliquots of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ ($4.06 \text{ } \mu\text{M}$, $500 \text{ } \mu\text{L}$) were added to the apoFtn solution over a 150 min period at 30 min intervals targeting with a goal of having an average loading of each Ftn cage to be 2000 iron atoms. For each Fe(II) addition, the solution was shaken slowly and was exposed to air for 30 min. The initially colorless solution slowly turned to a yellowish-orange, and a homogeneous solution was observed without any visible precipitation. Excess Fe(II) and other excess ions were removed by dialysis (molecular porous dialysis membrane, MWCO: 12-14 kDa) in fresh Tris buffer (pH 7.4, 0.1 M, 1000 mL) for 1.5 h intervals three times and stored in $4 \text{ } ^\circ\text{C}$.

Spherical-shaped AuNSps ($\sim 15 \text{ nm}$ diameter) were synthesized by a modified method based on prior work (Ji, X. et al., 2007, J. Am. Chem. Soc., 129, 13939; Frenz, G., 1973, Nat. Phys. Sci., 241, 20). First, an aqueous solution containing $\text{NaAuCl}_4 \cdot 2\text{H}_2\text{O}$ (0.25 mM, 100 mL) was boiled in a conical flask (250 mL) under continuous rapid stirring conditions (350 rpm). Upon boiling, 1% sodium citrate dihydrate (1 mL) was quickly added to the flask while stirring. The color of the solution initially turned green and then to bluish-purple, indicating the growth of the AuNSps. The boiling step was continued for another 30 min under vigorous stirring. Then the solution

rapidly changed to orange, followed by a stable wine-red color due to monodispersed spherical-shaped AuNSp formation. The final wine-red color AuNSp solution was stored at 4 °C.

A 20 ml volume of HSFtn ($\sim 10 \text{ mg mL}^{-1}$, $443000 \text{ g mol}^{-1}$) was transferred
5 to fresh phosphate buffer (0.1 M, 1000 mL, pH=7.4) by dialyzing (Molecular porous dialysis membrane, MWCO: 12-14 kDa) for 180 min intervals for a total of three times. Before the coupling reaction between SATA and HSFtn was initiated, a 5 mL sample of HSFtn was kept in an ice bath for 60 min. This cooling procedure helped to stabilize the protein during the reaction. Different concentrations on interest of SATA dissolved in
10 DMSO were then mixed with 5 mL of HSFtn. Individual SATA concentrations of 0.5, 2.6, 5.2, and 13.2 mg/mL were used, resulting in expected SATA: HSFtn ratios of 10, 50, 100, and 200. The AuNSp-HSFtn reaction mixtures were allowed to react overnight at 4 °C. After the SATA conjugation, excess SATA and DMSO were removed by dialysis with fresh phosphate buffer (0.1 M, pH=7.4, 1 mM EDTA) using a molecular porous
15 dialysis membrane (MWCO: 12-14 kDa) for 240 min intervals (a total of four dialysis cycles).

Deacylation of the protected thiol in the AuNSp-HSFtn was accomplished by placing the complex in a solution containing 2.5% hydroxylamine (25 mg mL^{-1} , 0.25 mL added to 5 mL of HSFtn) and TCEP (25 mg mL^{-1} , 0.10 mL added to 5 mL of HSFtn)
20 for 120 min at room temperature. The TCEP helped prevent disulfide bond. After the deacylation, the residual hydroxyl amine and diacylation products were removed by dialyzing it with fresh phosphate buffer (0.1 M, pH=7.4, 1 mM EDTA) in a molecular porous dialysis membrane (MWCO: 12-14 kDa) for 60 min.

AuNSp-HSFtn (100:1 SATA: HSFtn) was individually conjugated to
25 AuNSps at different concentrations ($[\text{AuNSp}] = 57.6, 115.2, 230.4 \text{ and } 460.8 \mu\text{M}$). The final AuNSp-HSFtn bioconjugates were transferred to Tris-HCl buffer (0.1 M, pH=7.4) by dialyzing in a molecular porous dialysis membrane (MWCO: 12-14 kDa) for 180 min (for three dialysis cycles) to remove excess reactant.

For the quantification of thiolation with Ellman's reagent, the absorbance
30 of L-cysteine (3.1 mL) at varying concentrations (0.0625, 0.125, 0.25, 0.5, 1.0, and 2.0 μM) in phosphate buffer (0.1 M, pH=7.4, 1 mM EDTA) was measured at 412 nm. The

relationship between absorbance and L-cysteine concentration was plotted to establish a correlation, and an equation was derived to quantify the -SH groups attached to HSFTn. To prepare Ellman's reagent, a solution of 4.0 mg L⁻¹ was prepared in sodium phosphate buffer (0.1 M, pH=8, 1 mM EDTA). Subsequently, 20 µL of Ellman's reagent was added to SATA-modified HSFTn, thoroughly mixed, and incubated for 15 min at room temperature. Following the incubation, UV-Vis absorbance at 412 nm was measured. The calibration curve established with L-cysteine was used to determine the total number of solvent-exposed free thiol residues in the SATA-modified HSFTn bioconjugates. Further details and visual representations are found in Figure 58.

Characterization

The concentration of HSFTn was quantitatively determined by the Precision Red advanced protein assay (Kikuchi, D. S. et al., 2019, J. Neuroinflammation, 16, 241) and the A280 method (Desjardins, P. et al., 2010, J. Vis. Exp., 33, 33). A 150 W UV-Vis spectrophotometer (Thermo-Fisher Scientific, UL 61010-1) was used to measure the absorbances. The Precision Red protein assay accurately measures protein concentrations from 0.25 to 50 mg/mL. Following the manufacturer's instructions, 200 mL of the assay was added to 1800 mL of protein and incubated for 1 min at room temperature. The protein concentration was determined by measuring the absorbance at 600 nm. The A280 method uses the molar absorptivity of a protein at 280 nm to determine its concentration.

To capture images of AuNSps and AuNSp-HSFTn, a JOEL JEM-1400, 120 kV TEM was used. All AuNSp and AuNSp-HSFTn samples were prepared on copper mesh-supported holey carbon TEM grids from Ted Pella and allowed to dry as thin films. To visualize the protein shell of Ftn, some samples were negatively stained with a 2% PTA solution at pH 7.

A Malvern Zetasizer particle size analyzer was used to measure the hydrodynamic particle size distribution of AuNP and AuNSp-HSFTn bioconjugates. 1 mL solutions were used in a disposable plastic cell (DTS0012), for the particle size analysis and Z.P measurements.

ICP-OES (Thermo-Scientific, iCAP 7400-ASX520) was used to determine the concentration of gold ([Au]) in AuNSp. AuNSp samples were diluted with DI water to 10 mL and 2% by volume HNO₃ was added to acidify the solution. AuNSp were mixed with 5 mL of concentrated aqua regia (25% HCl, 75% HNO₃), digested for at least 24 h, and then diluted the final samples to 10 mL with DI water before analyzing them for Au content using ICP-OES.

Photochemical Batch Reactions

Photochemical experiments were carried out on solutions of interest in a 1 cm² quartz cuvette (Thor Labs). A 200 W Xe lamp (Oriel Instruments, 66002/58) was utilized as the radiation source. Before exposing the sample to simulated solar radiation (SSR), the beam was filtered using an optical long-pass filter ($\lambda > 475$ nm), and the incident absorbance spectrum (800-200 nm) was acquired at zero-time. Then, at designated time points (t=5, 10, 20, 30, 40, and 60 min) absorbance spectrum corresponding to the iron release ($\lambda=562$ nm) and Cr(VI) degradation ($\lambda=372$ nm) was measured. All reactions had a total volume of 3.2 mL and were individually illuminated for a total of 60 min. To conduct the iron release studies, solutions containing AuNSp-HSFtn (~1.12 μ M HSFtn, ~58 μ M AuNSps), 32 mM sodium potassium tartrate, and 80 mM Fz in 0.1 M Tris buffer (pH 7.4) were utilized. For Cr(VI) reduction experiments, solutions containing 0.100 mM K₂Cr₂O₇ (0.2 mM [Cr(VI)]) (no Fz was present) were used.

Results and Discussion

Figure 59A exhibits a TEM image of citrate-capped AuNSps (AuNSp-Citrate) in DI. The majority of the particles have a spherical shape, although some triangular-shaped structures are present. Transferring the AuNSp-Citrate particles to Tris HCl buffer medium resulted in significant changes to the AuNSp morphology. In particular, the AuNSps became elongated and spherical and exhibited aggregation (Figure 59B). Figure 59C shows an unstained TEM image of the AuNSp-HSFtn bioconjugates, wherein the Fh core of the Ftn is visible near the AuNSps. Finally Figure 59D exhibits a stained TEM image of the AuNSp-HFtn system to highlight the position

of the protein cage of Ftn relative to the AuNSps. The image emphasizes that the protein cage of Ftn (white circular structures) is in contact with the AuNSps, consistent with the presence of AuNSp-HSFtn conjugates.

The distribution of particle sizes shown in the TEM images for the AuNSps and AuNSp-HSFtn was also shown by using ImageJ software (Figure 60A and Figure 60C). The average surface area of the AuNSps was $210.7 \pm 134.6 \text{ nm}^2$ and for AuNSp-HSFtn the average surface area was $270.3 \pm 574.4 \text{ nm}^2$ (Figure 60B and Figure 60D). The 30% increase in average surface area (and associated large standard deviation) for AuNSp-HSFtn is due to the elongation of the AuNSps and the aggregation of the AuNSps during the AuNSp-HSFtn modification. The wine-red colored AuNSps were stable and homogeneously dispersed in citrate with an average diameter of $13.6 \pm 2.5 \text{ nm}$ (Figure 59E). DLS for the AuNSps and AuNSp-HSFtn were also carried out. The DLS results show that the solution of AuNSp-Citrate exhibits a single peak at a 22.7 nm (hydrodynamic diameter). In contrast, AuNSp-HSFtn is associated with three peaks at 11.5, 67.5, and 266.5 nm (Figure 61). This result indicates particle aggregation and enlargement. At least for the peak observed at 11.5 nm, the DLS peak was attributed to free HSFtn. It is likely not due to AuNSps since they are closer to $\sim 14 \text{ nm}$.

Figure 62A exhibits the UV-vis absorbance spectra of AuNSp-Citrate, HSFtn and AuNSp-HSFtn. The zoomed in spectral area of the UV-Vis spectra at 400-800 nm is shown in Figure 62B. The absorbance peak of 520 nm is due to the LSPR of AuNSps. There is also a small UV absorbance feature at 250-300 nm which is unique to AuNSps (Capomaccio, R. et al., 2016, Biointerphases, 11). AuNSp-HSFtn exhibits two distinct peaks at 538 nm and 280 nm, corresponding to the LSPR band of the AuNSp and HSFtn protein absorbances, respectively. It is mentioned that the LSPR of the AuNSps attached to HSFtn is shifted 12 nm relative to the AuNSps alone. The redshift in the LSPR absorbance peak of the AuNSp-HSFtn was attributed to an increase in size of the AuNSps of the conjugate.

SATA is a well-known molecule that can modify primary amine groups into terminal sulfhydryl groups (-SH) (Joshi, P. R. et al., 2009, J. Biomed. Mater. Res. Part B Biomater., 91B, 128). Figure 63 and Figure 64 illustrate the chemical reaction between SATA and the primary amine-containing amino acid in HSFtn. These

nucleophilic primary amines can be available at the N-terminus of the polypeptide chain and in the side chain of Lys amino acid residues and positively charged at physiologic pH (McGee, W. M. et al., 2012, J. Am. Chem. Soc., 134, 11412). SATA can be bound to Lys by releasing the bulk N-hydroxysuccinimide group and forming an amide bond. The modified Lys from the reaction with SATA yields a protected terminal thiol. Hydroxylamine can deprotect the SATA-modified Lys to generate terminal sulfhydryl, and TCEP stabilizes the sulfhydryl by preventing the disulfide bond formation (Boutz, D. R. et al., 2007, J. Mol. Biol., 368, 1332). DTNB is selective for -SH groups at neutral pH. As shown in Figure 65, it reacts with a free -SH group to yield a mixed disulfide and yellow color 2-nitro-5-thiobenzoic acid (TNB²⁻). This yellow-colored product can be quantified using UV-vis spectroscopy based on its strong absorbance at 412 nm ($\epsilon = 13,600 \text{ M}^{-1}\text{cm}^{-1}$) (Tyagi, S. C. et al., 1998, Mol. Cell. Biochem., 181, 107). A series of optimization experiments were conducted to determine the ideal SATA: HSFtn molar ratio for achieving maximum thiol bond formation while maintaining the homogeneity of the AuNSp-HSFtn. Data presented in Figure 66 showed that increasing the concentration of SATA per HSFtn molecule resulted in a proportional increase in the number of primary amine residues modified to thiol per HSFtn subunit. However, the SATA conjugation reached a plateau at around SATA:HSFtn molar ratio of 100:1. At this ratio it was determined that there were approximately 335 thiol groups per HSFtn protein molecule (~14 thiols per HSFtn subunit). According to prior literature, ~9 Lys and 2 Cys amino acids are present in each horse spleen apoFtn subunit (Heuterspreute, M. et al., 1981, FEBS Lett., 129, 322).

Experiments were carried out to investigate the photochemistry of AuNSp-HSFtn. Previous research demonstrated that the visible light excitation of the plasmon resonance of AuNSp attached to HSFtn led to the excitation of the HSFtn core material (Fh) through the LSPR effect of gold (Cerkez, E. B. et al., 2019, Environ. Sci. Nano, 6, 85). These prior experiments grew AuNSps on HSFtn via a photochemical process. During the reaction, Fz was employed as a chelating agent for Fe(II). The formation of the Fe(II)-Fz complex resulted in a solution that exhibited a visible purple color with an absorbance peak at 532 nm. ($\epsilon=27,900 \text{ L.mol}^{-1} \text{ cm}^{-1}$) (Lapenna, D. et al., 2002, Biochem. Pharmacol., 64, 1661).

Data shown in Figure 67A show that the excitation of AuNSp-HSFtn with SSR, using a 475 nm long pass filter, showed that 3.9 μM of iron was released from the bioconjugate in 60 minutes. However, when tartrate was absent from the reaction medium, iron release decreased by almost 60% to 1.6 μM . This observation confirms the role of tartrate as a hole scavenger for the photochemical reaction. Fe(II) release from the HSFtn was not detected when AuNSps were absent from the reaction mixture, and an insignificant amount of Fe(II) release was observed ($\sim 0.2 \mu\text{M}$) when the AuNSp-HSFtn bioconjugate was kept in the dark for 60 min. In a control study, where AuNSp were added to a solution containing unmodified HSFtn showed 1.0 μM Fe(II) release under irradiation, about 75% less than when AuNSp were chemically attached to HSFtn in the bioconjugate. It was suspected that when AuNPs are simply added to a solution containing HSFtn, that there is some conjugation between the AuNP and HSFtn, albeit it is likely electrostatic in nature. It is mentioned that proteins can be bio-conjugated to gold either by passive conjugation (non-covalent interactions) or active covalent conjugation. Passive conjugation involves proteins binding to gold particles through van der Waals and ionic forces. But it has stability limitations and requires specific conditions. The AuNSp-HSFtn bioconjugate system contains a stronger covalent Au-S interaction which enhances the photochemical release of iron through LSPR (Gao, J. et al., 2012, Langmuir, 28, 4464).

Studies that investigated the photochemistry of AuNSp-HSFtn (using a long pass filter with a wavelength of $\geq 475 \text{ nm}$) as a function of AuNP concentration ($[\text{AuNSp}] = 57.6, 115.2, 230.4, \text{ and } 460.8 \mu\text{M}$) on the release of Fe(II) were conducted. The findings demonstrated a direct correlation between $[\text{AuNSp}]$ concentration and Fe(II) release rate, with the rate increasing as the concentration of AuNSp attachment to HSFtn increased. Nevertheless, a certain point was reached where the Fe(II) release rate stabilized and gradually declined. The research indicated that the optimal $[\text{AuNSp}]$ concentration for the highest Fe(II) release rate was 115.2 μM (Rate = $920.8 \mu\text{M min}^{-1} \text{ g}^{-1}$) (Figure 67B and Table 3).

Table 3. The Average Fe(II) release rate from the AuNSp-HSFtn with varying $[\text{AuNSp}]$

[AuNSp] (μM)	Average Fe release rate ($\mu\text{M min}^{-1} \text{g}^{-1}$)
57.6	668.6
115.2	920.8
230.4	627.5
460.8	357.5

In addition to iron release from AuNSp-HSFtn the photochemistry of the bioconjugate in the presence of aqueous chromate (Cr(VI)) was investigated. Previous research showed that the reduction of Cr(VI) to Cr(III) could be accomplished by exposing photochemically grown Au spheres on HSFtn to light with wavelength (λ) > 475 nm while in the absence of Au, HSFtn needed to be exposed to $\lambda < \sim 475$ nm to facilitate Cr(VI) reduction. Figure 68 shows data for the reduction of Cr(VI) when Au spheres are linked to a thiolated HSFtn using SATA. Exposure of this bioconjugate to SSR with $\lambda > 475$ nm leads to the reduction of aqueous Cr(VI). Specifically, the exposure of the AuNSp-HSFtn bioconjugate to 0.2 mM of Cr(VI) in the presence of wavelengths $\lambda > 475$ resulted in a conversion of 36% Cr(VI) to Cr(III) in 120 min. A control reaction carried out in the dark did not show any reduction of Cr(VI) (Figure 68).

The Ftn core of laboratory-prepared HSFtn contains Fh that can release Fe(II) through the bandgap excitation mechanism using a photon energy > 2.6 eV (Pavithra, S. et al., 2023, Inorg. Nano-Metal Chem., 53, 152). The long pass filter used in present experiments ($\lambda < 475$ nm) eliminates photons that would be able to directly excite the bandgap of the Fh core material and no Fe(II) release occurs in this circumstance. Figure 69 exhibits UV-visible absorbance spectra for Fh and AuNSps. Fh has a wide absorbance band at 430 nm (Liu, Y. et al., 2017, Appl. Catal. B Environ., 213, 74), which overlaps with the plasmonic band of AuNPs in the visible region at 520 nm. As a result, the PIRET mechanism may be viable because of the spectral overlap between the gold and small bandgap semiconductor Fh (Subramanyam, P. et al., 2022, J. Photochem. Photobiol. C Photochem. Rev., 51, 100472), even though the AuNSp and semiconductor core is separated by a 2 nm thick HSFtn protein shell. In PIRET, energy is transferred

from the plasmonic metal, and this induces charge separation in the semiconductor via a dipole-dipole interaction. Also, AuNSps can generate hot electrons through intra-band excitation or inter-band transitions (d-s) (Li, C. et al., 2022, Appl. Catal. B Environ., 303, 120901). Plasmonic gold has an inter-band transition energy of ~ 2.3 eV (Govorov, A. O. et al., 2014, Nano Today, 9, 85). Hence, the maximum energy used in the SATA functionalized AuNSp-HSFtn is >2.6 eV (<475 nm) which is sufficient to generate hot electrons by the intra-band excitation mechanism. The n-type semiconductor, Fh, could conceivably accept these electrons in the unfilled states of its conduction band. If this mechanism is operative, it would appear that the plasmonic hot electrons are able to cross the S.B (PHETr mechanism) or tunnel (PHETu mechanism) through the ~ 2 nm HSFtn protein shell between that separates the AuNP from the Fh core.

Future Applications

Nanoparticle probes are being investigated for the detection and treatment of deadly diseases in humans. Previous research has generally shown that AuNSps are suitable for the treatment of humans due to their biocompatibility and stability. In the context of contrast agents, AuNSps have been investigated as a viable alternative to commercial contrast agents. Bioconjugates made by stabilizing and functionalizing AuNSps with biomolecules, such as proteins, have demonstrated outstanding potential for biomedical imaging applications (DeLong, R. K. et al., 2010, Nanotechnol. Sci. Appl., 3, 53; Anik, M. I. et al., 2020, Nano Sel., 3, 792). Clustering AuNSps together promotes interparticle plasmon coupling, which is also has been proposed to be potentially advantageous for imaging. With regard to Ftn, previous research has shown that gold nanoclusters (GNC) incorporated into apo HFtn can serve as a NIR fluorescent probe for kidney targeting and imaging (Sun, C. et al., 2015, Bioconjug. Chem., 26, 193). The present work has shown that when AuNPs are attached to SATA functionalized HSFtn there is a redshift of the plasmonic AuNSps. This is likely due to aggregation of bioconjugates. This type of cluster formation, if controllable in vivo, could make AuNSp-HSFtn potentially useful for biomedical imaging.

Another potential application of the present AuNSp-HSFtn bioconjugate is in cancer treatment. Prior research has shown that HSFtn has a strong affinity for TfR1

receptors, commonly found in cancer cells. This suggests that the AuNSp-HSFtn bioconjugate system investigated in present research could potentially be precisely directed toward cancer cells, making it a versatile tool for both cancer cell imaging and tumor suppression. If this targeting is successful, it could be investigated whether the exposure to visible photons could release Fe(II) into the cancer cells, triggering ferroptosis, a process where the generation of ROS due to the presence of Fe(II) destroys cellular membranes.

Conclusion

The primary objective of this chapter was to develop AuNSp-HSFtn bioconjugates by enhancing the surface-exposed thiol content of component HSFtn using the linker molecule SATA. AuNSp-Citrate were chemically synthesized, exhibiting a UV-vis absorbance peak at 520 nm. The average surface area of the AuNSps was measured to be $210.7 \pm 134.6 \text{ nm}^2$, while for the AuNSp-HSFtn bioconjugates, it increased to $270.3 \pm 574.4 \text{ nm}^2$. The wine-red colored AuNSps showed an average diameter of $13.6 \pm 2.5 \text{ nm}$. DLS results indicated that the AuNSp-Citrate solution exhibited a single HD of 22.7 nm. In contrast, the AuNSp-HSFtn bioconjugates displayed three peaks at 11.5 nm, 67.5 nm, and 266.5 nm, indicative of particle aggregation and enlargement during the bioconjugation process. Optimization reactions were conducted to determine the optimal SATA: HSFtn molar ratio for achieving the maximum thiol content per HSFtn protein molecule. The highest Thiol: HSFtn ratio of 335:1 was attained at a SATA: HSFtn molar ratio of 100:1. Moreover, the AuNSp-HSFtn bioconjugate system demonstrated the release of Fe(II) under SSR irradiation ($\lambda > 475 \text{ nm}$), which is below the bandgap energy of the Fh core material in HSFtn. The results indicated that $3.9 \mu\text{M}$ Fe(II) was released during 60 min of light exposure ($\lambda > 475 \text{ nm}$), suggesting that the localized surface plasmon resonance (LSPR) effect of AuNSps attached to HSFtn could induce Fe(II) release from the core. The release of Fe(II) was found to be dependent on the concentration of AuNSps, with the maximum release observed at $115.2 \mu\text{M}$. Furthermore, Cr(VI) reduction studies revealed that the AuNSp-HSFtn bioconjugate system effectively converted 36% of Cr(VI) to Cr(III) within 120 min under light exposure ($\lambda > 475 \text{ nm}$). The mechanistic approach to Fe(II) release from HSFtn attached

to AuNSps was discussed, emphasizing the viability of the PIRET mechanism due to the spectral overlap of the Fh semiconductor and AuNSps, as well as the potential for PHETr/PHetu between AuNSps and Fh. Finally, future modifications of the system and its simultaneous utilization for cancer therapeutics and imaging were discussed.

5

The first part of the present research investigated for the first time the photochemistry of CuFtn. The primary goal of this study was to shed light on whether electrons resulting from the bandgap excitation of the inorganic core could transverse the protein shell of Ftn to induce chemistry in the aqueous phase. It was shown that Cr(VI) in solution could be reduced via the photo-excitation of the core, lending strong support to a mechanistic pathway that includes electron transfer through the Ftn shell. This is an important contribution to the scientific community since this study is the first study to confirm under well-controlled conditions that this electron transfer process occurs in photoexcited Ftn. Furthermore, the study investigated the wavelength-dependence of the Cr(VI) reduction by photoexcited CuFtn system and showed that photons able to excite electrons across the bandgap of the semiconductor core were a prerequisite for reaction. Characterization of the copper hydr(oxide)-based core material was carried out by using techniques that included XRD, XPS, TEM, and UV-Vis. The CuFtn has the potential as a photocatalytic redox system for water remediation applications.

Bioconjugation chemistry has gained significant importance in biomedical applications due to its ability to integrate diverse properties of its components into a unified system. The present work is focused on , in part, investigating the synthesis and photochemistry, respectively, of a novel bioconjugate system made from HFtn and AuNRs (i.e., AuNR-HFtn). A synthetic protocol for the bioconjugation of anisotropic gold nanostructures, specifically AuNRs, with HFtn is shown by utilizing solvent-exposed cys on HFtn as attachment points. These novel bioconjugates allowed Ftn to extend its photochemistry to longer wavelengths, specifically in the NIR region. This bioconjugate, the Fh semiconducting material within the Ftn can be activated via the NIR excitation of the LSPR of the attached anisotropic AuNRs. Support for this contention came from the experimental observation that NIR excitation of the bioconjugate was able to reduce solution phase Cr(VI) while NIR (at 850 nm) excited AuNRs alone were not

able to reduce Cr(VI) in solution. The surfactant CTAB can also be removed from the AuNRs to form the bioconjugate. Removal of the CTAB also ensured that the AuNRs were non-toxic and suitable for biological cell studies, which were investigated in a later chapter. The bioconjugate systems were characterized with characterization techniques, including UV-Vis, DLS, and Z.P measurements to assess surface charge properties. TEM imaging was utilized to support the assertion that the AuNRs were conjugated to the HFtn. These findings hold great promise for the application of these types of bioconjugates for various biomedical applications, which include targeted drug delivery, imaging, and therapeutic interventions.

The present work further relates to the investigation of the utilization of the AuNR-HFtn bioconjugate system for a potential biomedical application. Studies using the PC3 cancer cell line were used to investigate the effect of the NIR-excited AuNR-HFtn on the viability of the cancer cells. While more experiments in this area need to be carried out, the results that were obtained do underscore the potential of bioconjugates for the advancement of novel biomedical therapeutics and for addressing critical healthcare challenges.

The present work further relates to the bioconjugation of another anisotropic gold nanostructure, AuNSs, with HFtn through the utilization of exposed cysteine on the protein surface (AuNS-HFtn). A research achievement in this study was the successful removal of the toxic surfactant TX-100 (confirmed by ATR-FTIR analysis) that was employed during AuNS synthesis. Removal of this cytotoxic surfactant allows the AuNS-HFtn bioconjugates to potentially be used for biomedical applications. Like the attachment of AuNRs to HFtn, the attachment of AuNSs extended the photochemistry of HFtn to longer wavelengths (in the NIR region).

Another objective of the present work was to modify the external surface of HSFtn to allow the attachment of AuNSps (i.e., AuNSp-HSFtn). HSFtn was first modified with SATA to introduce solvent-exposed -SH groups on the surface of HSFtn. The resulting AuNSp-HSFtn bioconjugate exhibited the release of Fe(II) under longer wavelengths of light ($\lambda > 475$ nm) than if the AuNSp was absent. A future direction would be to attach AuNRs to this modified HSFtn so HSFtn could, along with

AuNR/AuNS-HFtn, find potential applications in the biomedical or environmental chemistry research area.

Overall, it was believed that the present work made important contributions to the understanding of the photochemistry of Ftn and AuNP-Ftn bioconjugates. It was showed in particular that the interesting photochemical and chemical properties of the bioconjugates might be useful for future applications in the environmental and biomedical fields. A future direction should certainly include more detailed investigations of the photochemical mechanisms occurring in the bioconjugate systems. Transient absorption spectroscopy would be useful, for example, to shed light on electron transfer between the AuNR/AuNS and the inorganic core of Ftn. Understanding the energy/electron transfer mechanisms will provide valuable insight into the photochemistry of bio-inorganic hybrid materials and may lead to the development of innovative applications in various fields. By employing confocal microscopy, detailed, high-resolution images of the initial contact between cancer cells and the AuNR/AuNS-HFtn system can be captured to track the internalization and trafficking of the AuNR/AuNS-HFtn system within the cancer cells, and evaluate any potential cellular damage caused by the interaction.

Experimental Techniques

Transmission Electron Microscopy (TEM)

TEM imaging is a powerful technique with a magnification (< 50 million) that allows atomic-level spatial resolution (~ 0.2 nm). The technique is essential to visualize the structure of materials at the nanoscale (Schrand, A. M. et al., 2010, Nat. Protoc., 5, 744). In TEM, a beam of electrons is transmitted through an ultrathin (>100 nm) specimen, and the resulting interactions between the electrons and the sample provide detailed information about its morphology, crystal structure, and elemental composition (Inkson, B. J., 2016, Materials characterization using nondestructive evaluation (NDE) methods, pp 17-43). The high atomic-scale spatial resolution of TEM allows for the examination of fine details within materials (Muller, D. A., 2009, Nat. Mater., 8, 263). By manipulating the electron beam, various imaging modes can be employed, including bright-field imaging (Findlay, S. D. et al., 2010, Ultramicroscopy,

110, 903), dark-field imaging, and high-resolution imaging (Otten, M. T., 1993, Ultramicroscopy, 48, 77), each providing unique contrast mechanisms. TEM imaging has found widespread applications in materials science, nanotechnology, biology, and other scientific fields, enabling researchers to explore and understand the intricate features and properties of nanomaterials and biological specimens with exceptional detail (Smith, D. J. et al., 2010, Microelectron. Reliab., 50, 1514).

All the TEM images for the research were collected using a JOEL JEM-1400 TEM operating at 120 kV. All Ftn protein samples were prepared on copper mesh-supported holey carbon TEM grids (Ted Pella) and allowed to dry as a thin film. A 10 μ L aliquot of sample was deposited on the grid and allowed to sit for 10 min. Excess liquid was removed by filter paper, and the grid was then rinsed with 10 μ L of water. Some samples were negatively stained with a 2% phosphotungstic acid (PTA) solution at pH 7 to allow the visualization of the Ftn protein shell. In order to stain the sample, 2 μ L of the PTA solution was deposited, allowed to sit for 2 min, and the excess was removed by drawing the liquid droplet from the surface using filter paper. A 2% PTA staining process was followed to image the Ftn protein outer shell. Usually, the negative staining process wraps the particles with electron-dense material and allows the stained area to be highlighted. This highlighting occurs from the contrast between the stained area (dark) and unstained areas (light). TEM images of Ftn typically show the dense inner core as black spheres, whereas when stained, they look like Cheerios since the protein shell becomes visible (Figure 70). The morphology of the AuNPs, AuNRs, and AuNSs were characterized throughout this work with TEM (Figure 70C – Figure 70F).

Selected Area Electron Diffraction (SAED)

SAED is a powerful technique used in materials science and electron microscopy to study the crystal structure and orientation of materials at the nanoscale (Brodusch, N. et al., 2013, J. Microsc., 250, 1). It involves directing a beam of high-energy electrons onto a small selected area of a sample. The electrons interact with the crystal lattice of the material, causing them to scatter (Snyder, G. J., 2008, Nat. Mater., 7, 105). The scattered electrons then form a diffraction pattern, which is captured by a detector. The diffraction pattern provides information about the arrangement of atoms

within the material and can be used to determine the crystal structure, crystal symmetry, and lattice parameters. SAED is particularly useful for analyzing crystalline materials such as metals, alloys, semiconductors, and minerals. It allows researchers to obtain valuable insights into the microstructure, phase composition, and defects present in the material. SAED is commonly employed in TEM and scanning transmission electron microscopy (STEM) techniques, enabling researchers to investigate the atomic structure and properties of materials with high spatial resolution (Liu, X., 2018, Small Methods, 2, 1800006). By interpreting the diffraction patterns, a deeper understanding of the materials' properties and behavior can be developed, facilitating advancements in materials design, characterization, and engineering.

UV-Vis Spectroscopy

UV-Vis is used to measure the UV or visible light absorbed by, or transmitted through, a sample with respect to a reference. Absorbance(A) under the different UV-Vis wavelength ranges can be calculated from the transmittance (T) (Poh, J.-J. et al., 2021, Sensors Actuators A Phys., 325, 112698). Absorbances can be converted to concentrations via Beer–Lambert's equation (Parnis, J. M., 2013, J. Photochem. Photobiol. A Chem., 267, 6). Hence, the UV-Vis spectrum shows the different absorbances of the compound of interest at different wavelengths (Cerdeira, V. et al., 2022, TrAC Trends Anal. Chem., 157, 116772).

$$A = \varepsilon C l = \log_{10} \left(\frac{I_0}{I} \right) = \log_{10} \left(\frac{1}{T} \right) = \log_{10}(T) \quad (1)$$

where, A= absorbance, ε = molar absorptivity of the absorbing species, C= concentration of the absorbing species, l= length of the light path, I_0 = Intensity of incident light, I= intensity of the transmitted light, and T= Transmission.

Either a single high intensity xenon lamp, which can simultaneously generate UV and visible light, or two lamps consisting of a tungsten or halogen lamp for visible light and a deuterium lamp for UV light are used as radiation sources. A monochromator and dispersive element allow specific wavelengths to be selected (Figure 71). The light passes through the sample and the transmitted radiation is detected by a photoelectric semiconducting material to generate a readable signal. The output of UV-

vis is typically shown as absorbance, optical density, or transmittance vs. the wavelength (Figure 71).

Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES)

5 ICP-OES is a powerful analytical technique to determine the elemental composition of samples. The technique is based upon the spontaneous emission of photons from the gaseous atoms and ions in the sample that are excited by Argon (Ar) plasma and relaxed back to the ground state. The unique wavelength corresponding to the emitted photons can be used to determine the elements (Hou, X., 2000, Encyclopedia of
10 analytical chemistry, pp 9468-9485).

In the ICP-OES setup, a peristaltic pump helps to deliver the sample solution to the nebulizer. There, the sample fluid decomposes into droplets by the Ar gas flow. The downstream spray chamber can break the larger sample droplets. The atomized sample aerosol is transferred to the plasma-generating chamber filled with Ar. A RF
15 power supply coupled to the plasma coil generates a high-frequency (40 MHz) electric current, which transfers energy to the Ar gas. The resulting plasma is generated from the ionized Ar ($T = 6000\text{--}10,000\text{ K}$) and is mixed with the sample. The dichroic spectral combiner (DSC) enables synchronizing between the radial and axial measurement modes. The polychromator consists of prisms, gratings, and mirrors, and separates the light that
20 enters into the multiple component wavelengths simultaneously, and the dispersed spectrum generated in the polychromator is focused onto the detector. A charge-injection device (CID) sensor or charged coupled device (CCD) sensor is utilized to detect the signal (Figure 72).

25 *Dynamic Light Scattering (DLS)*

DLS, also called photon correlation spectroscopy, is utilized to investigate the hydrodynamic size of nanostructures (Pecora, R. et al., 2000, J. Nanoparticle Res., 2, 123). The DLS technique is a non-invasive technique that requires only minimal sample preparation. DLS measurements can be influenced by various factors such as solvent
30 type, sample homogeneity, sample concentration, color, fluorescence, and the shape of the particles being studied (Bhattacharjee, S., 2016, J. Control. Release, 235, 337).

Nanostructures dispersed in solution scatter incident light proportional to the 6th power of their radii (Rycenga, M. et al., 2011, Chem. Rev., 111, 3669). The fluctuation rate in scattered light is related to the diffusion rate of the particles in the solvents and their hydrodynamic radii (Stetefeld, J. et al., 2016, Biophys. Rev., 8, 409). The time-dependent fluctuation in the scattered light is measured in the DLS technique (Hassan, P. A. et al., 2015, Langmuir, 31, 3). Hence, the fluctuation rates can be manipulated to obtain the diffusion coefficients and the particle sizes via the Stokes-Einstein equation (Bevan, M. A. et al., 2000, J. Chem. Phys., 113, 1228) (2)

$$D_t = \frac{K_B T}{6\pi\eta r_h} \quad (2)$$

where D_t is the diffusion coefficient, K_B is Boltzmann's constant, T is the temperature in Kelvin, η = absolute viscosity, and r_h = hydrodynamic radii

For DLS experiments for the present work, 1 mL of transparent and homogeneous samples were used in a clean, square cuvette made of translucent plastic. The schematic diagram of the DLS instrument is shown in Figure 73. The laser source provides a steady beam of coherent monochromatic light, whereas the attenuator can adjust the laser power. The laser beam transfers through the sample to the detector via a focusing lens. The detectors are placed at 90° and 173° angles. The detector at 173° angle can detect the backscattering and filter excess light scattering. After several data fittings and analyses, the final size distribution can be obtained. The Malvern Zetasizer particle size analyzer was used to measure the hydrodynamic particle size distribution throughout the dissertation.

25 *Attenuated Total Reflectance – Fourier Transform Infrared Spectroscopy (ATR-FTIR)*

ATR-FTIR was used to confirm the removal of CTAB and TX-100 surfactants from AuNPs, respectively. All experiments were conducted by using a Nicolet Magna 750 FTIR spectrometer with a single bounce diamond crystal ATR cell (Specac™) and mercury cadmium telluride A (MCTA) detector cooled by liquid N₂.

30 ATR-FTIR is a tool that can probe *in situ* chemical reactions and the structure (Functional groups) of single or multiple layers of adsorbed or deposited species at a

solid or liquid interface. The IR beam is internally reflected at the diamond crystal-sample interface while traveling through the crystal. During the internal reflection process, part of the IR beam can penetrate into the sample ($\sim 0.50\text{-}5\ \mu\text{m}$) as an evanescent wave and be absorbed (Figure 74). The penetration depth of the evanescent wave can be
 5 calculated by the following equation (Equation 3) (Zaca-Moran, P. et al., 2018, Laser Phys., 28, 116002)

$$D_p = \frac{\lambda}{2\pi n_{crystal} [(\sin^2 \theta) - (\frac{n_{sample}}{n_{crystal}})^2]^{0.5}} \quad (3)$$

where, D_p = depth of penetration, λ = wavelength of the light, $n_{crystal}$ = The refractive index of the diamond crystal, n_{sample} = The refractive index of the sample, and θ = the
 10 angle of incidence of the light.

The attenuated IR beam is directed to the FTIR detector from the diamond crystal. The detector records the IR beam as an interferogram signal, and Fourier transforms it to generate an IR spectrum.

15 *Fluorimeter*

A Fluorimeter is an analytical instrument commonly used in scientific research and various industries to measure the fluorescence properties of samples. It operates on the principle of exciting a sample with a specific wavelength of light and then detecting the emitted fluorescence (Lichtman, J. W., 2005, Nat. Methods, 2, 910). The
 20 instrument consists of an excitation source, such as a high-intensity lamp or laser, which emits light of a specific wavelength onto the sample. The sample absorbs the excitation light energy and re-emits it at a longer wavelength, known as fluorescence emission. The emitted light is then collected by a detector, typically a photomultiplier tube or a photodiode, which converts the light signal into an electrical signal. The intensity of the
 25 emitted fluorescence is measured and recorded, providing valuable information about the sample's properties, such as its chemical composition, molecular structure, and concentration. Their sensitivity, accuracy, and versatility make fluorimeters indispensable tools in scientific research and industrial applications.

30 *Powder X-Ray Diffraction (PXRD)*

The way that the incident X-ray beam interferes with crystalline solid surfaces diffraction occurs or not. Bragg's law expresses that when the incident X-ray beam hits the crystal surface of the solid, its incidence (θ) will reflect with the same angle of scattering (θ) (Adams, B. W., 2008, Rev. Sci. Instrum., 79) (Figure 75 and Figure 76).

- 5 If the path difference (d) is a whole number of wavelengths, constructive interference will occur and reflect the X-rays. If not, destructive interference will appear on the crystal surfaces. X-ray diffraction (XRD) is based on constructive interference of monochromatic X-rays and a solid crystalline sample. Bragg's equation is as follows (Bunaciu, A. A. et al., 2015, Cric. Rev. Anal. Chem., 45, 289) (Equation 4).

10
$$n\lambda = 2d \sin\theta \quad (4)$$

where, n = integer, λ =the wavelength of X-ray, d = spacing of the crystal layers (path difference), θ = incident angle (the angle between the incident ray and the scattering plane).

- PXRD is generally used to identify unknown crystalline materials such as
15 minerals and inorganic compounds. Besides, to determine the crystalline or amorphous nature and the purity of materials, to determine the unit cell dimensions, and to analyze the orientation of crystal grains in polycrystalline materials.

- X-rays are generated in a cathode ray tube by bombarding the target material with high-energy electrons. Copper is the most common target material (CuK α radiation = 1.5418Å) K α and K β core electrons of the target material are typically excited
20 to generate characteristic X-ray spectra. In a powdered sample, generally, all the particulates are randomly orientated. When the sample and the detector rotate through a range of 2θ ($2\theta = 0^\circ$ - 80°), if the geometry of the incident X-rays fulfills the Bragg law requirements, constructive interference occurs. The resulting X-ray signal can be
25 detected.

- All PXRD measurements in this dissertation were carried out by the Bruker Kappa APEX II DUO diffractometer using Mo K α radiation from a sealed molybdenum tube with a TRIUMPH monochromator. Pure CuCtrl, CuO, and Cu(OH) $_2$ commercial samples were finely ground and suspended in isopropyl alcohol. Prior to the
30 analysis, samples were drop cast and concentrated on Si low background sample holder (BrukerAXS) and dried.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. While this invention has been disclosed with reference to specific embodiments, it is
5 apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed is:

1. A composition comprising a Ferritin-metal nanoparticle bioconjugate,
wherein the Ferritin-metal nanoparticle bioconjugate comprises at least one metal nanoparticle and at least one Ferritin or a variant or a fragment thereof conjugated to the at least one metal nanoparticle; and
wherein the Ferritin-metal nanoparticle bioconjugate specifically targets at least one cell expressing a transferrin receptor 1 (TfR1).
2. The composition of claim 1, wherein the at least one Ferritin or a variant or a fragment thereof is selected from the group consisting of a human H-type Ferritin or a variant or a fragment thereof, horse spleen Ferritin or a variant or a fragment thereof, and any combination thereof.
3. The composition of claim 1, wherein the at least one metal nanoparticle is selected from the group consisting of a metal nanorod, metal nanosphere, metal nanostar, and any combination thereof.
4. The composition of claim 1, wherein the at least one metal nanoparticle is selected from the group consisting of a gold nanoparticle, iron nanoparticle, chromium nanoparticle, copper nanoparticle, and any combination thereof.
5. The composition of claim 1, wherein the at least one metal nanoparticle further comprises an oxide coating.
6. The composition of claim 5, wherein the oxide coating is selected from the group consisting of a Fe_2O_3 coating, SiO_2 coating, and any combination thereof.

7. The composition of claim 1, wherein the at least one Ferritin or a variant or a fragment thereof is conjugated to the at least one metal nanoparticle via a linker.
8. The composition of claim 1, wherein the linker comprises N-succinimidyl S-acetylthioacetate or a derivative thereof.
9. The composition of claim 1, wherein the Ferritin-metal nanoparticle bioconjugate has an average hydrodynamic diameter of between 5 nm to 1,000 nm.
10. The composition of claim 9, wherein the Ferritin-metal nanoparticle bioconjugate has an average hydrodynamic diameter of between 10 nm to 300 nm.
11. The composition of claim 1, wherein the Ferritin-metal nanoparticle bioconjugate absorbs light having a wavelength between 200 nm to 1100 nm.
12. The composition of claim 1, wherein the Ferritin-metal nanoparticle bioconjugate releases at least one iron ion when irradiated with light having a wavelength of between 200 nm to 1100 nm.
13. The composition of claim 1, wherein the metal nanoparticle is a gold nanorod, and
the Ferritin-metal nanoparticle bioconjugate has an average width of between 5 nm to 20 nm and an average length of between 30 nm to 70 nm.
14. The composition of claim 1, wherein the metal nanoparticle is a gold nanostar, and

the Ferritin-metal nanoparticle bioconjugate comprises between 3 to 7 spikes,
wherein the spikes have an average length of between 20 nm to 150 nm.

15. The composition of claim 1, wherein the metal nanoparticle is a gold nanosphere, and
the Ferritin-metal nanoparticle bioconjugate has a surface area of between 50 nm² to 900 nm².

16. The composition of claim 1, wherein the Ferritin-metal nanoparticle bioconjugate further comprises a targeting domain.

17. The composition of claim 1, wherein the composition further comprises a therapeutic agent.

18. The composition of claim 1, wherein at least one cell expressing a TfR1 is a cell overexpressing a TfR1.

19. The composition of claim 18, wherein the cell overexpressing a TfR1 is a cancer cell.

20. A method of treating a disease or disorder associated with the level or activity of a TfR1 in a subject in need thereof, wherein the method comprises:
administering to the subject the composition of claim 1, and
irradiating the subject with light having a wavelength of between 200 nm to 1100 nm.

21. The method of claim 20, wherein the disease or disorder is selected from the group consisting of cancer, prostate cancer, breast cancer, Alzheimer's disease, and any combination thereof.

23. A method of inhibiting proliferation of at least one cell expressing a TfR1, wherein the method comprises:

administering to the subject the composition of claim 1, and
irradiating the subject with light having a wavelength of between 200 nm to 1100 nm.

24. A method of inducing ferroptosis of at least one cell expressing a TfR1, wherein the method comprises:

administering to the subject the composition of claim 1, and
irradiating the subject with light having a wavelength of between 200 nm to 1100 nm.

25. The method of claim 24, wherein the irradiation selectively kills or inhibits the at least one cell expressing a TfR1.

26. The method of claim 25, wherein the at least one cell expressing a TfR1 is a cancer cell.

27. A method of making a Ferritin-metal nanoparticle bioconjugate, comprising the steps of:

providing a metal nanoparticle; and
dispersing the metal nanoparticle in a solution comprising Ferritin.

28. The method of claim 27, wherein the solution comprising Ferritin is a buffered solution.

Figure 1B

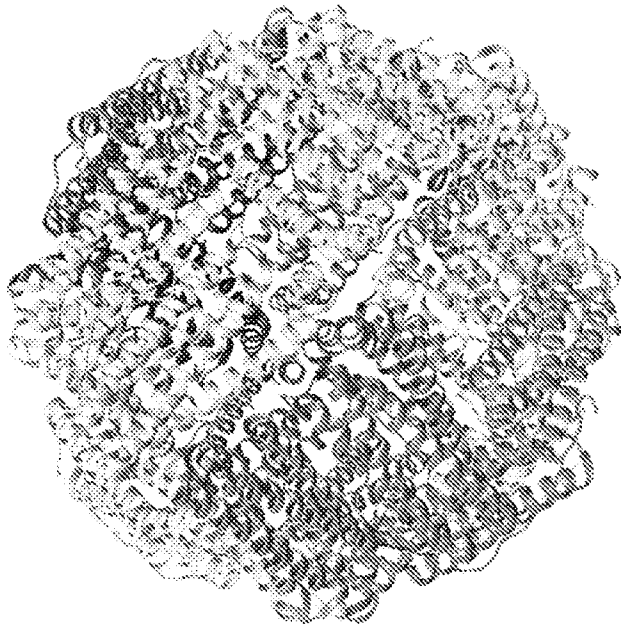


Figure 1A

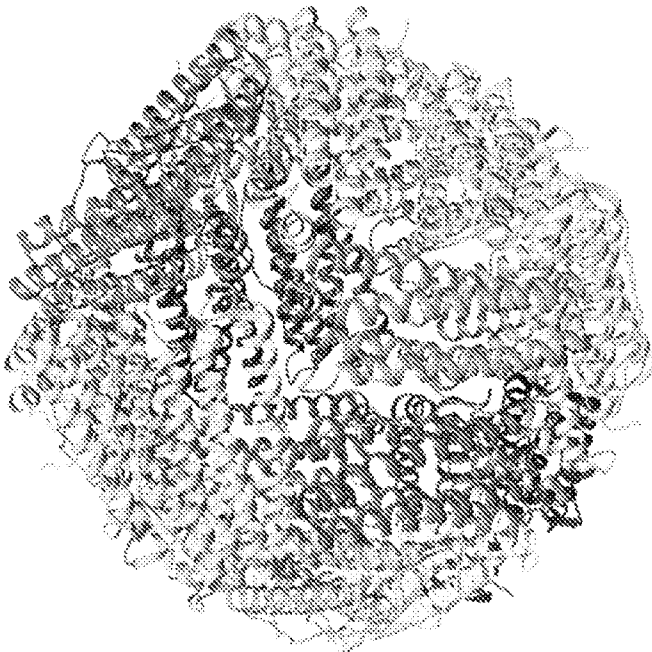


Figure 1

2/93

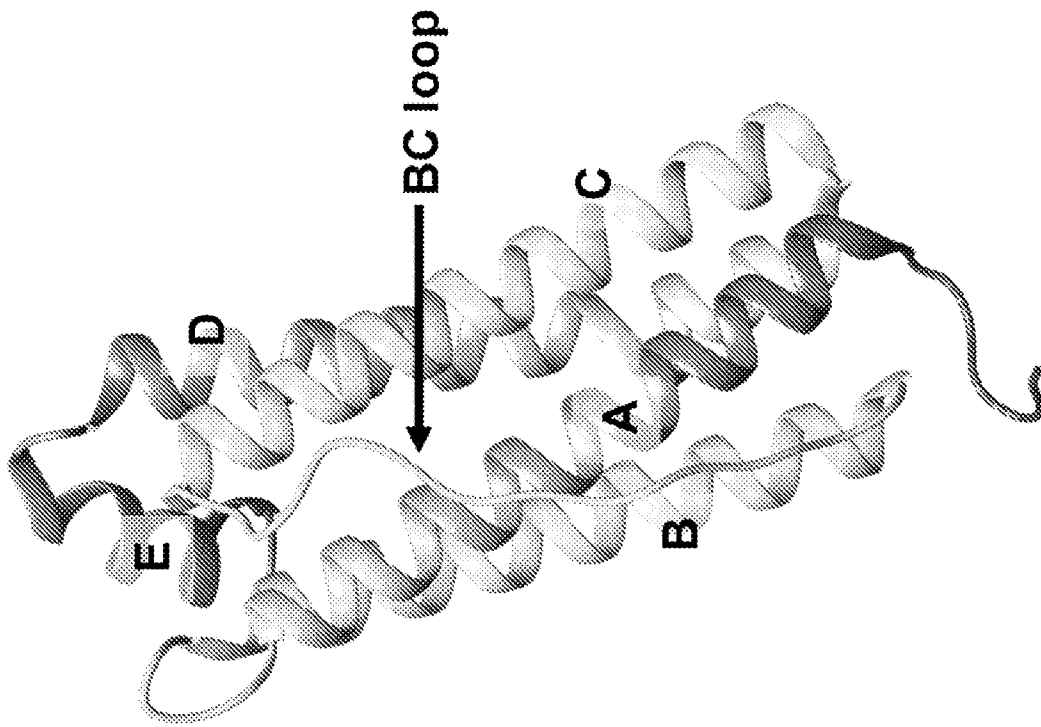


Figure 2

Figure 3A

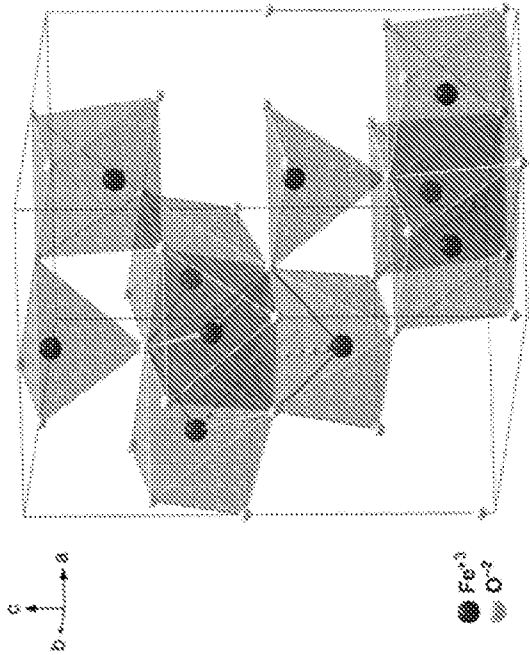


Figure 3B

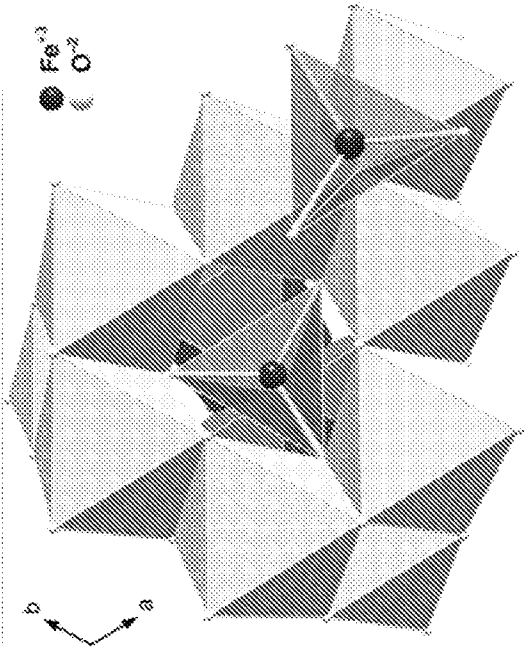


Figure 3

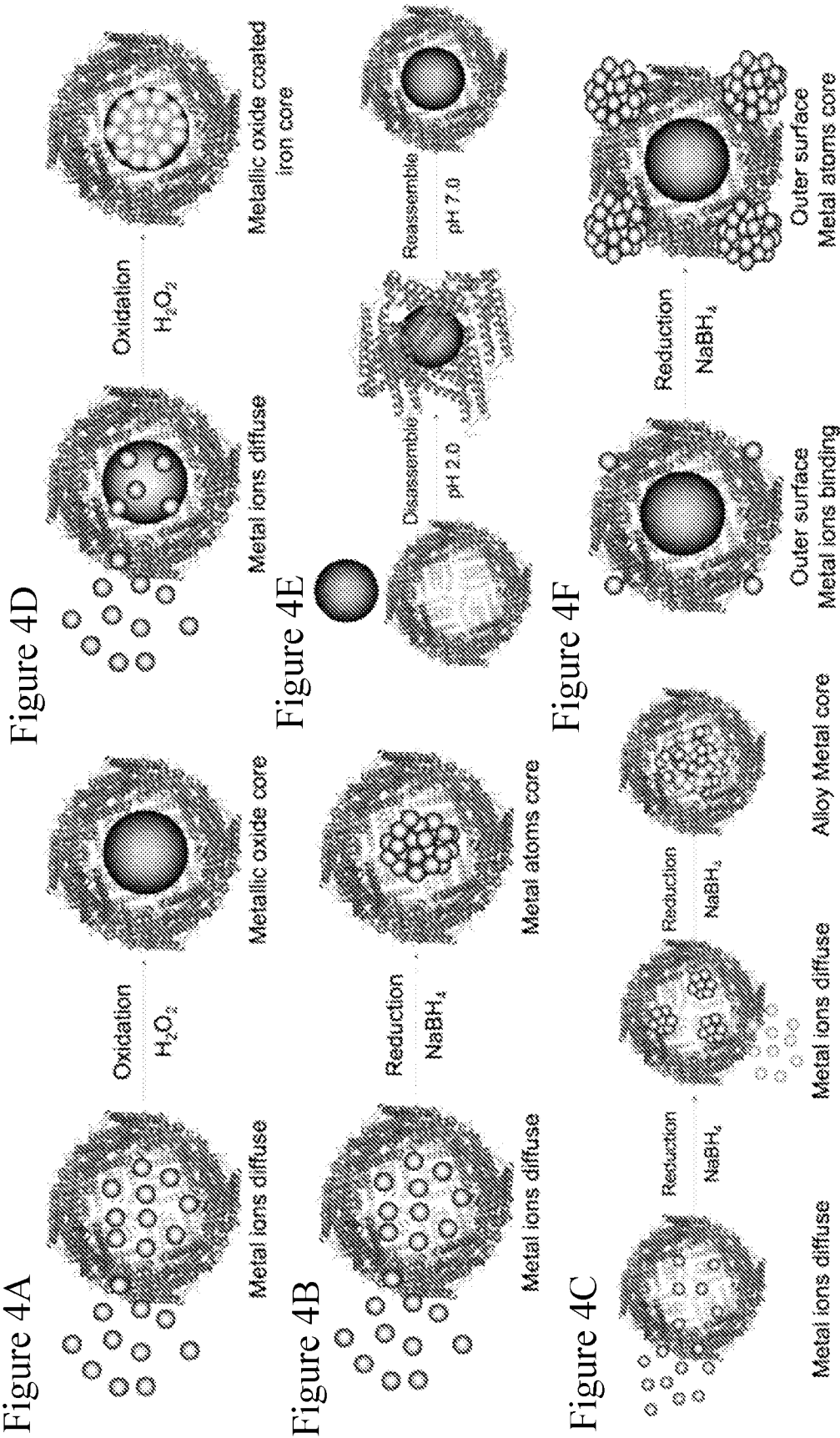


Figure 4

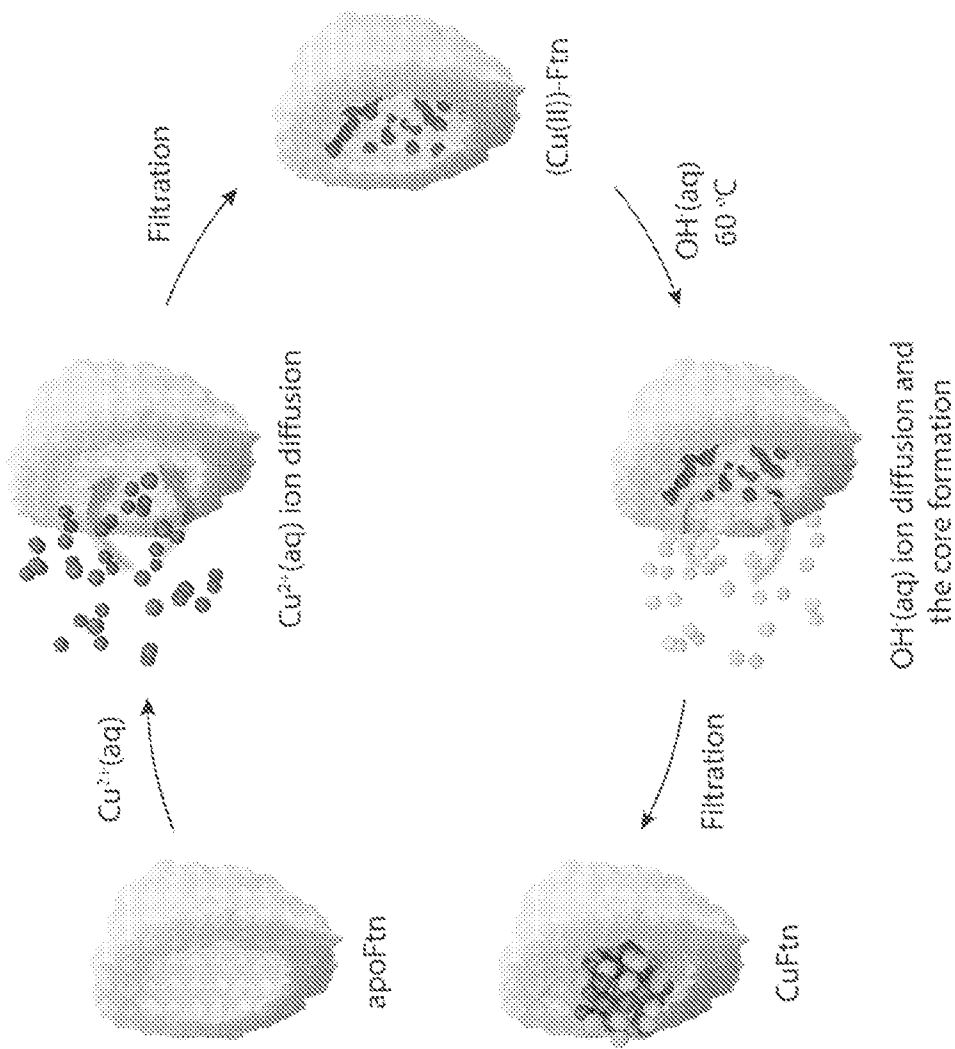


Figure 5

Figure 6A

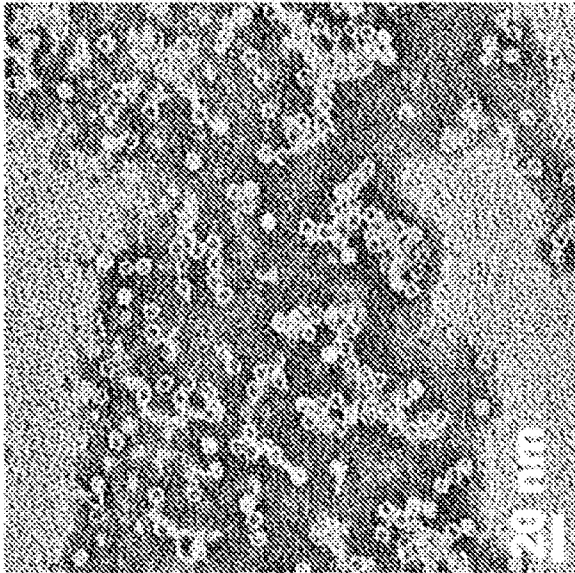


Figure 6B

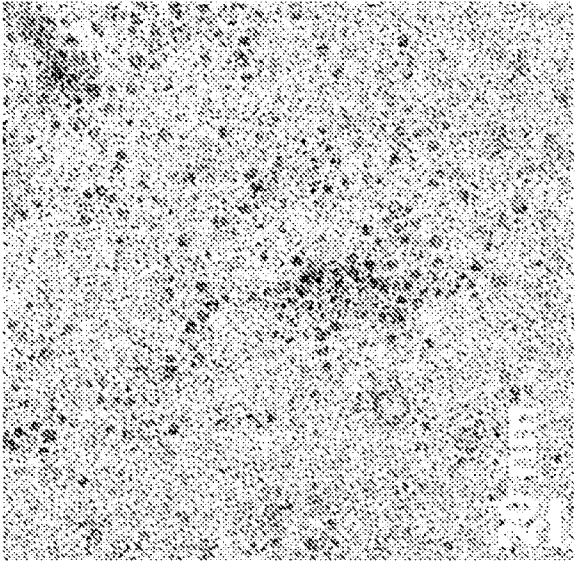


Figure 6C

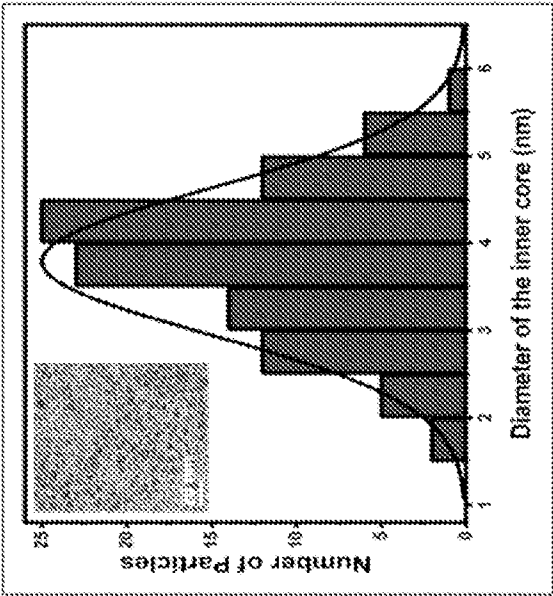


Figure 6D

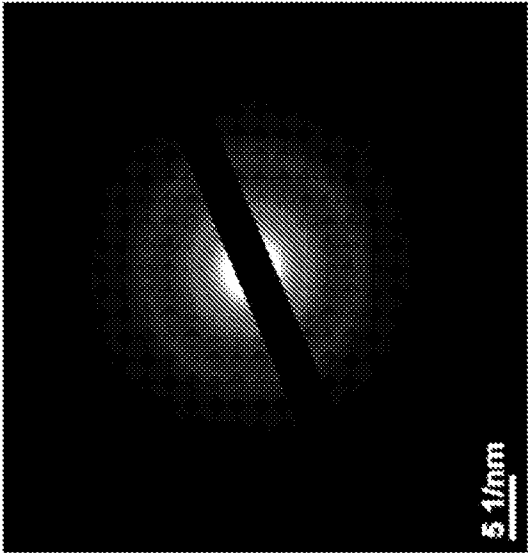


Figure 6

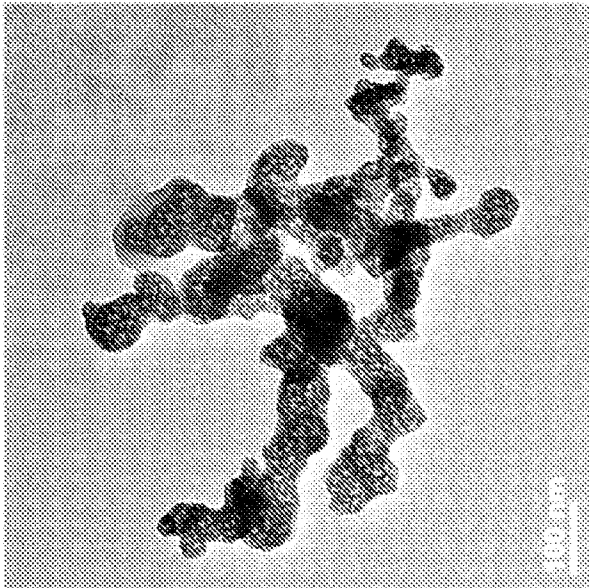


Figure 7A

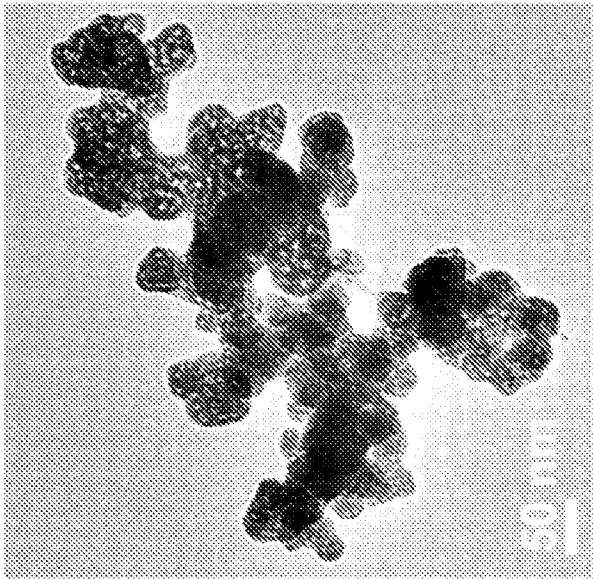


Figure 7B

Figure 7

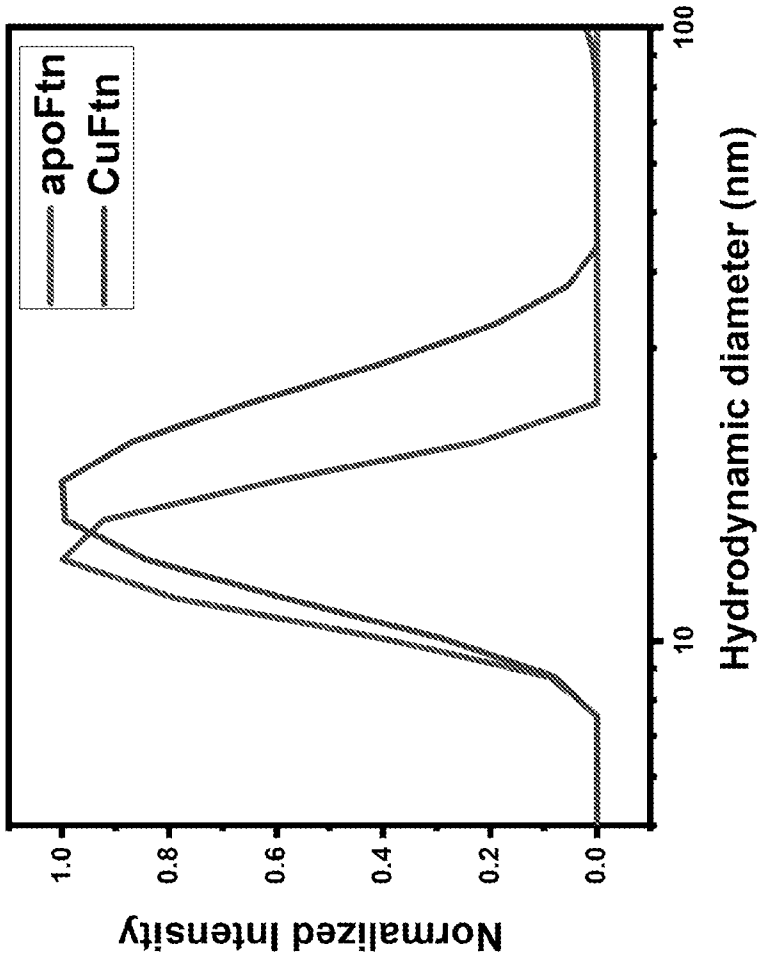


Figure 8

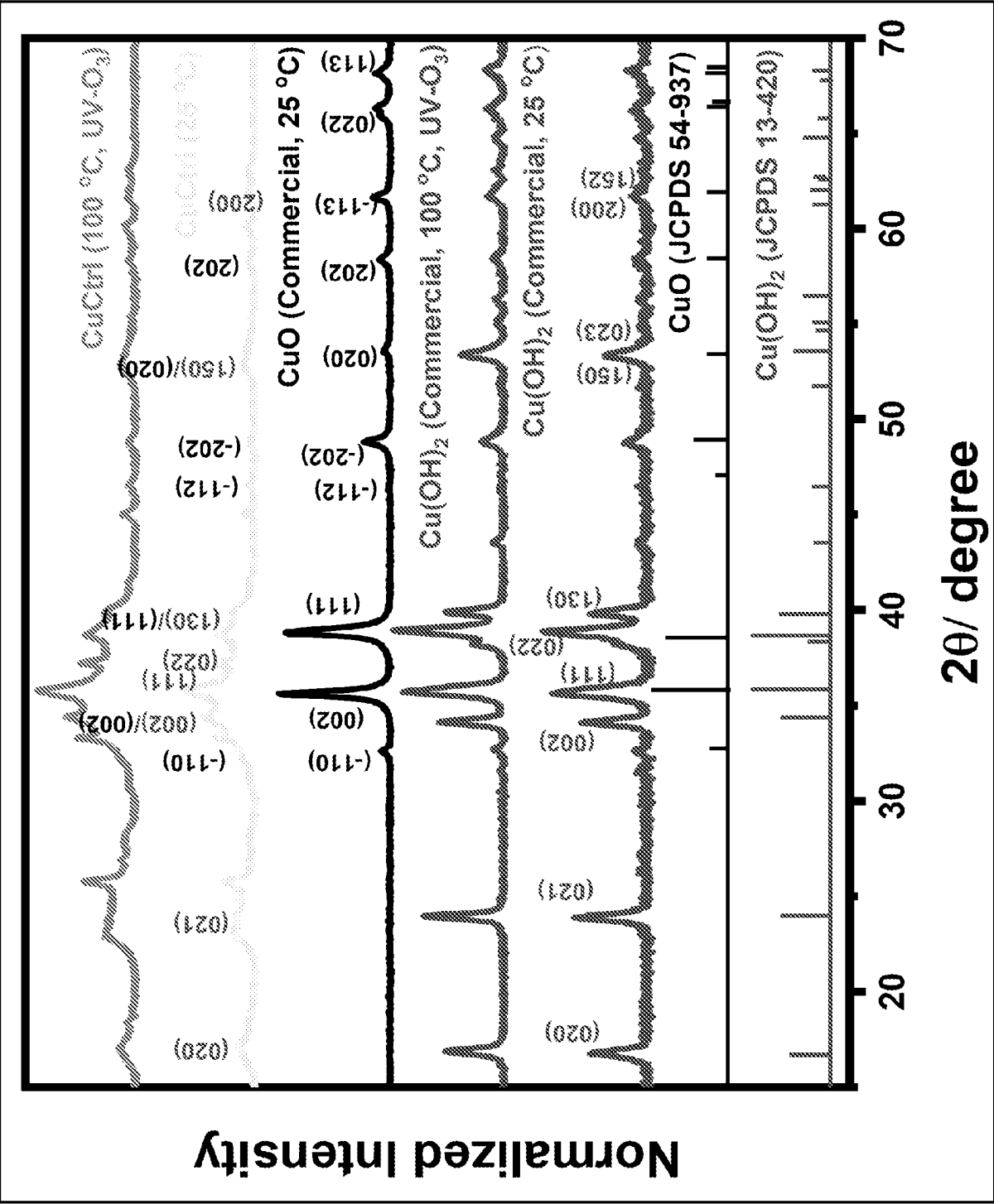


Figure 9

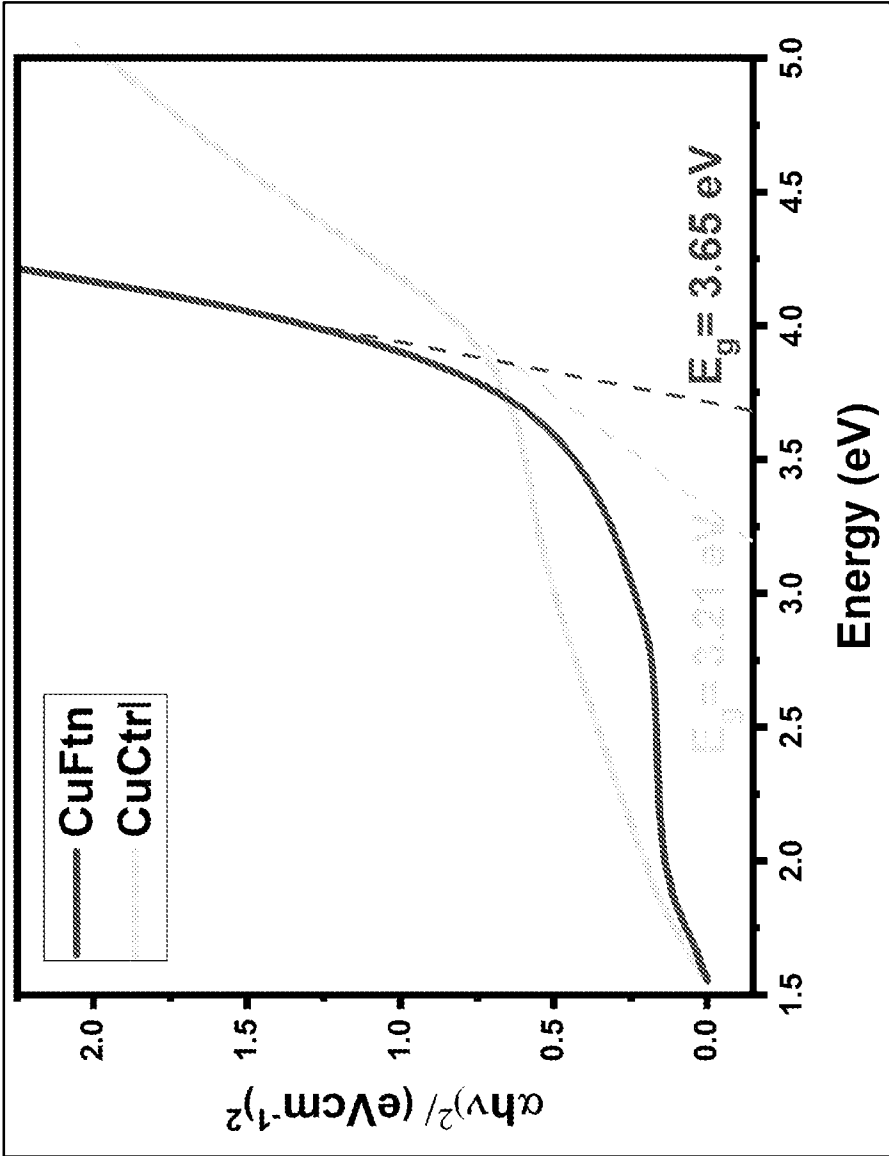


Figure 10

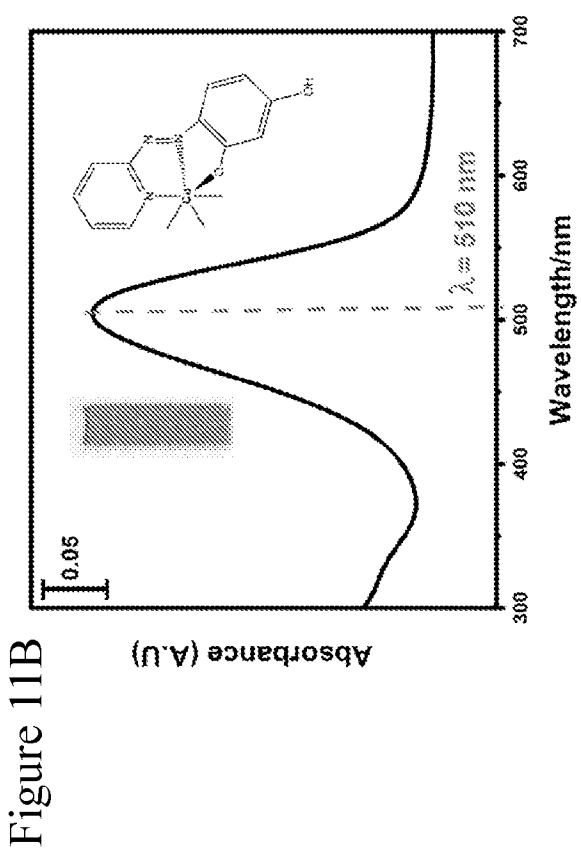
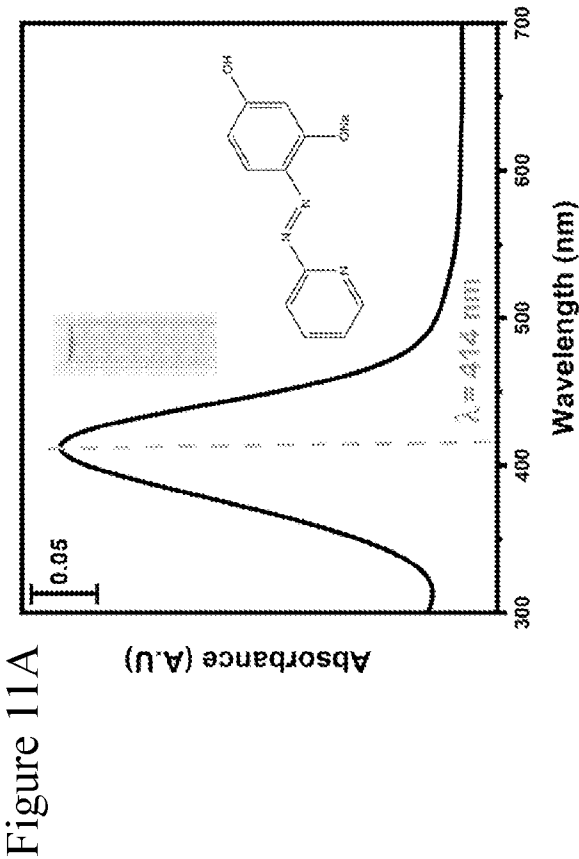


Figure 11

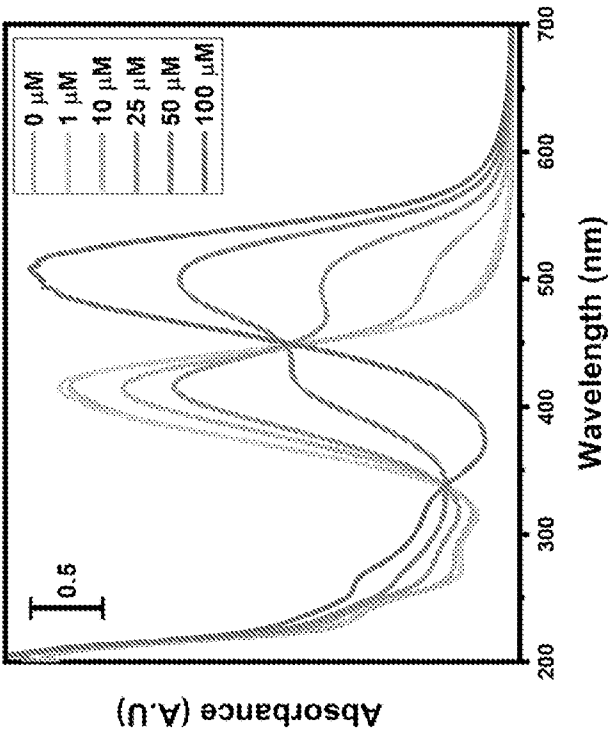


Figure 12A

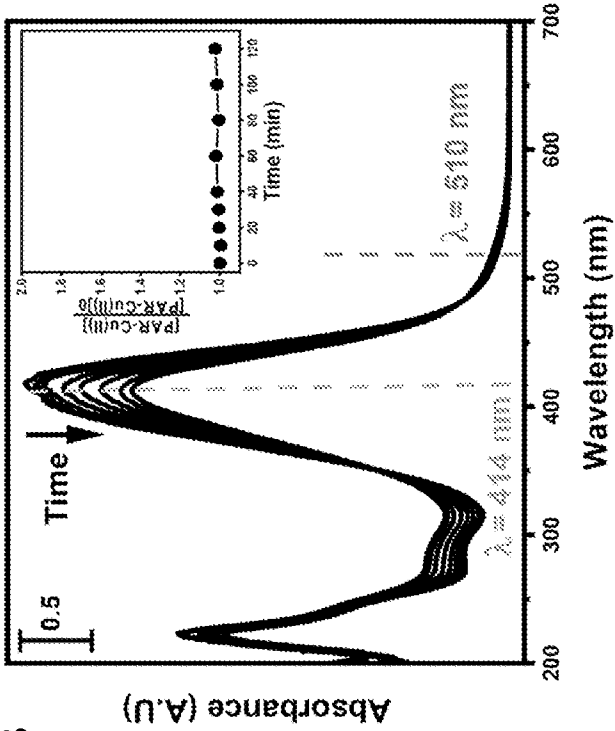


Figure 12B

Figure 12

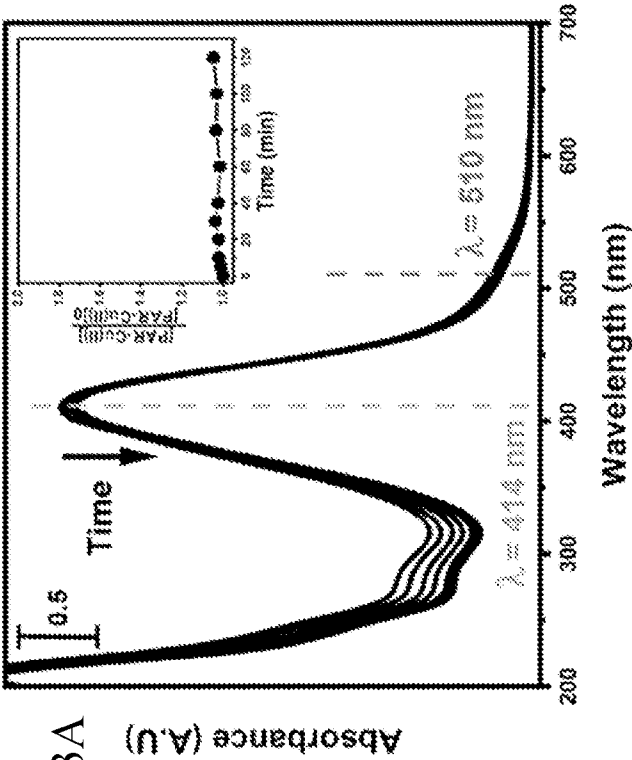


Figure 13A

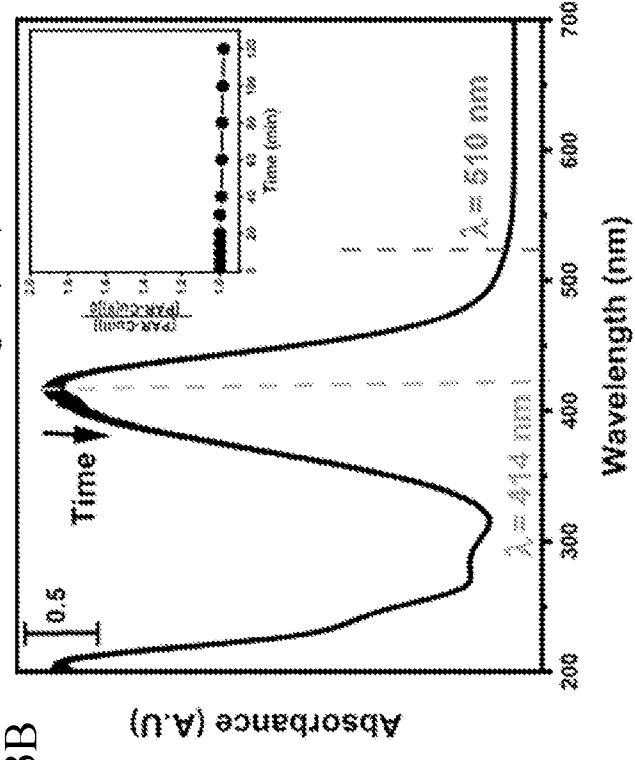


Figure 13B

Figure 13

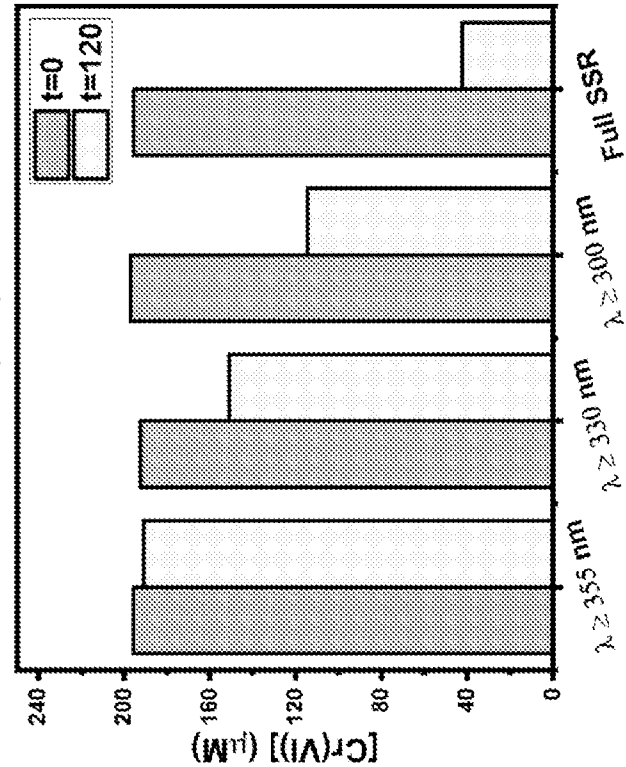
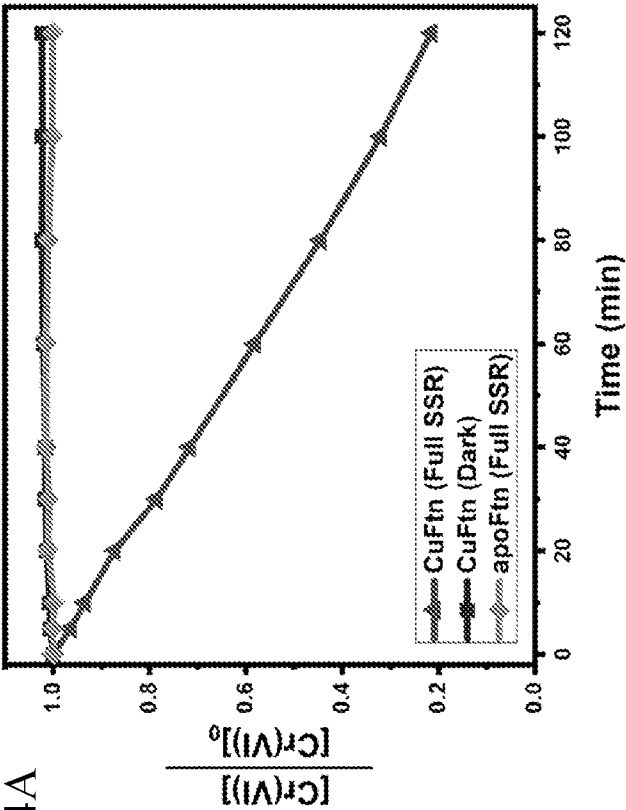


Figure 14

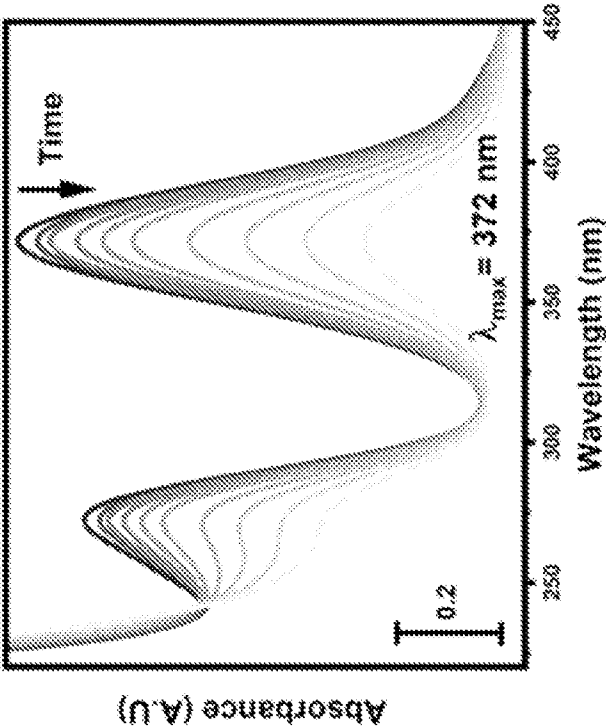


Figure 15A

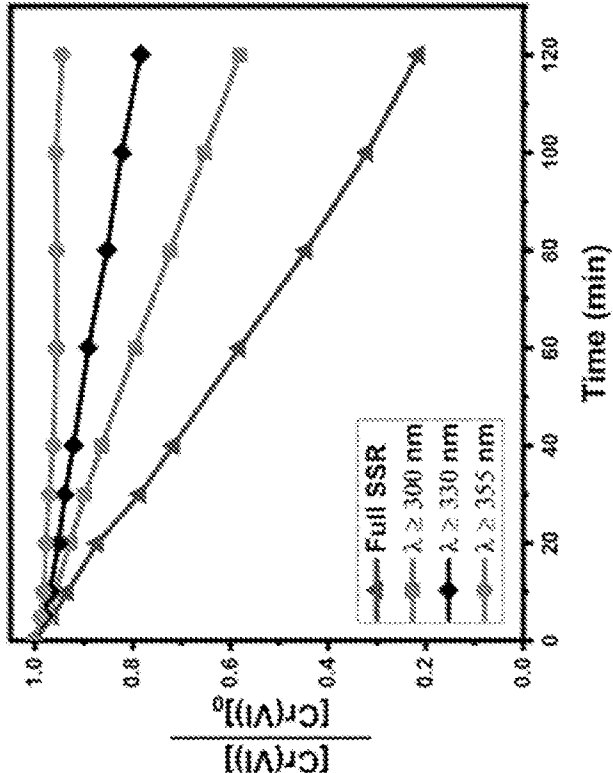


Figure 15B

Figure 15

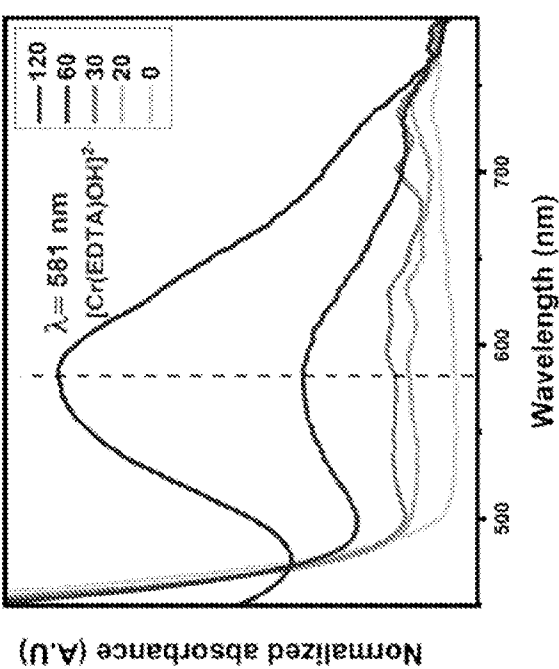


Figure 16A

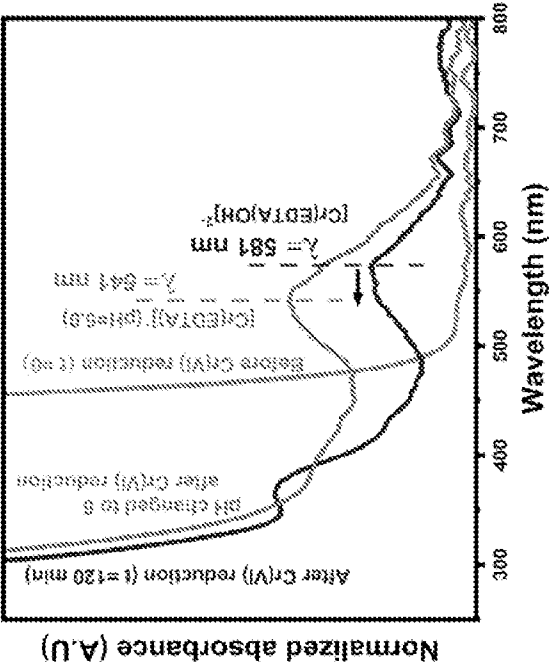


Figure 16B

Figure 16

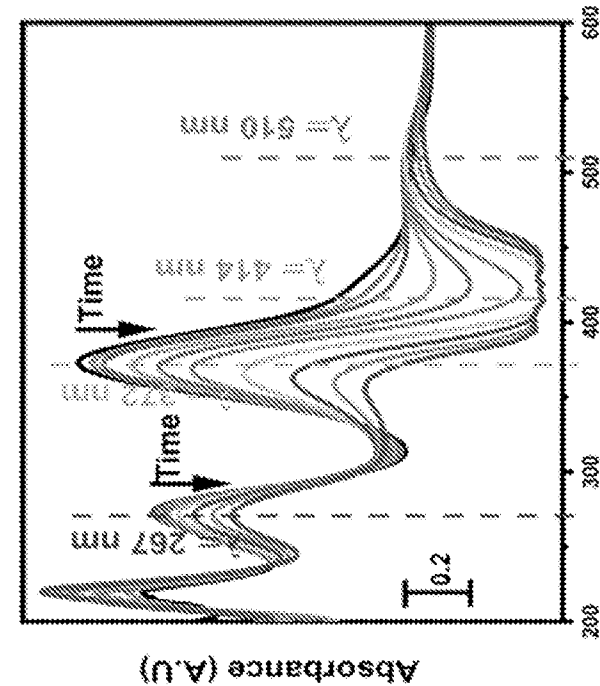


Figure 17A

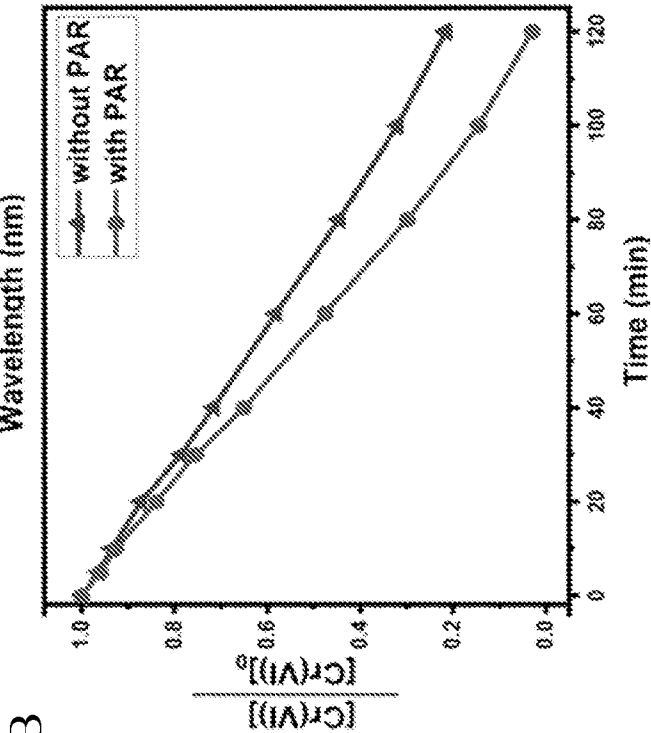


Figure 17B

Figure 17

Figure 18A

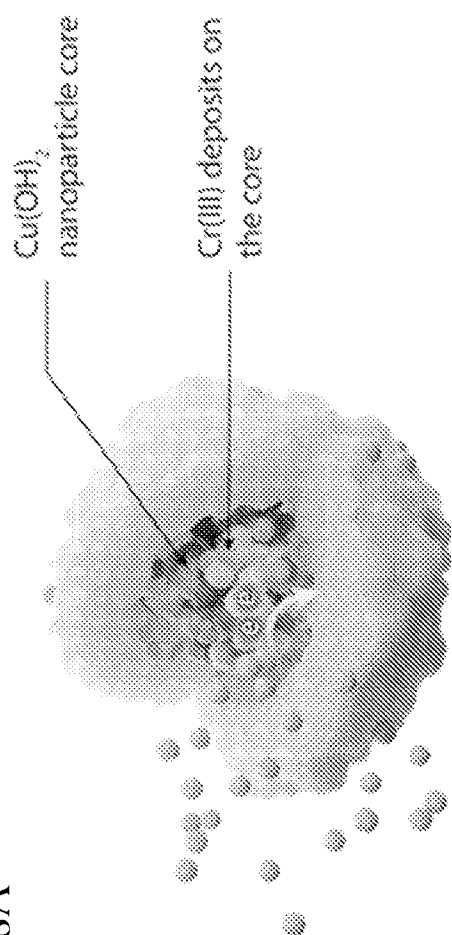


Figure 18B

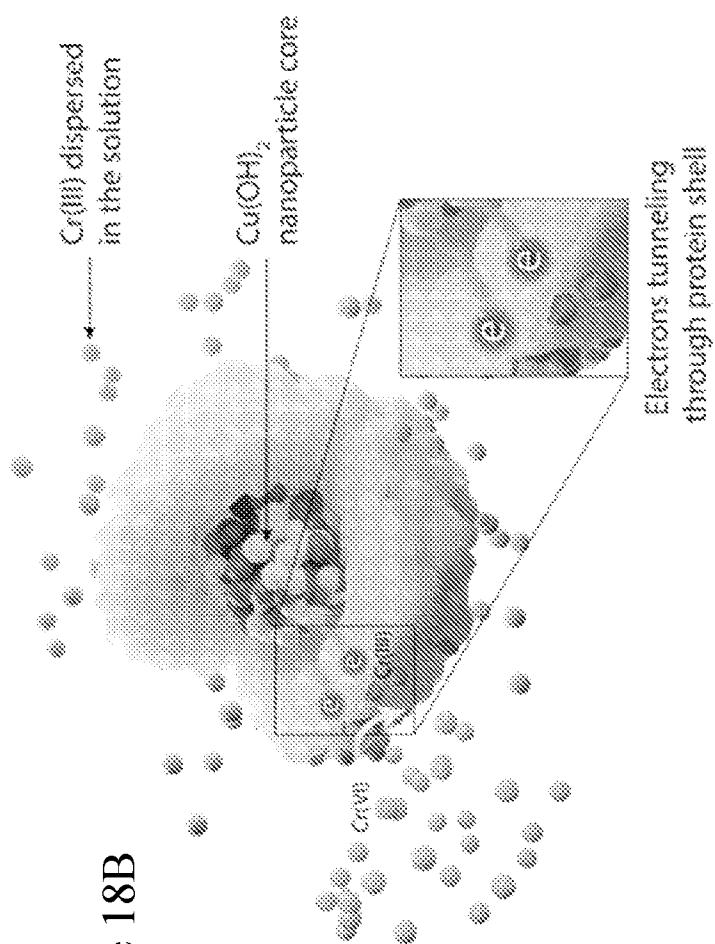


Figure 18

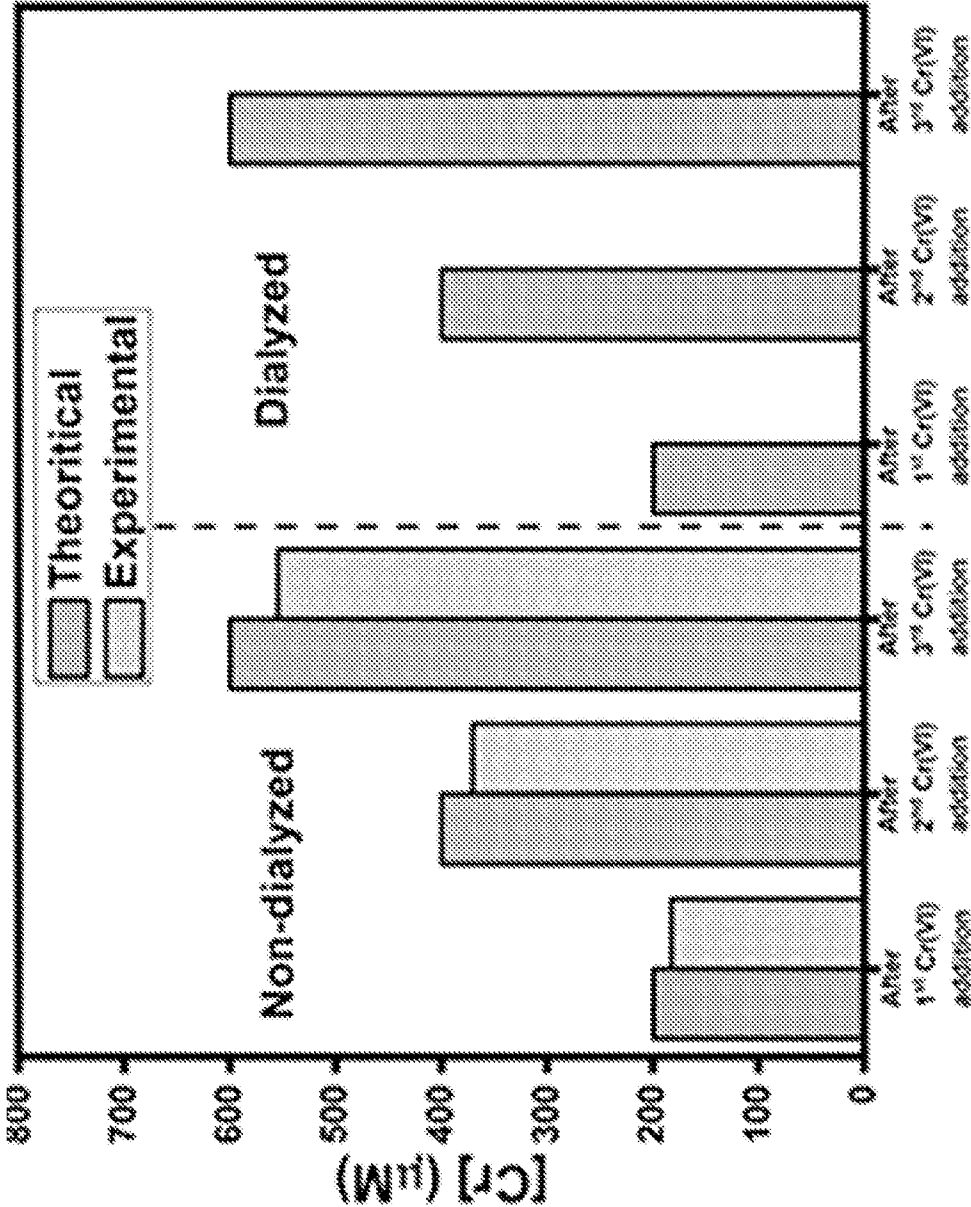


Figure 19A

Figure 19

Figure 19B

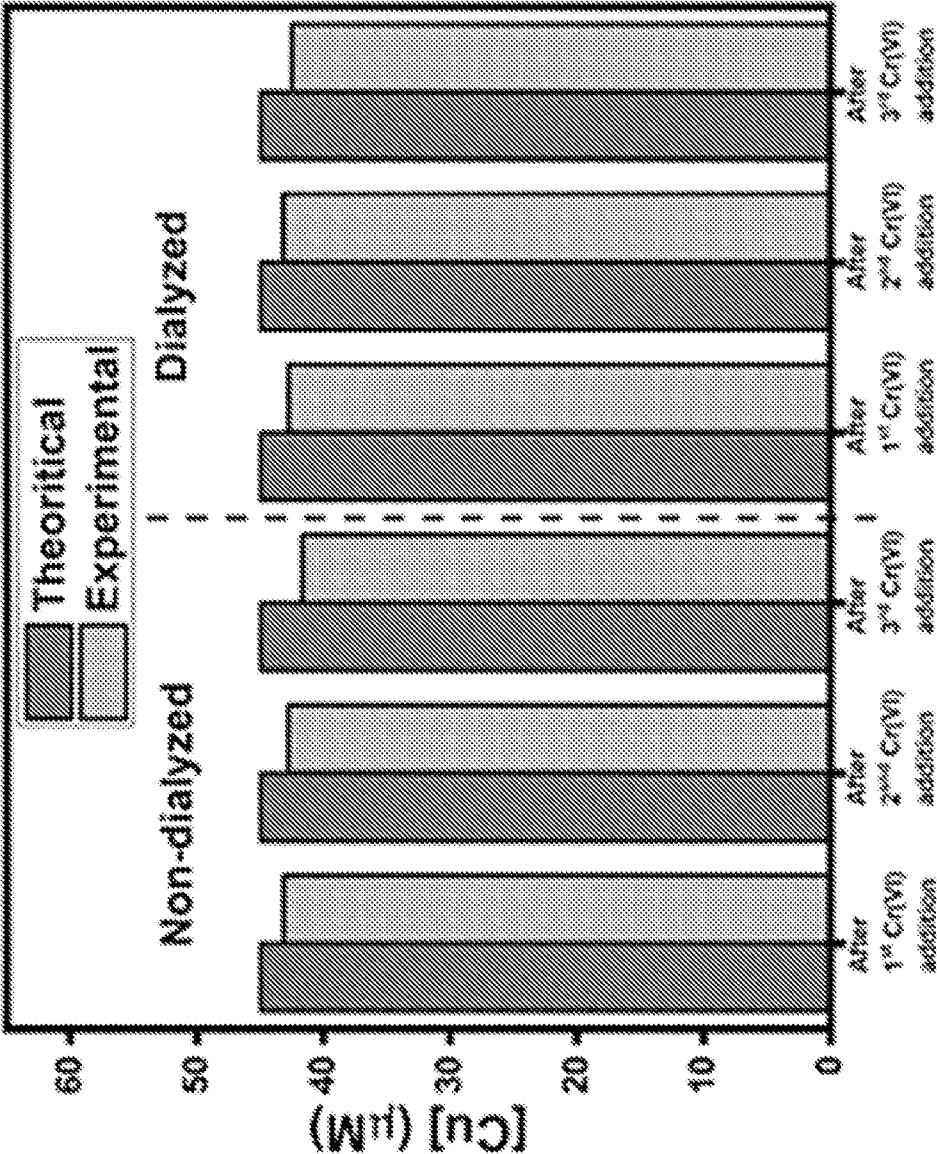


Figure 19 (cont.)

Figure 19D

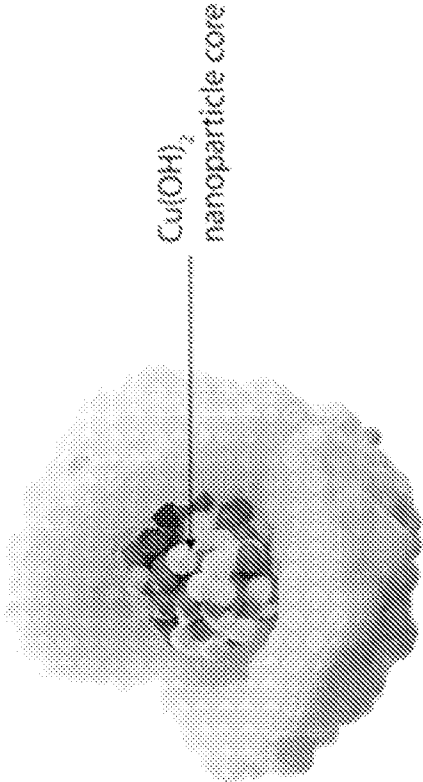


Figure 19C

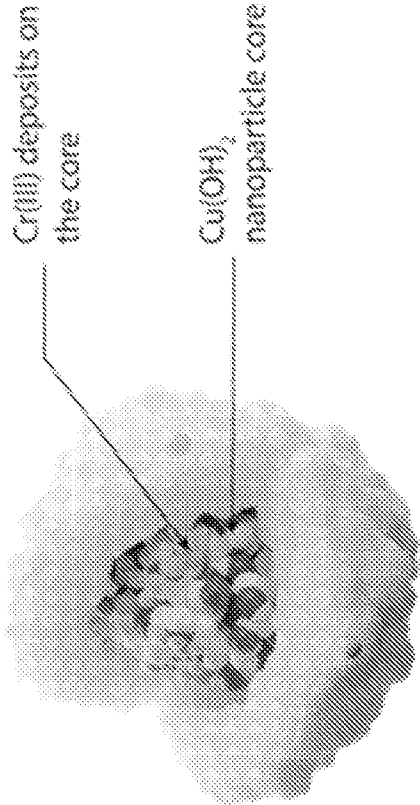


Figure 19 (cont.)

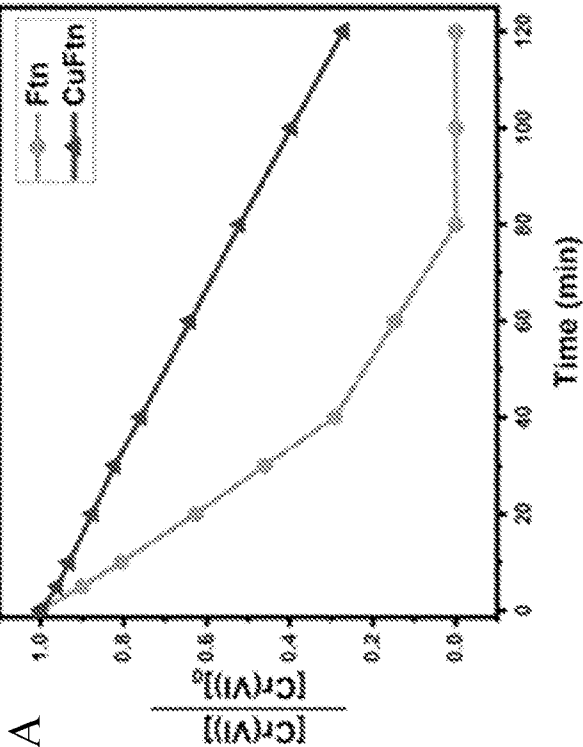


Figure 20A

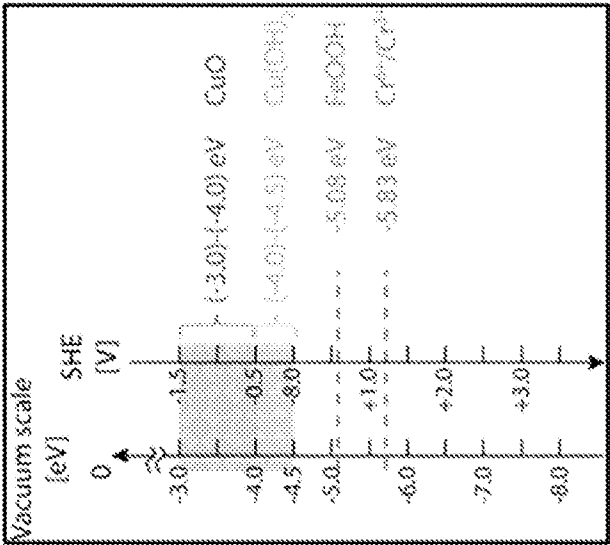


Figure 20B

Figure 20

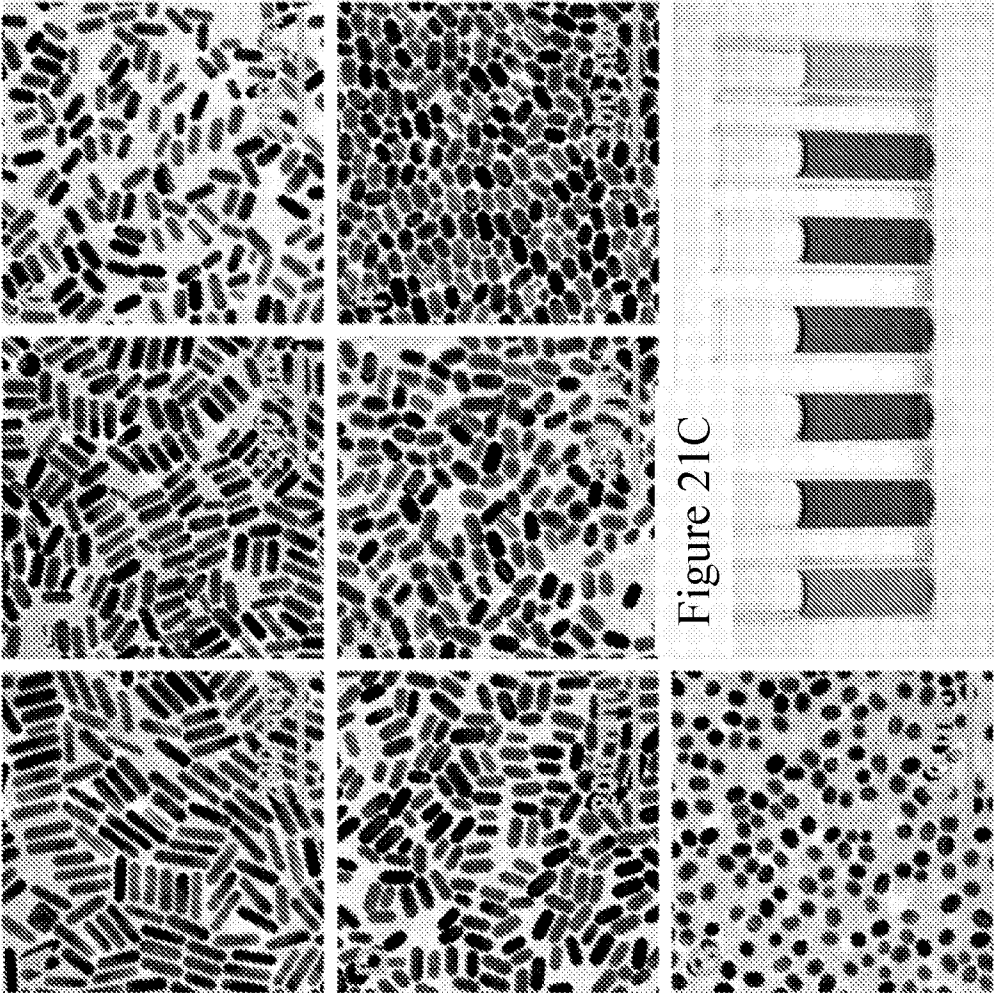


Figure 21

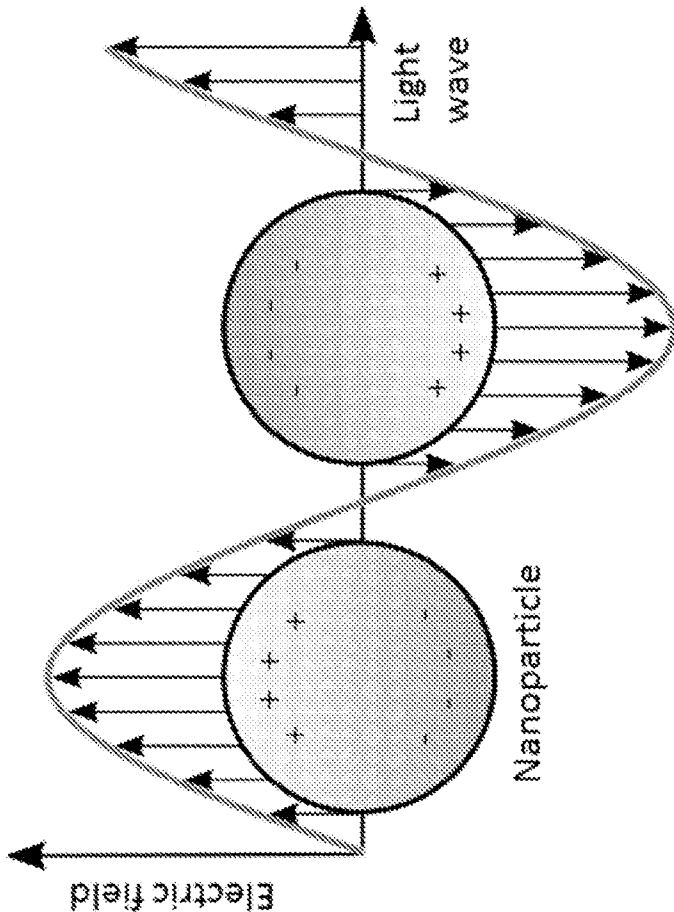


Figure 22

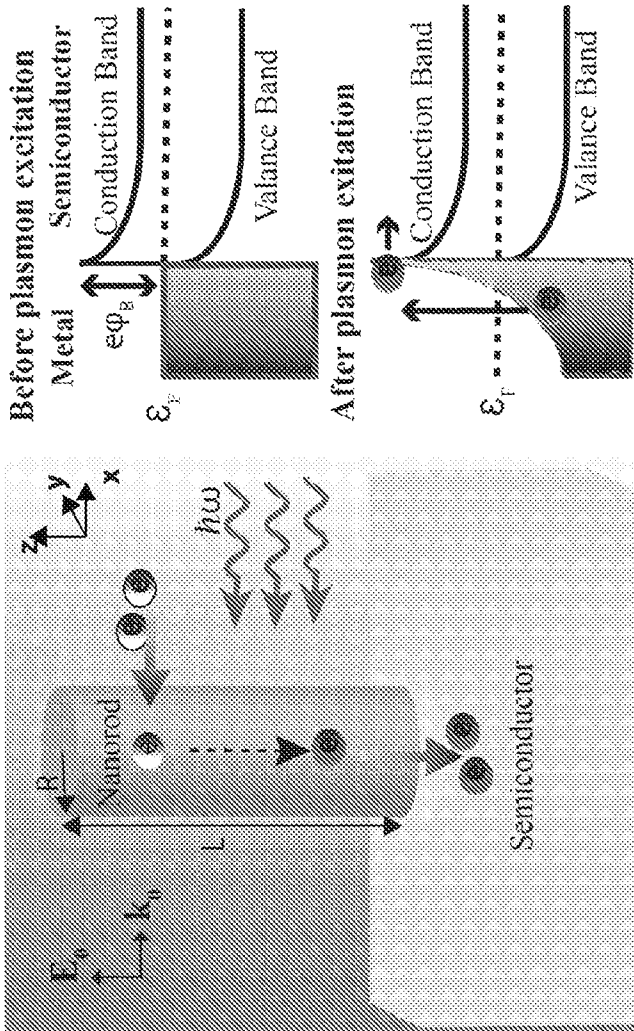


Figure 23

Figure 24B

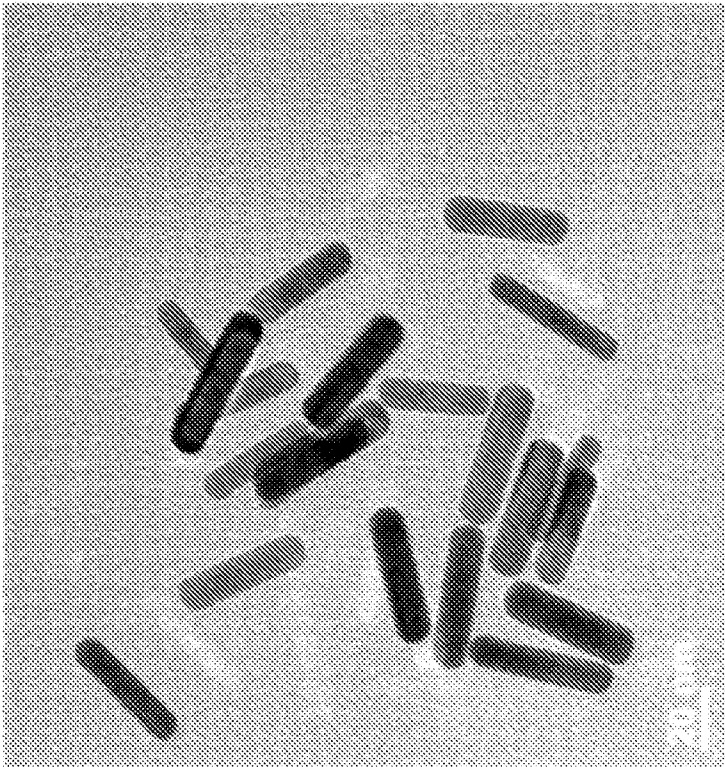


Figure 24A

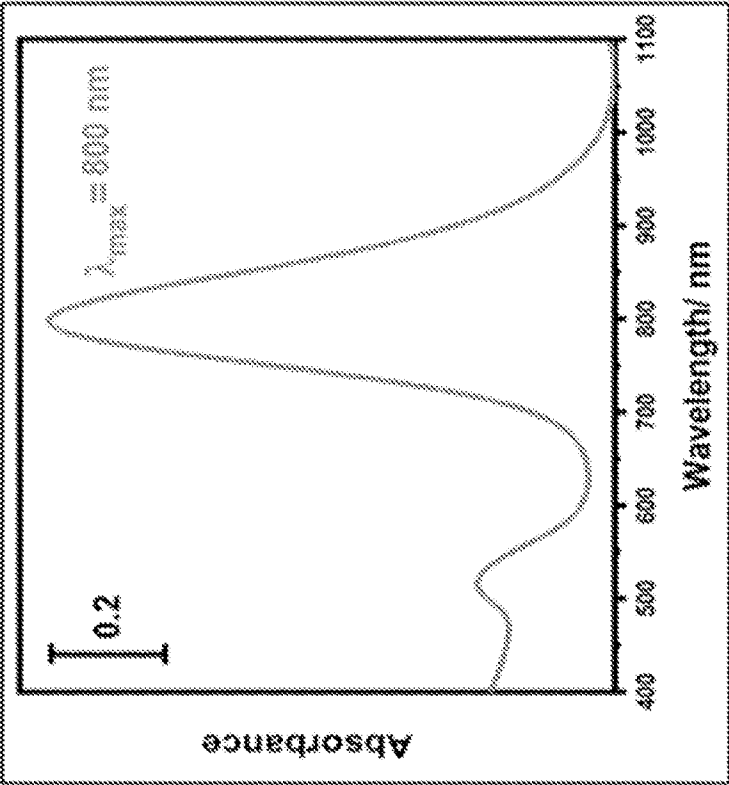


Figure 24

Figure 24D

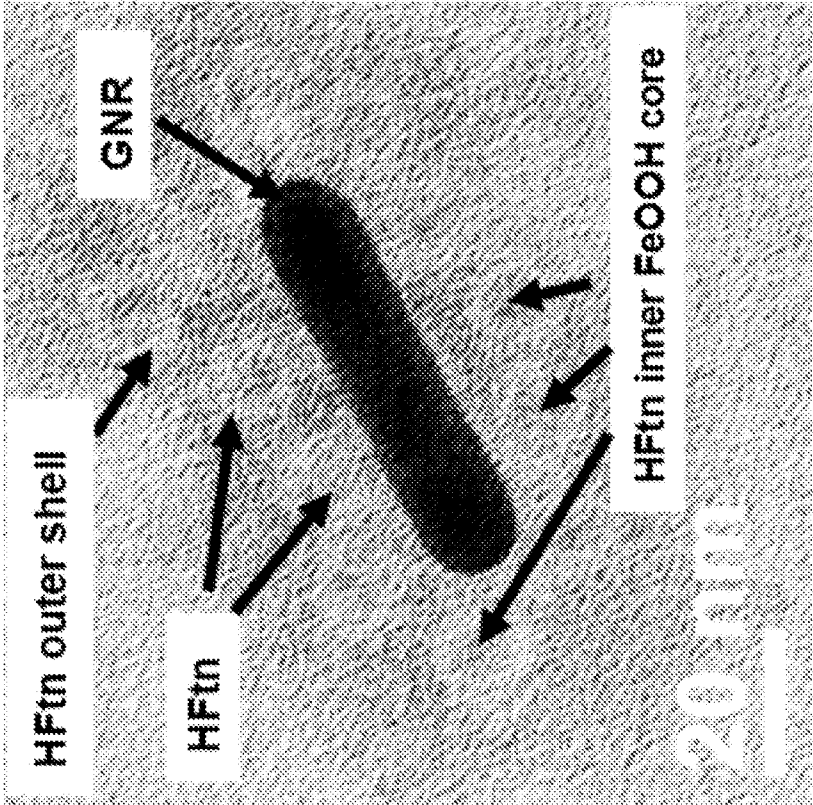


Figure 24C

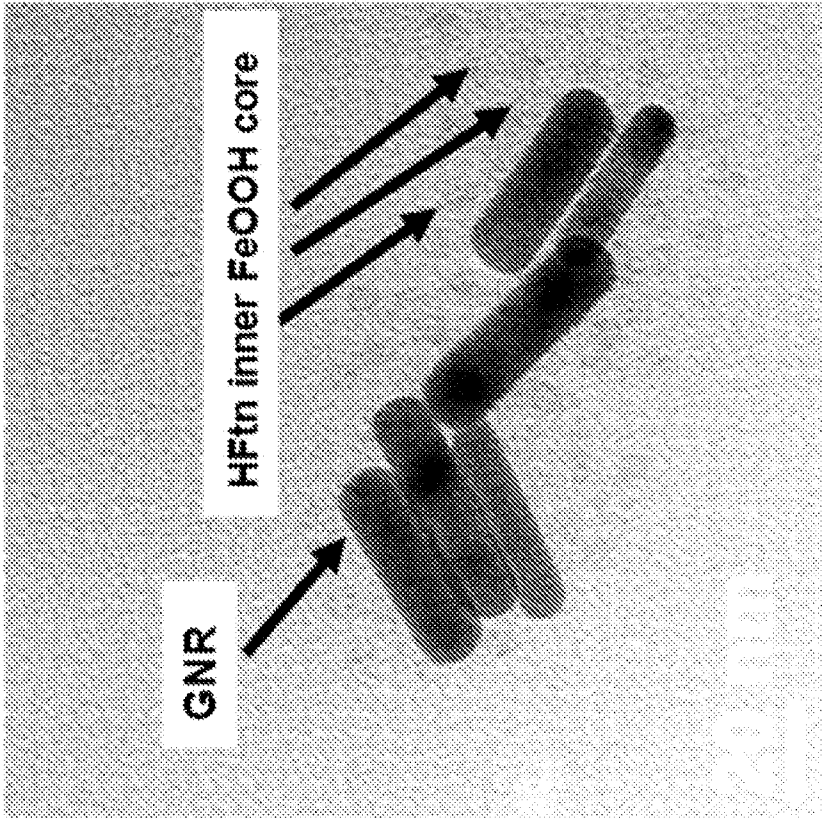


Figure 24 (cont.)

Figure 25B

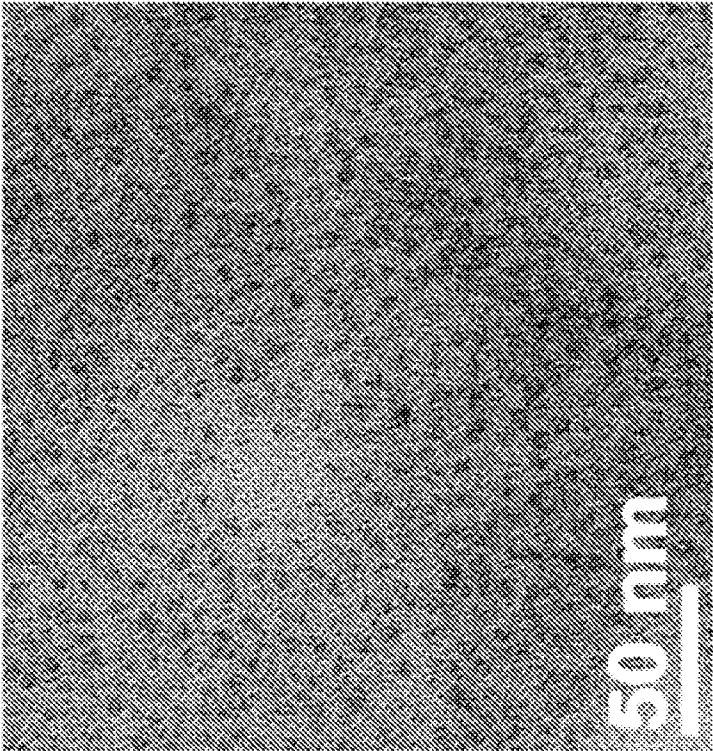


Figure 25A

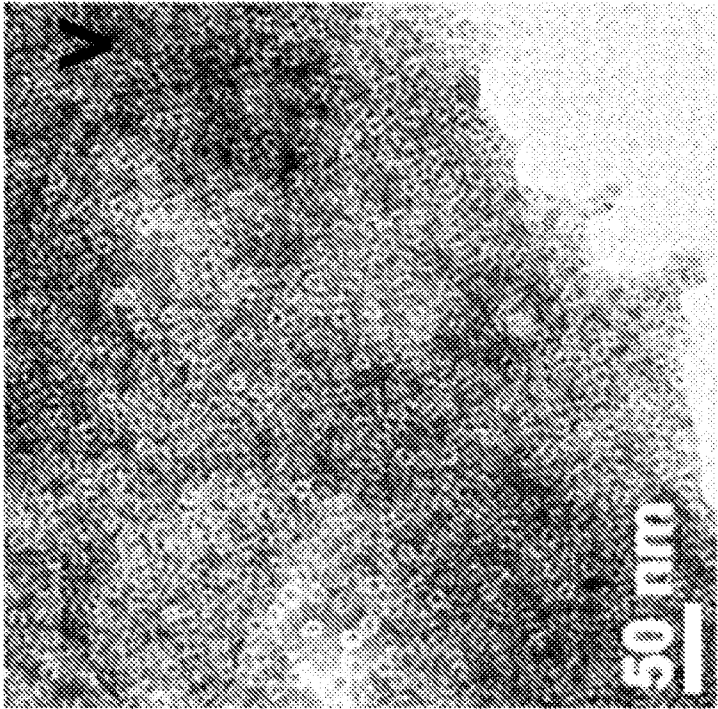


Figure 25

Figure 26B

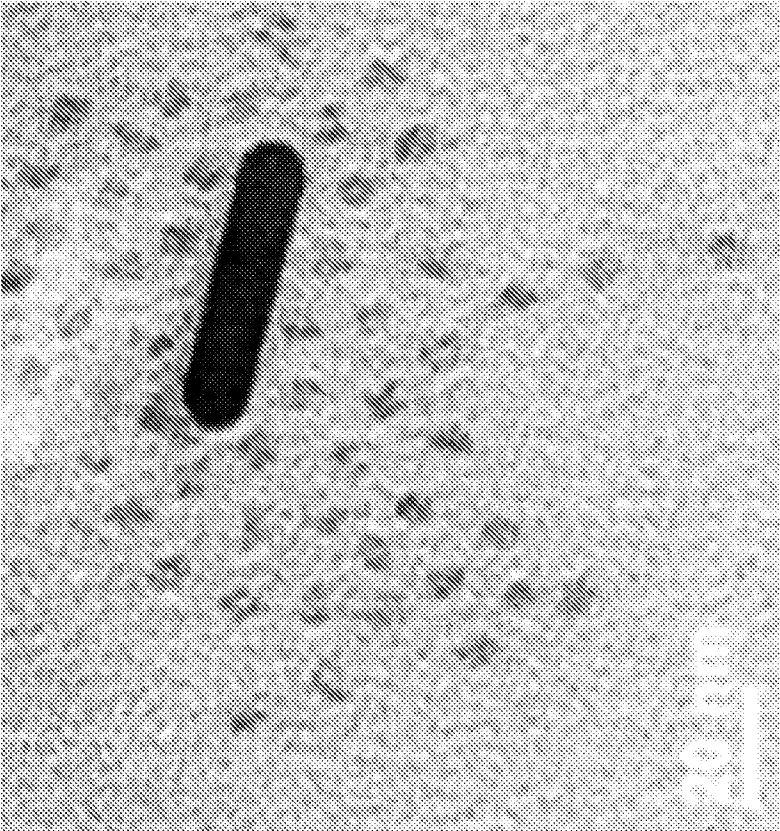


Figure 26A

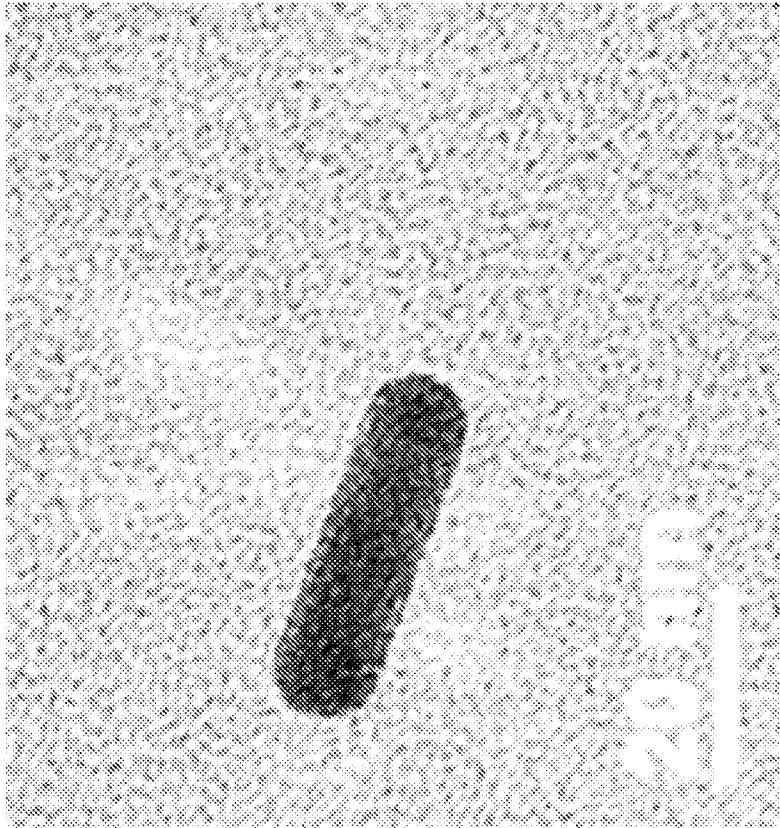


Figure 26

Figure 26C

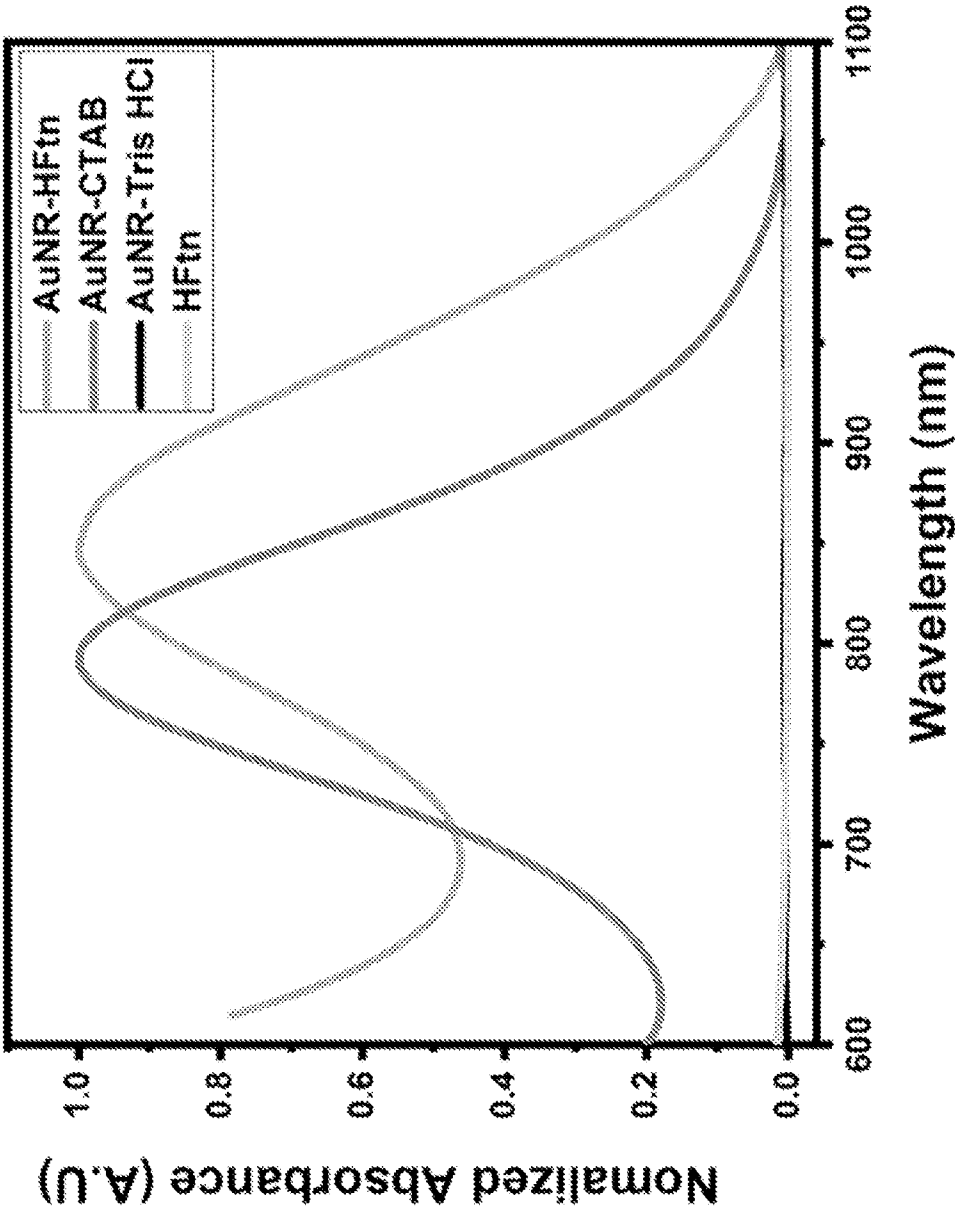


Figure 26 (cont.)

Figure 27B

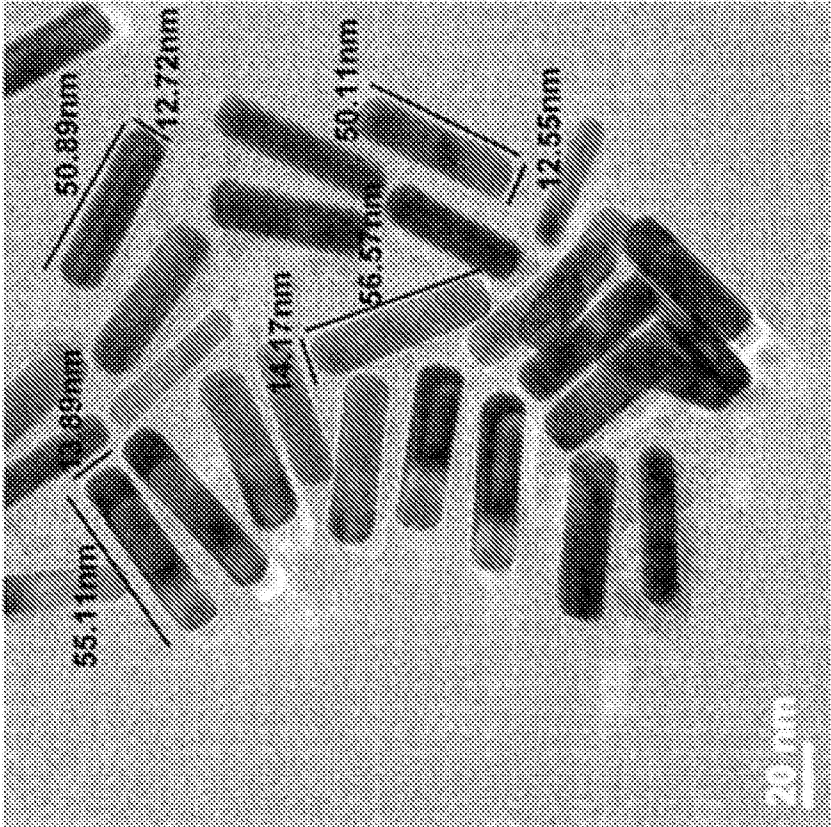


Figure 27A

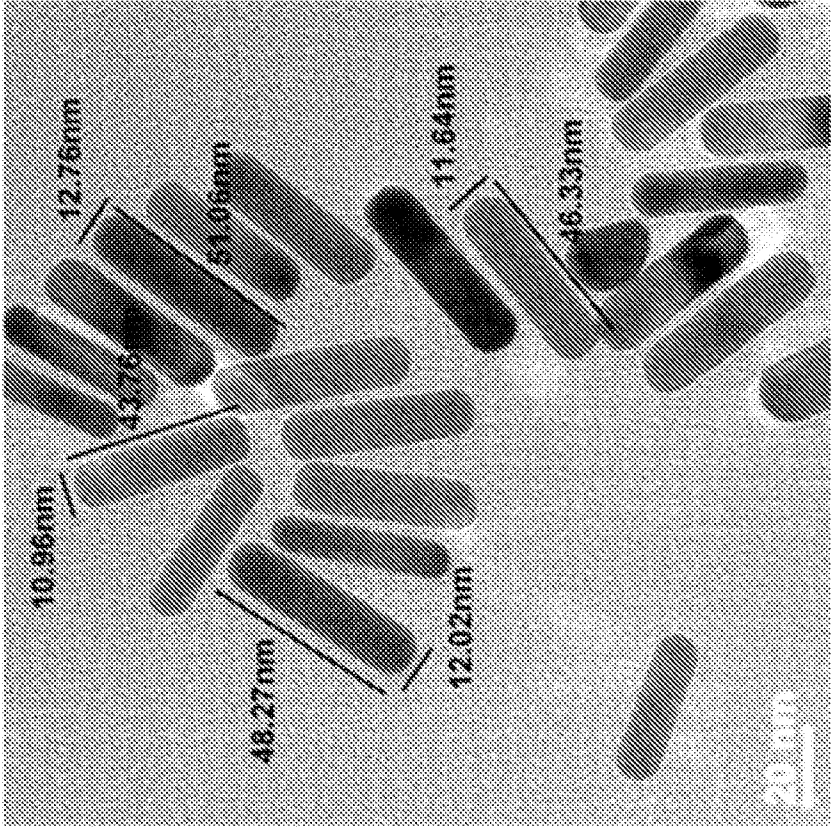


Figure 27

Figure 27D

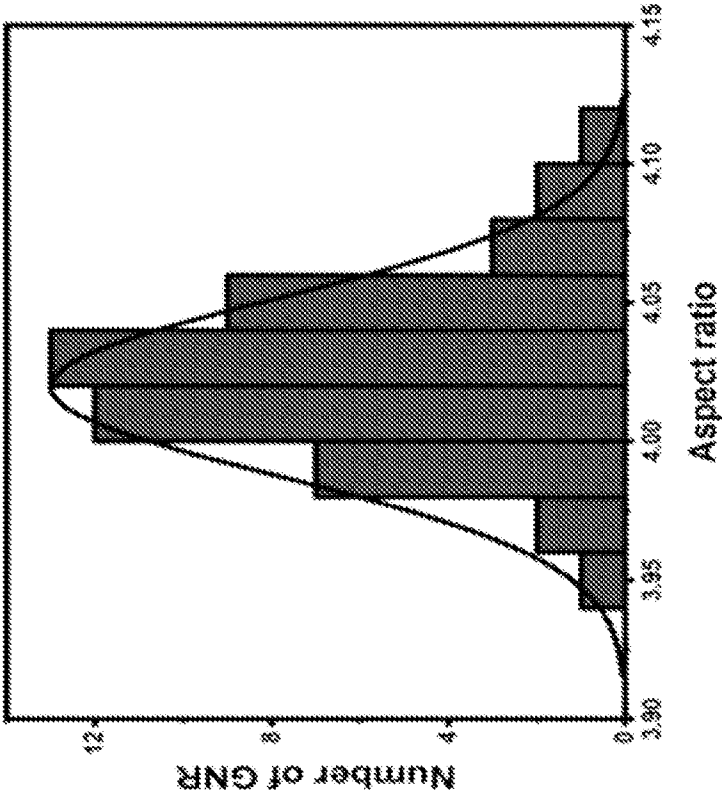


Figure 27C

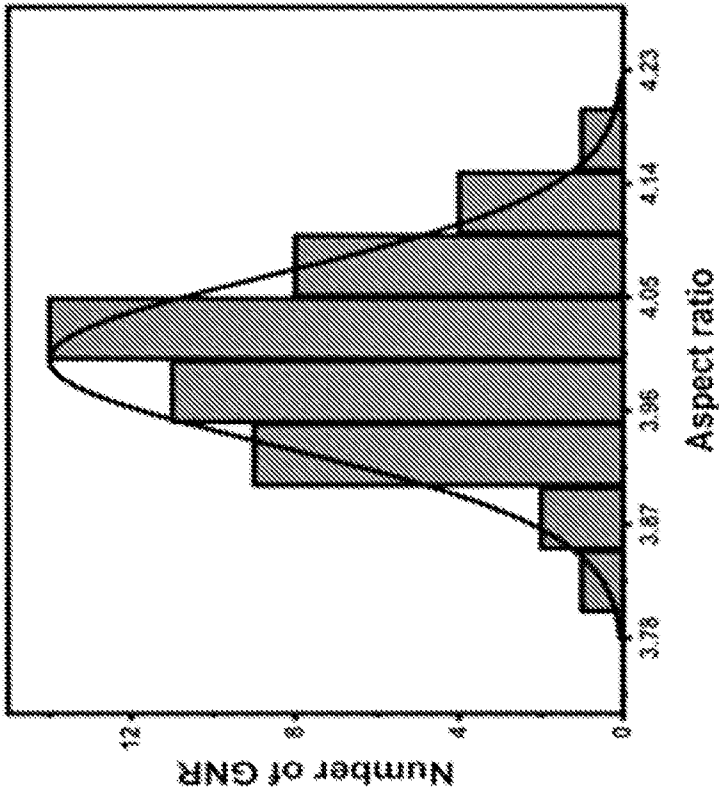


Figure 27 (cont.)

Figure 28A

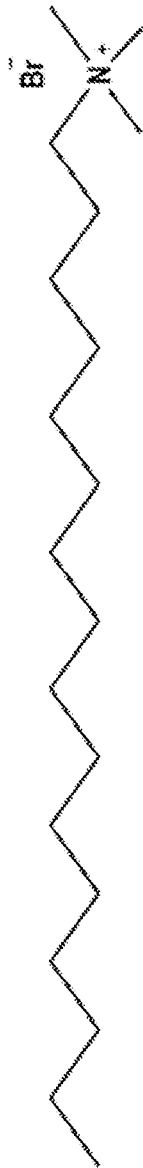


Figure 28B

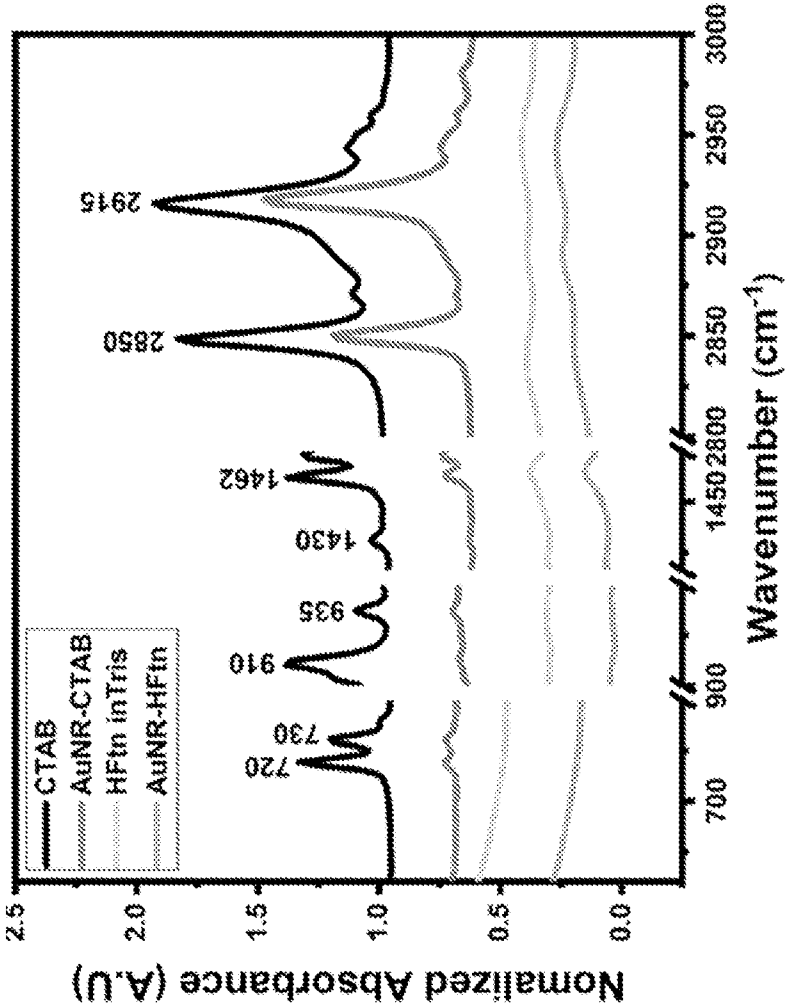


Figure 28

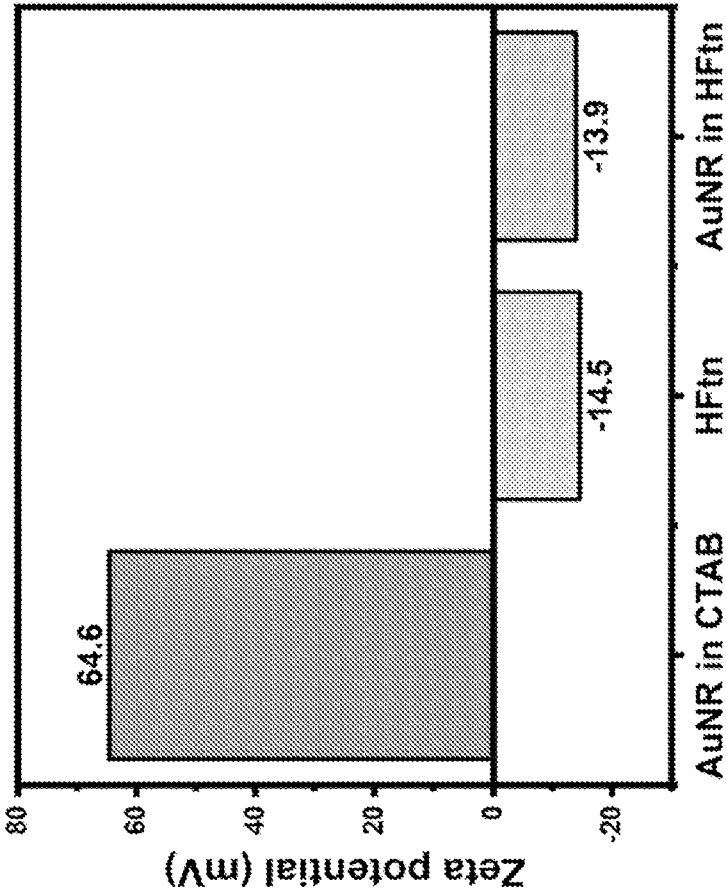


Figure 29

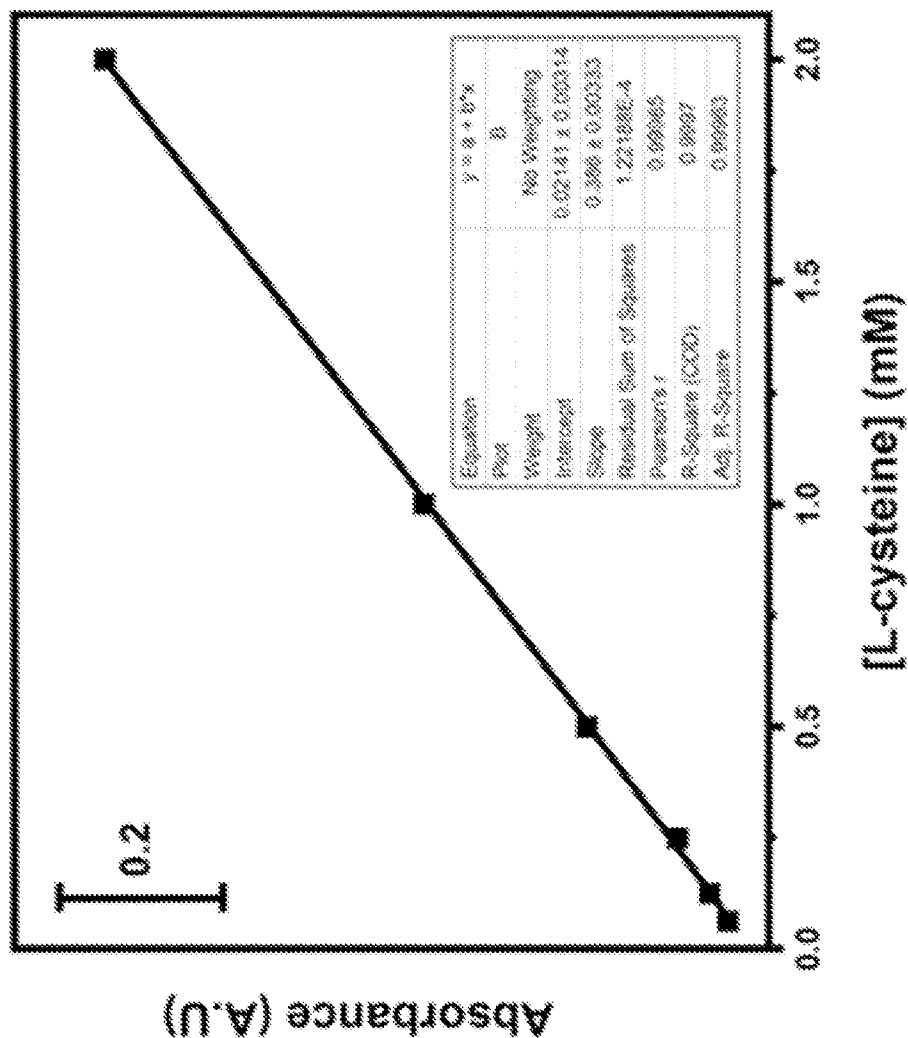


Figure 30

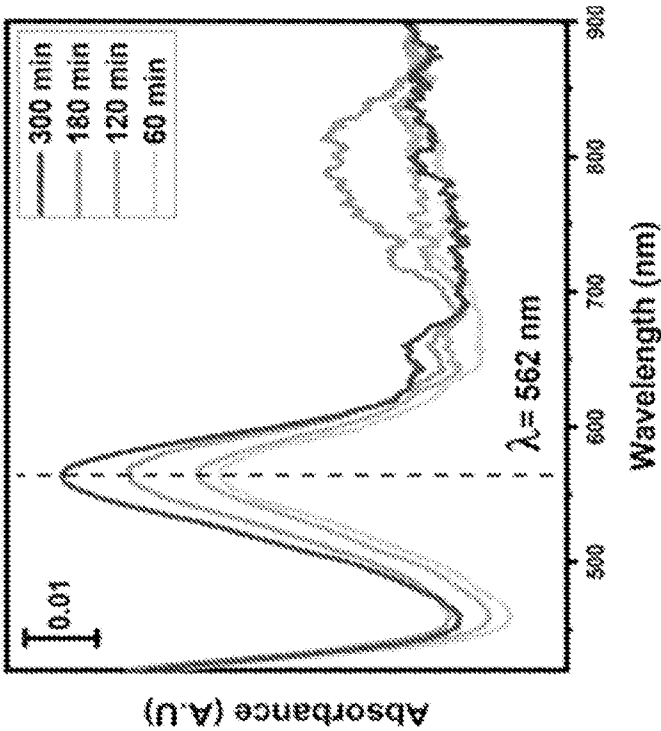


Figure 31A

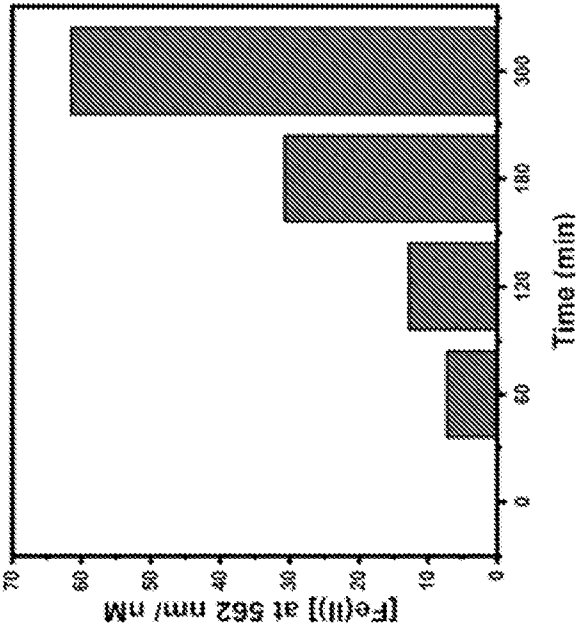


Figure 31B

Figure 31

Figure 32B

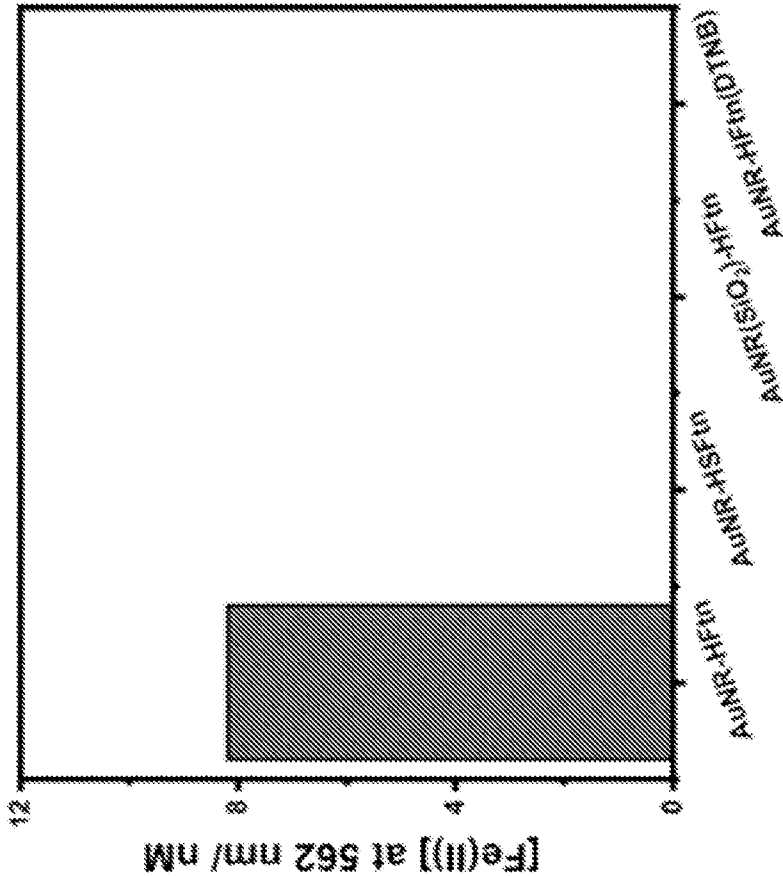


Figure 32A

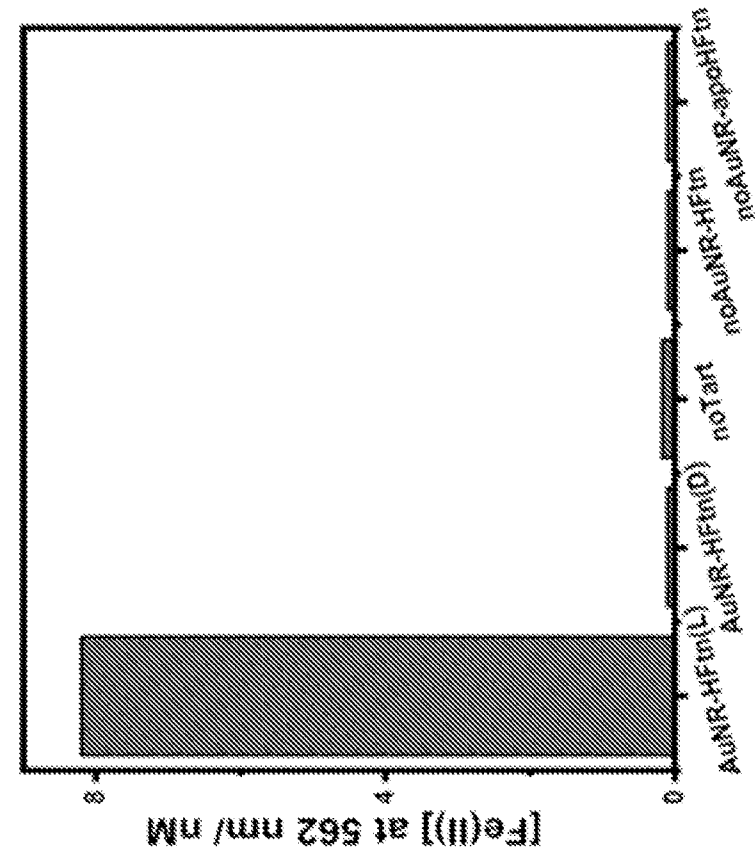


Figure 32

Figure 33B

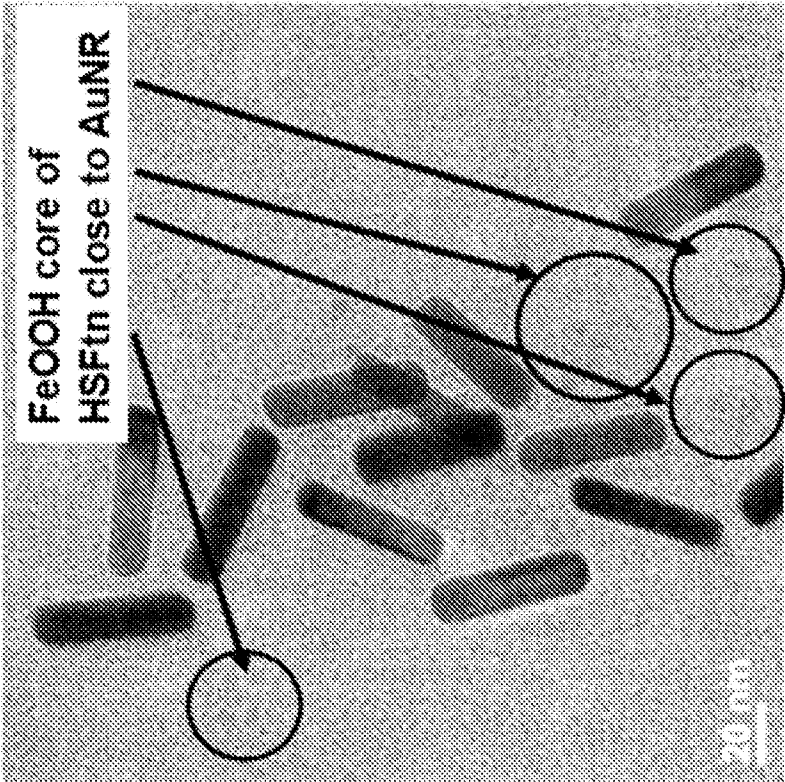


Figure 33A

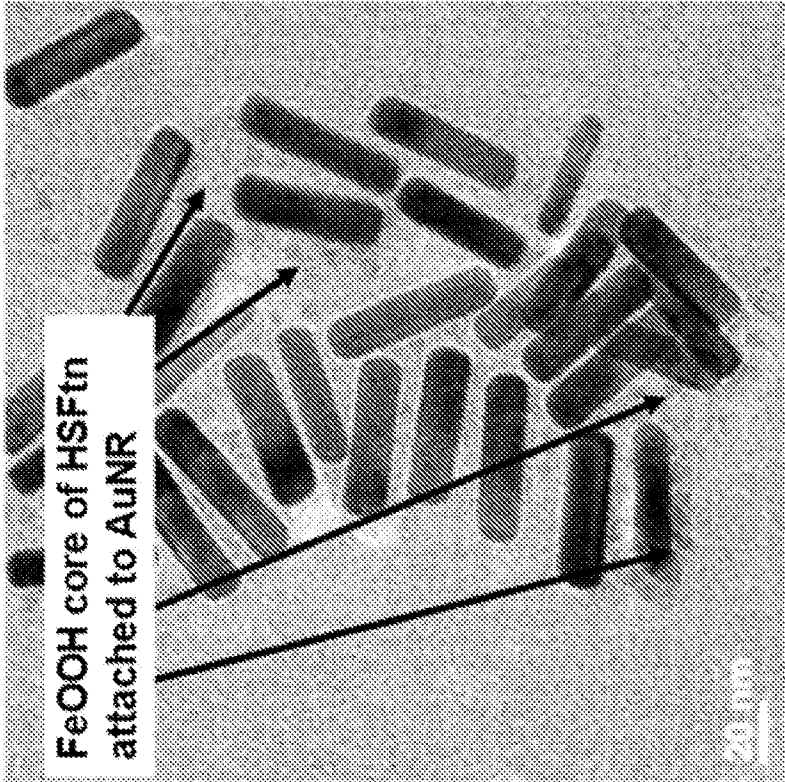


Figure 33

Figure 33D

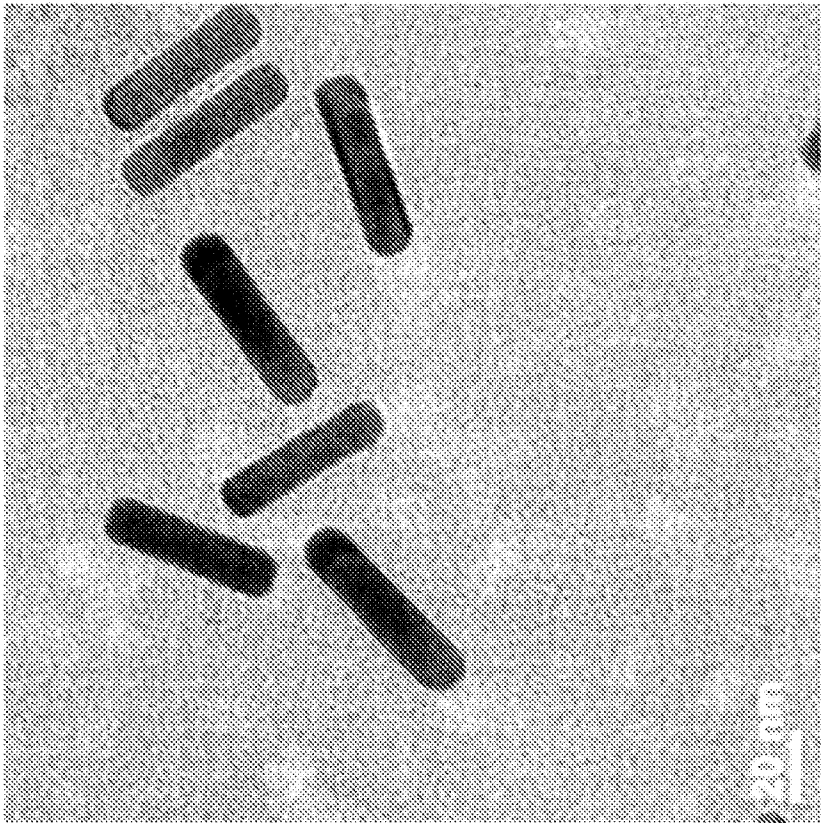


Figure 33C

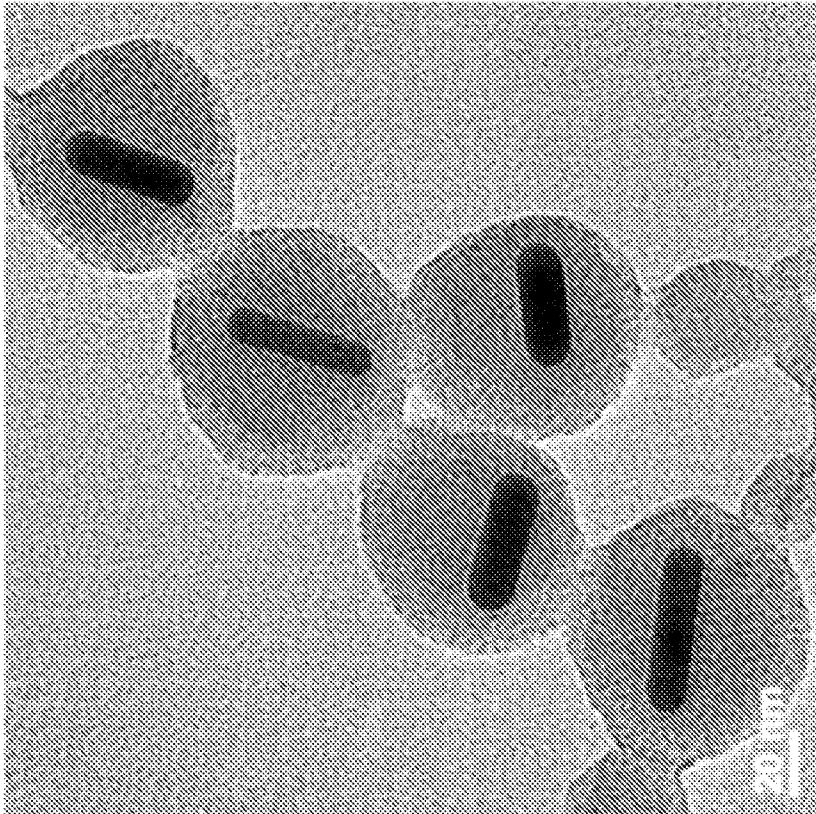


Figure 33 (cont.)

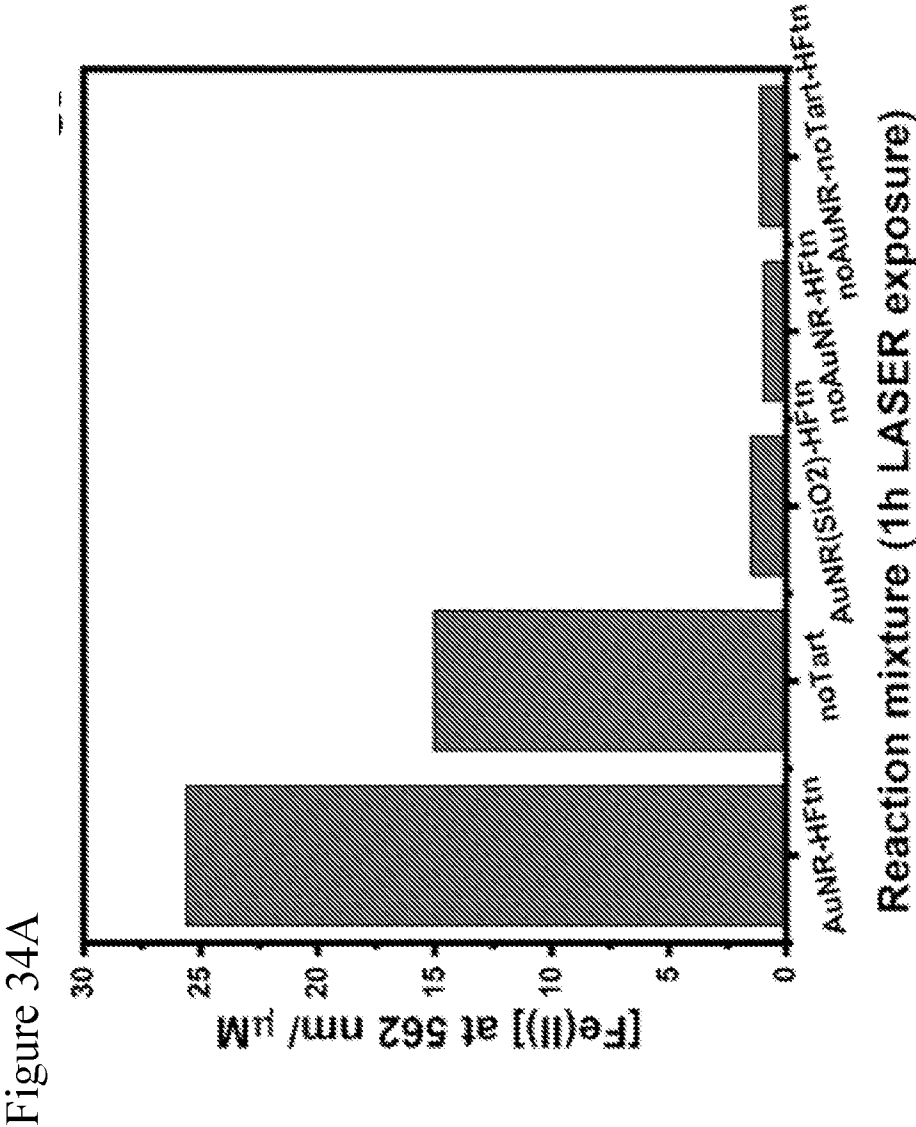


Figure 34

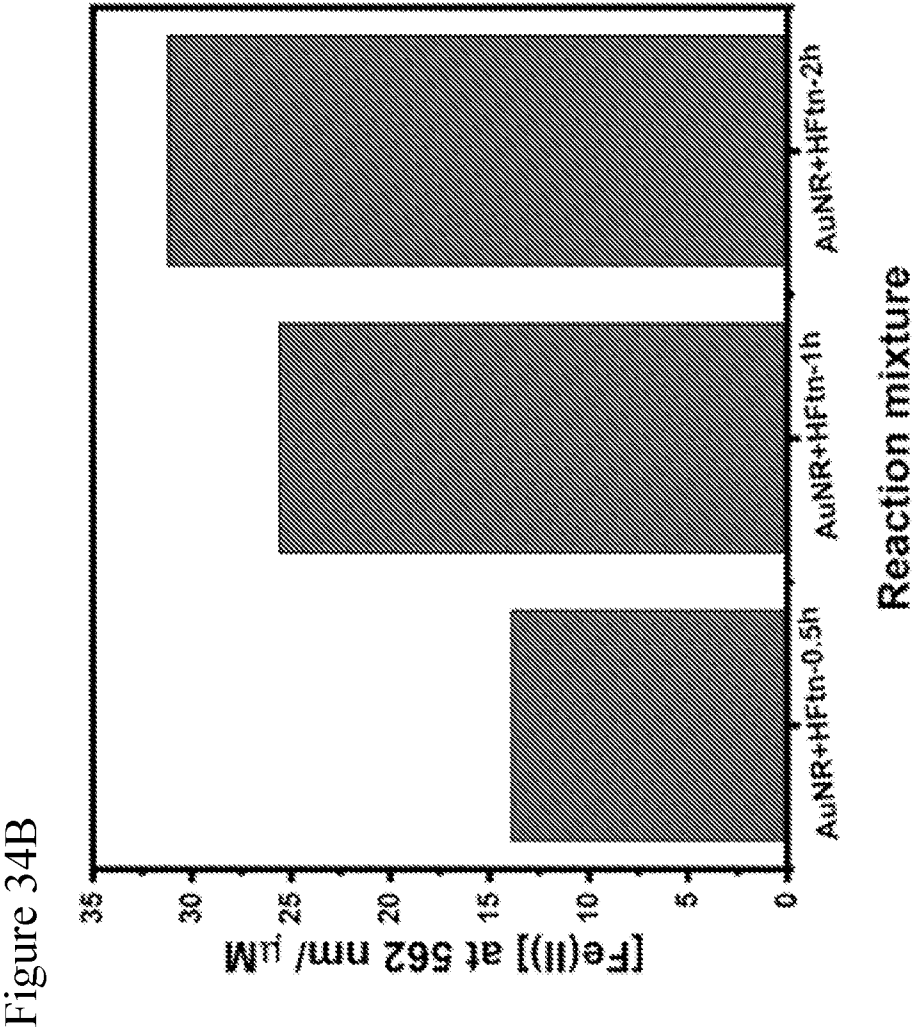


Figure 34 (cont.)

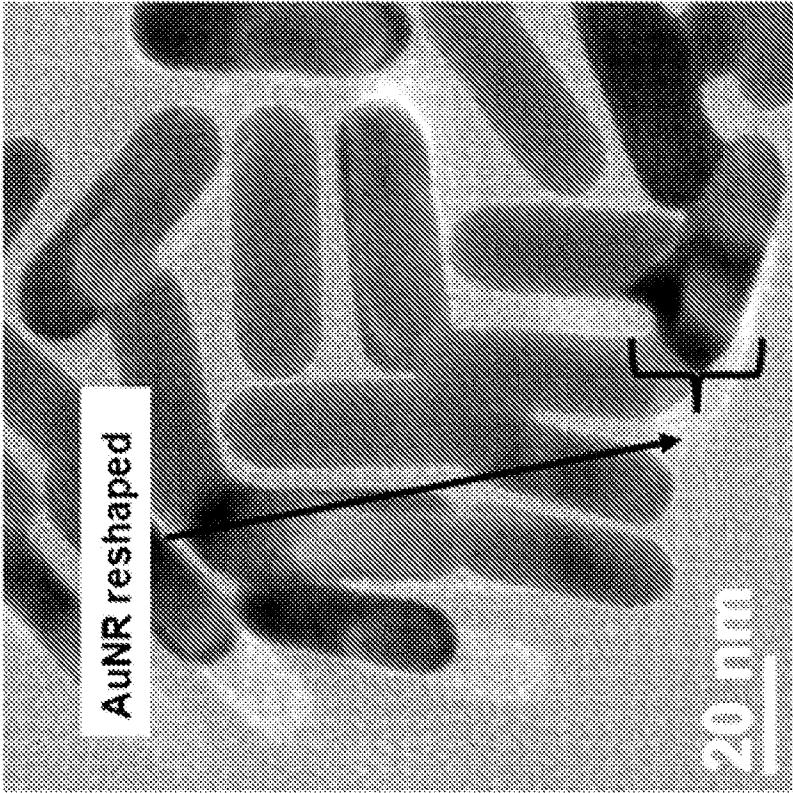
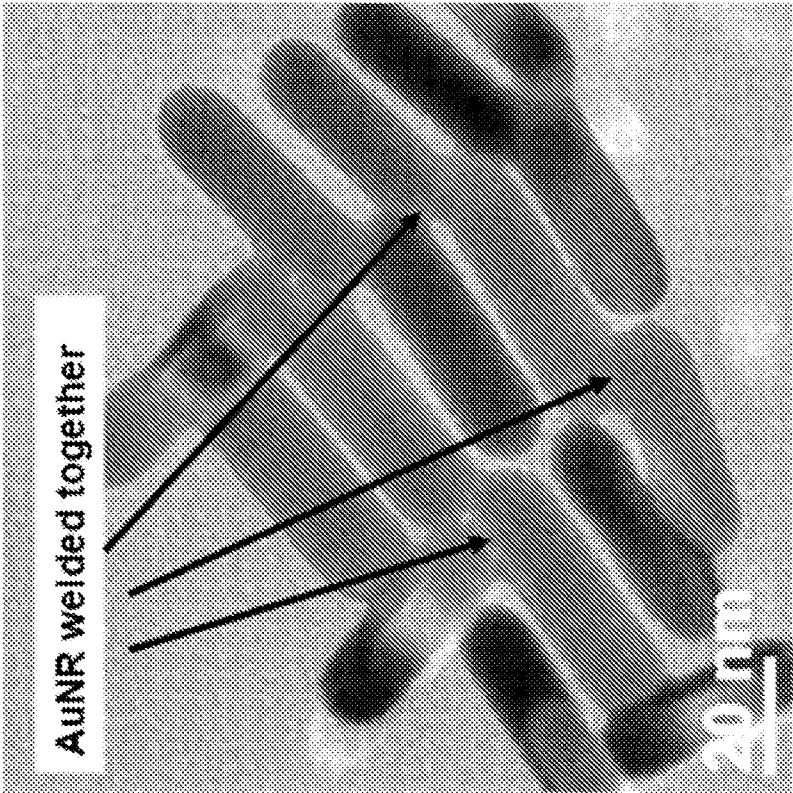


Figure 35B

Figure 35A

Figure 35

Figure 35C

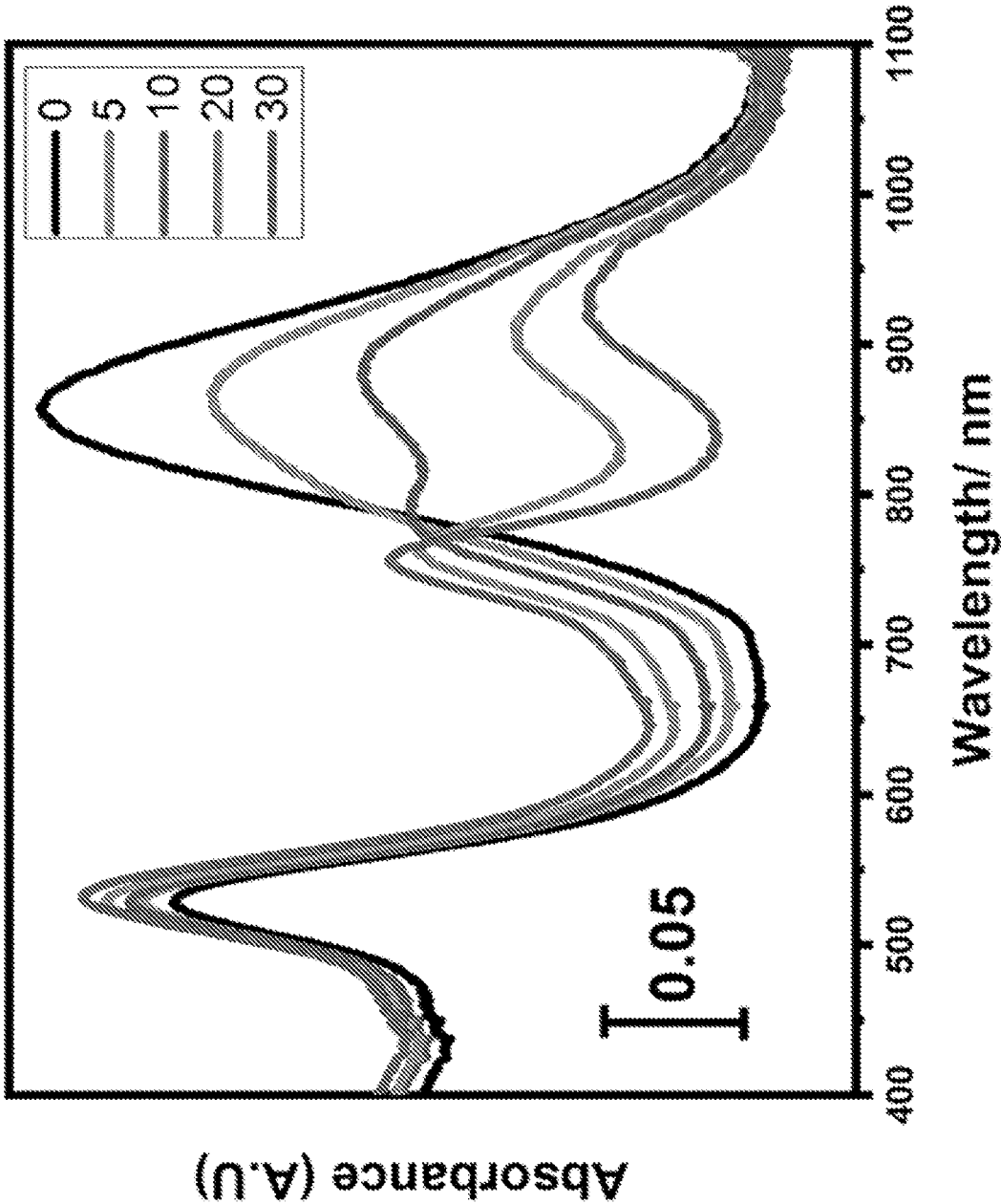


Figure 35 (cont.)

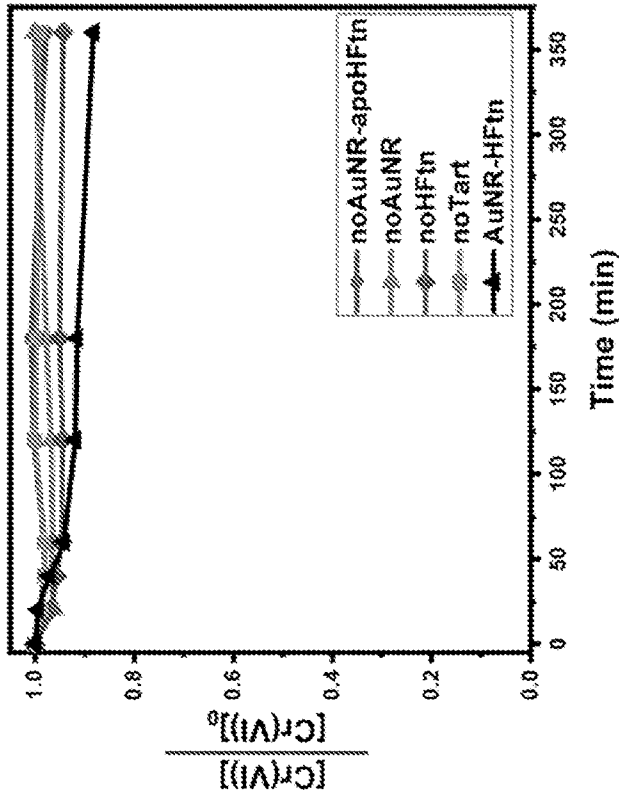


Figure 36A

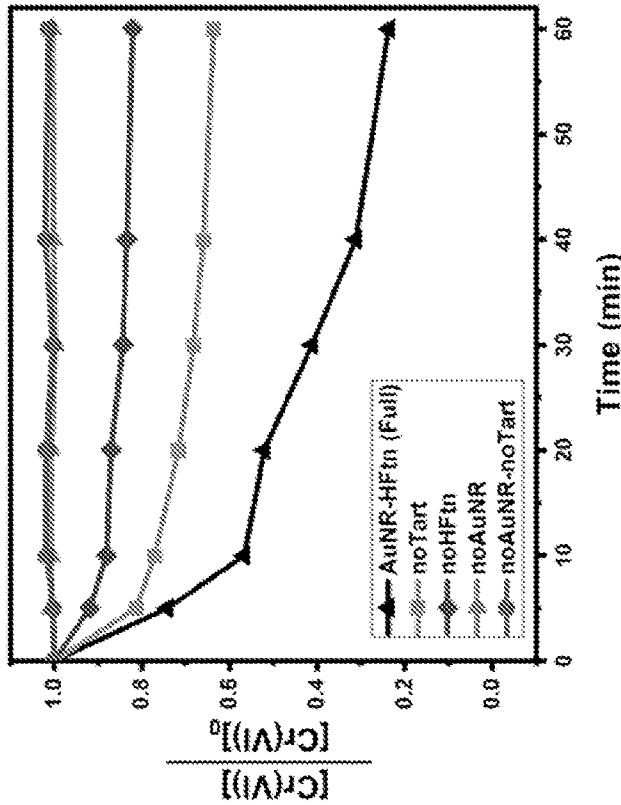


Figure 36B

Figure 36

Figure 37A

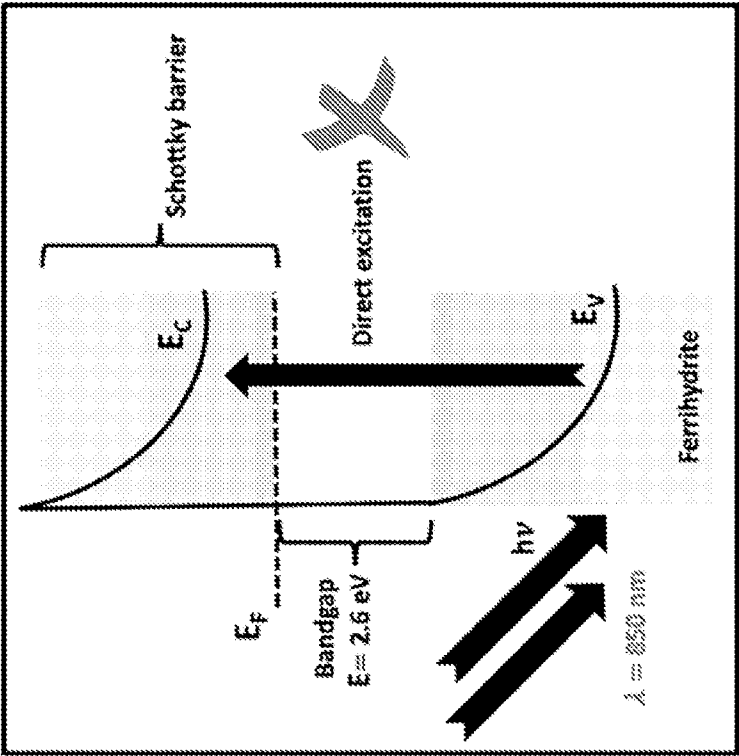


Figure 37

Figure 37B

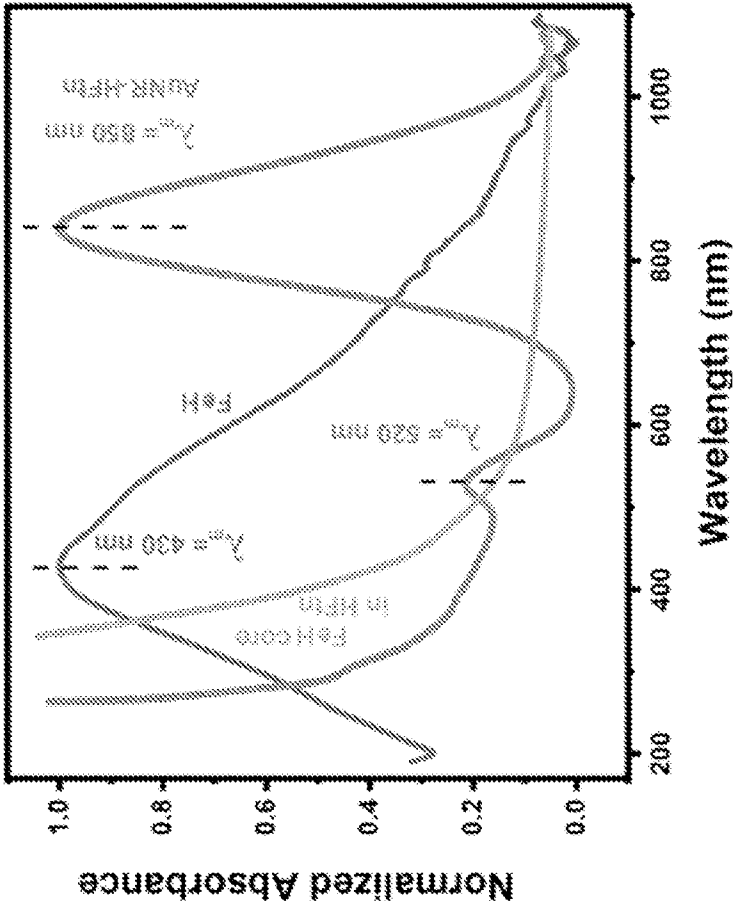


Figure 38B

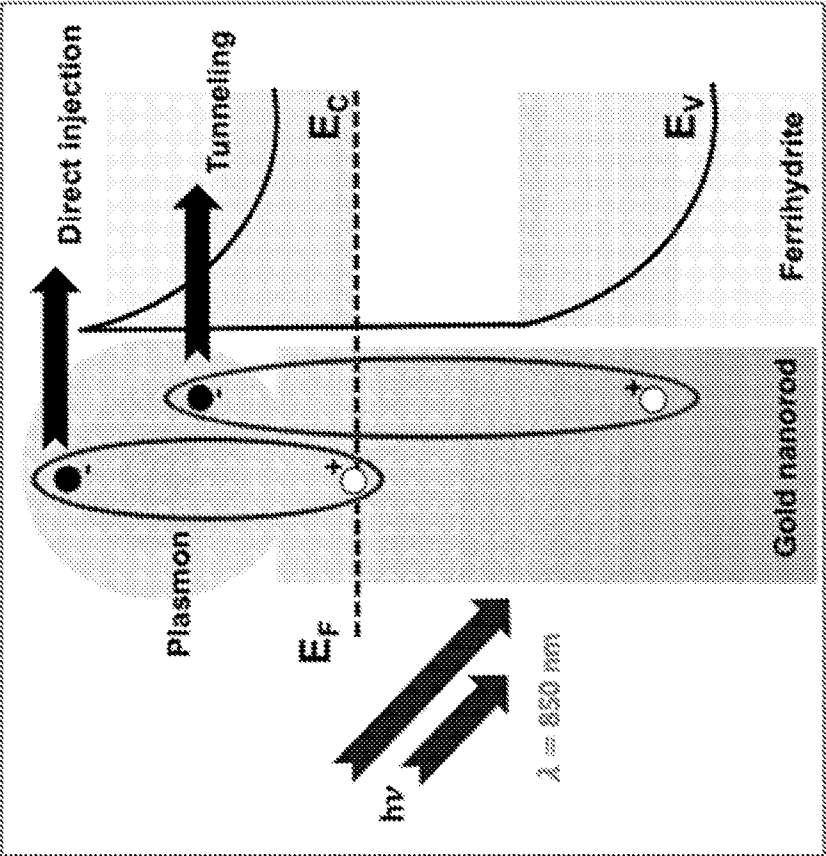


Figure 38A

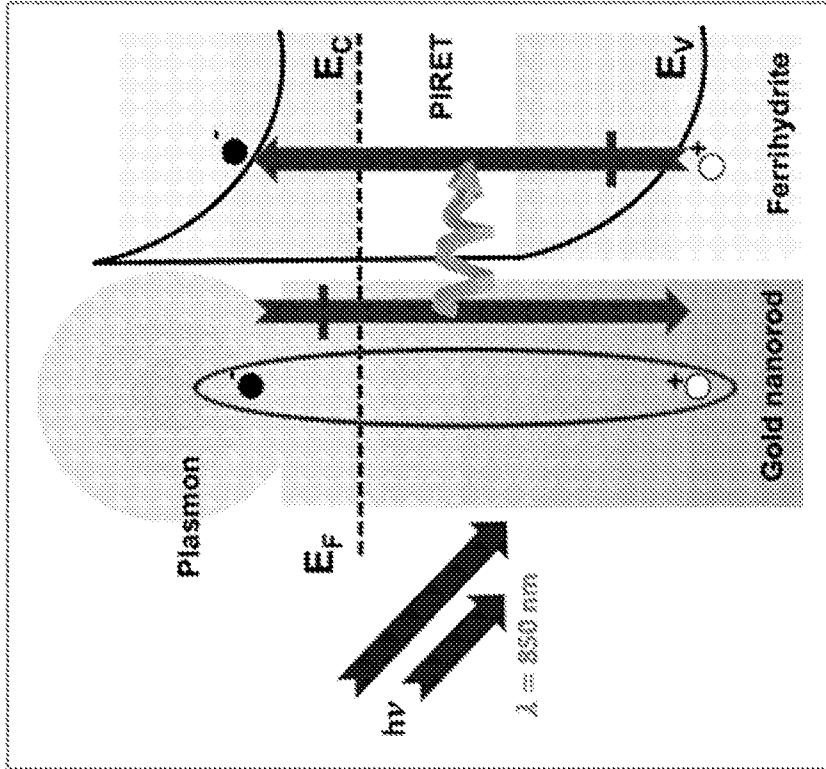


Figure 38

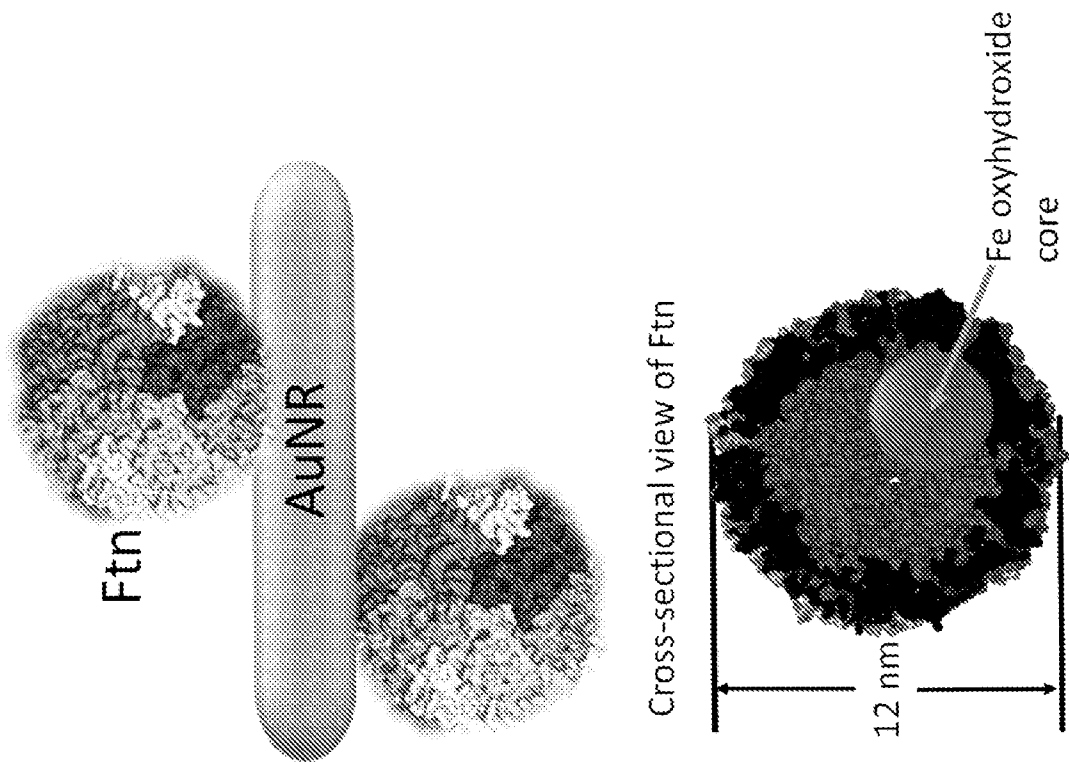


Figure 39

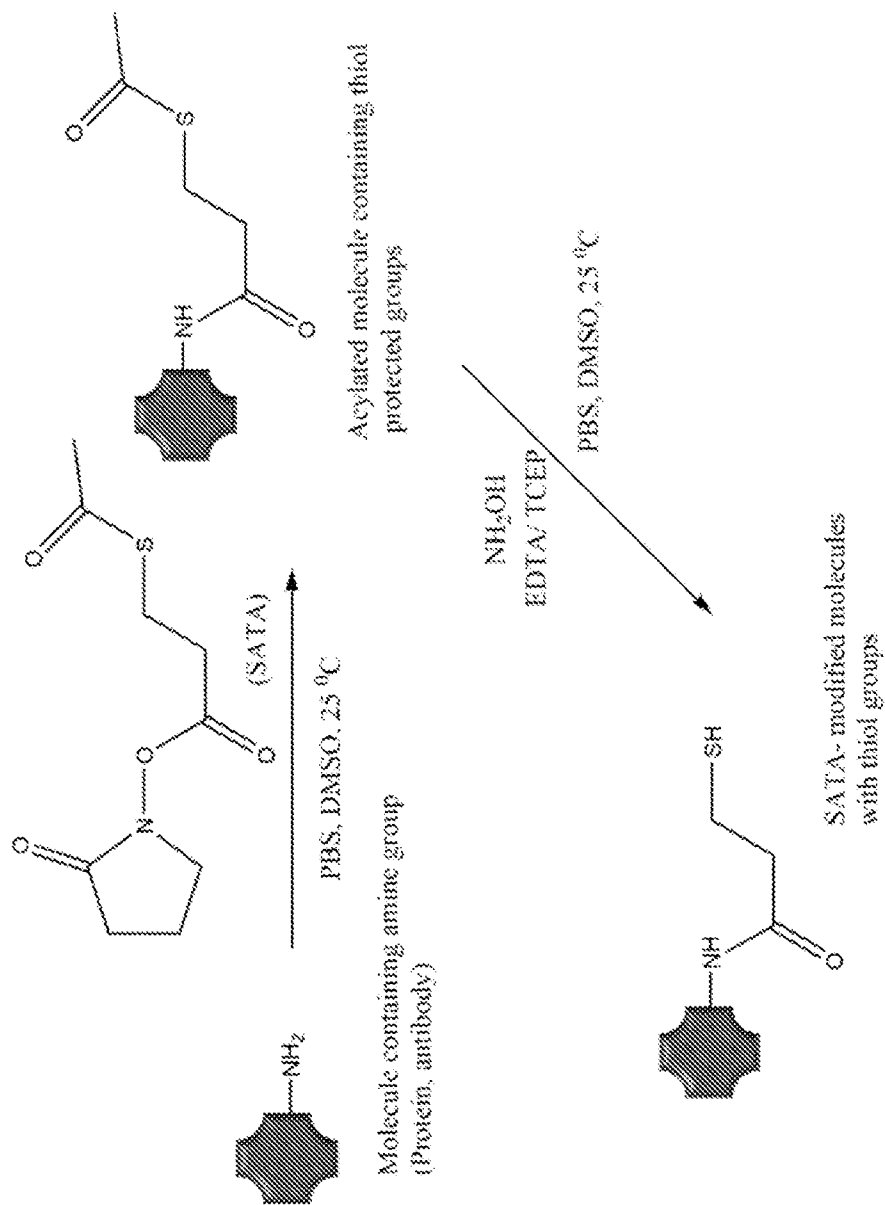


Figure 40

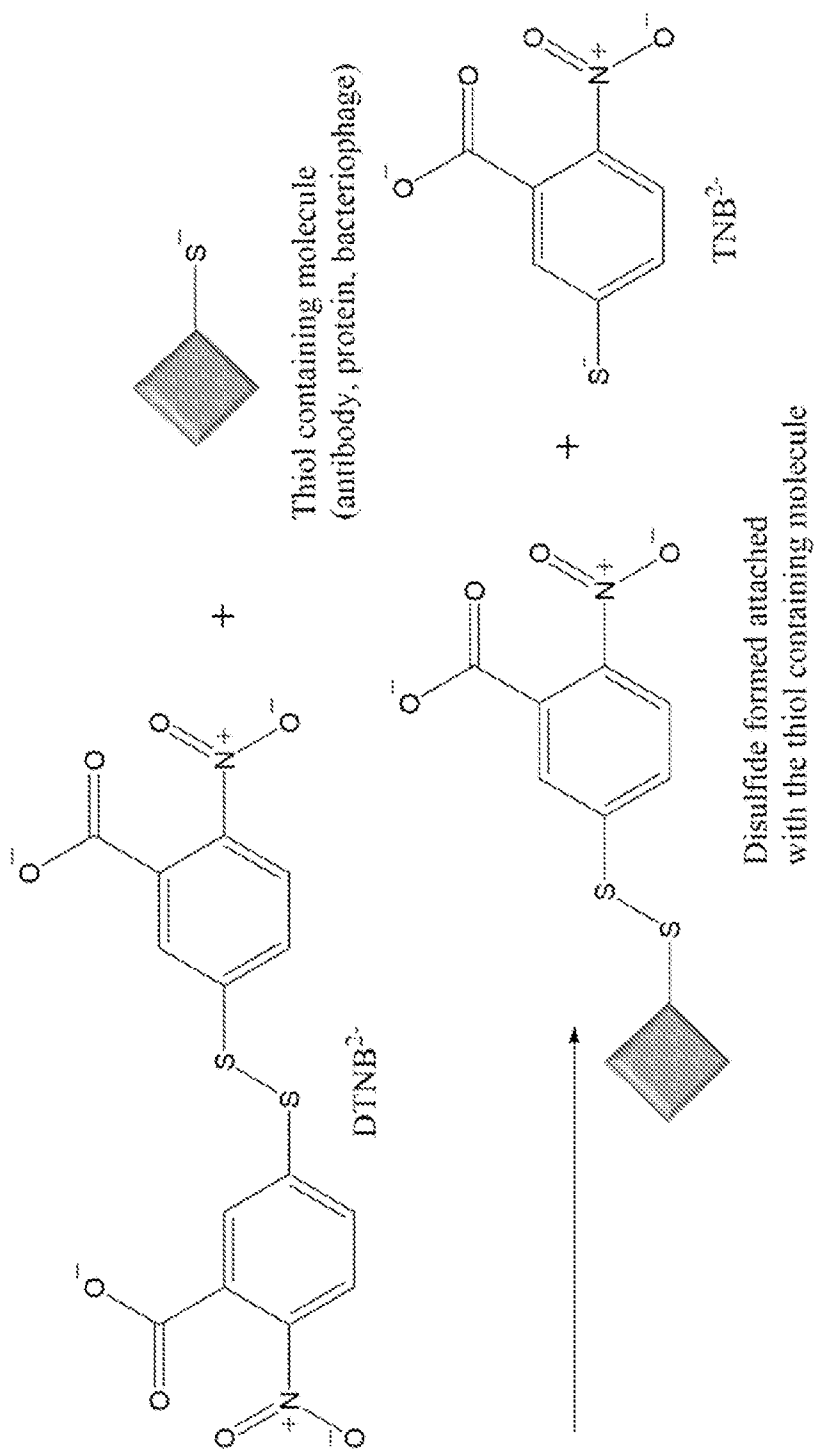


Figure 41

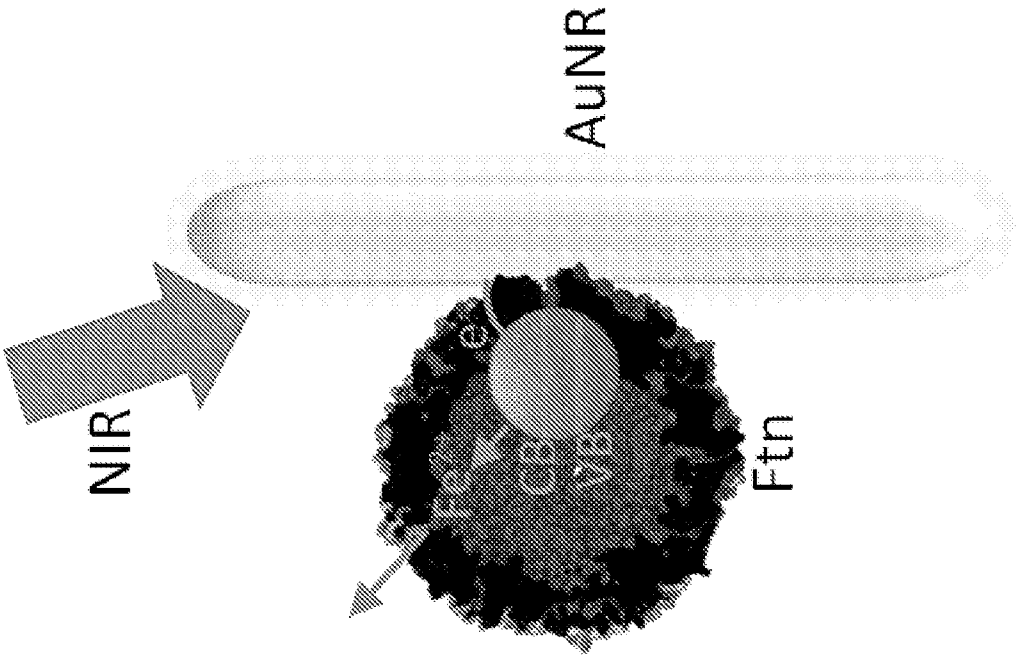


Figure 42

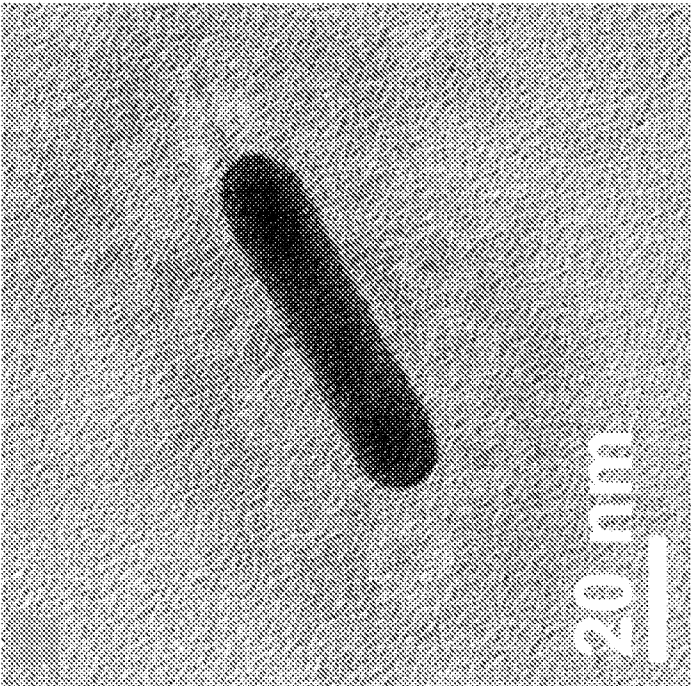


Figure 43A

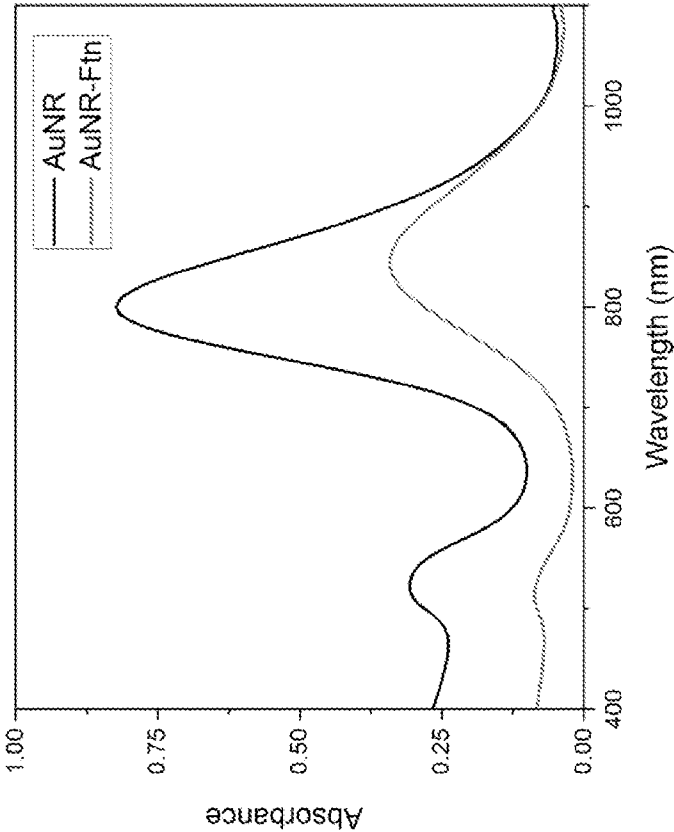


Figure 43B

Figure 43

Figure 44B

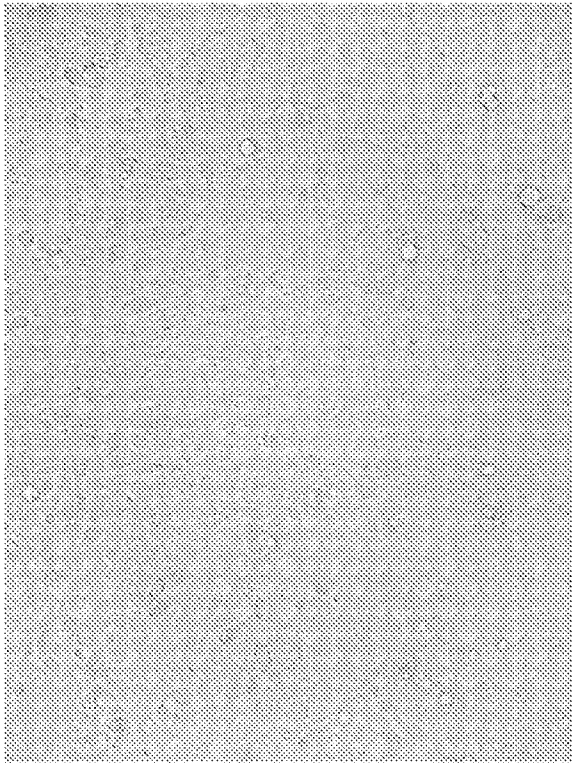


Figure 44D

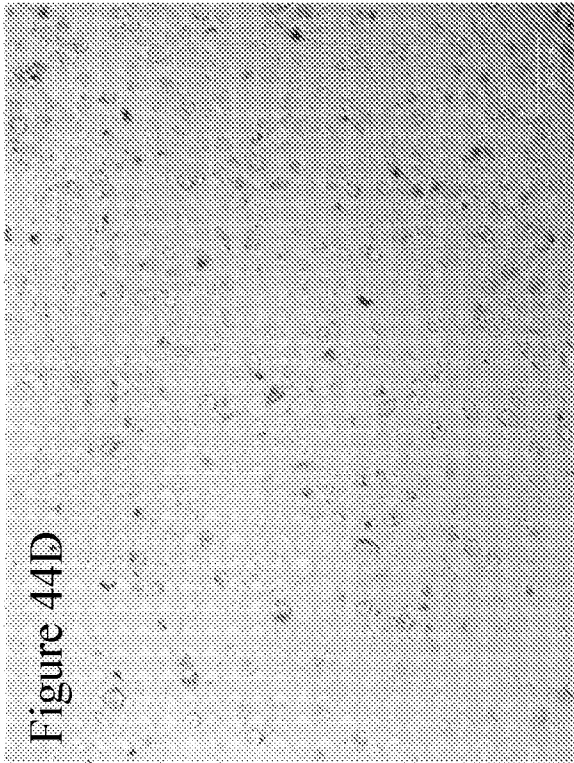


Figure 44

Figure 44A

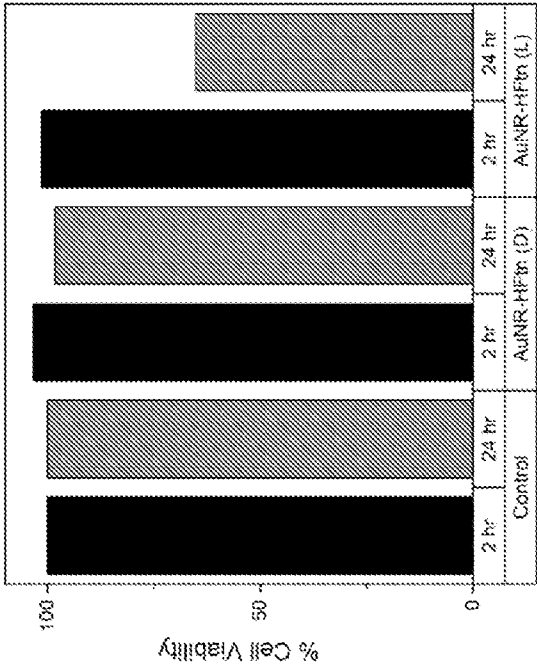


Figure 44C



Figure 45B

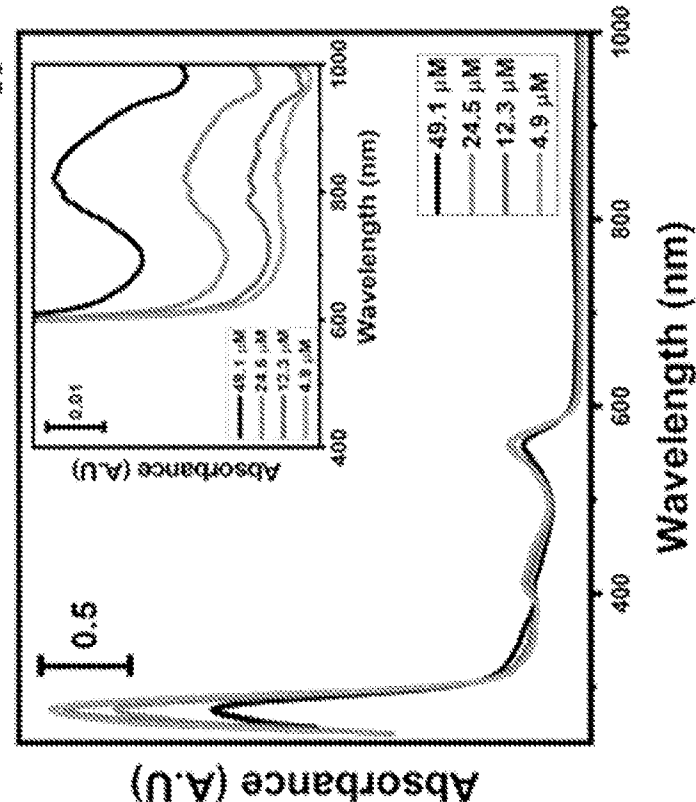


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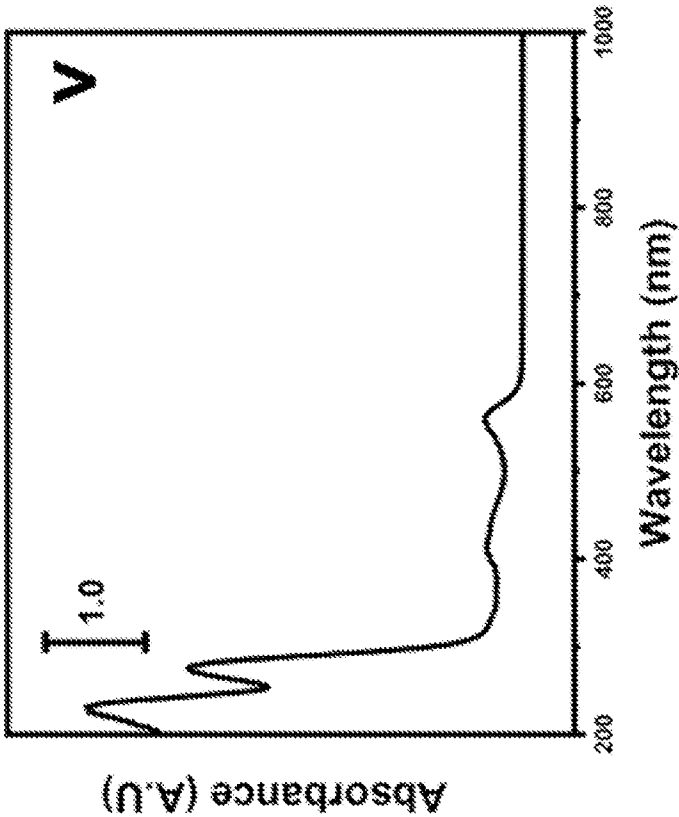


Figure 45

Figure 45D

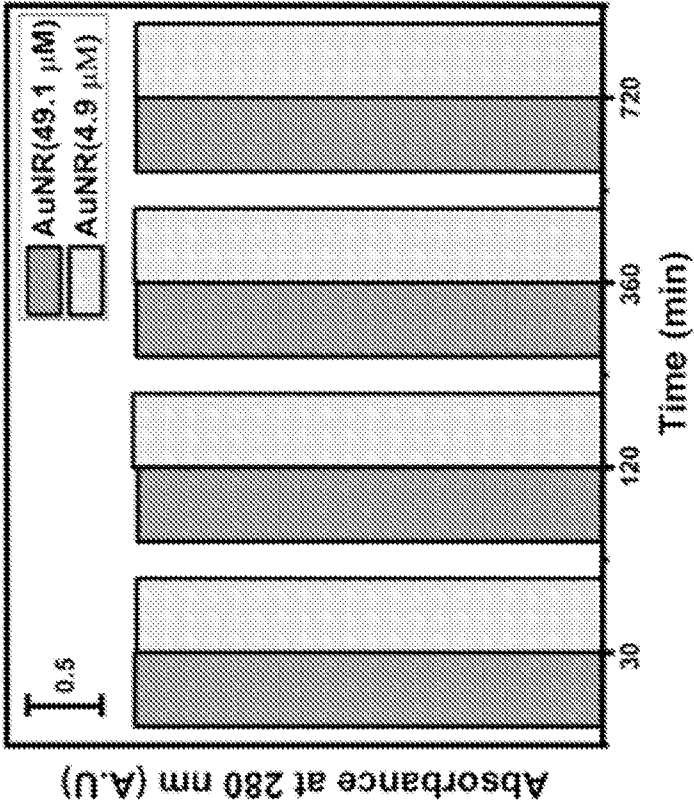


Figure 45C

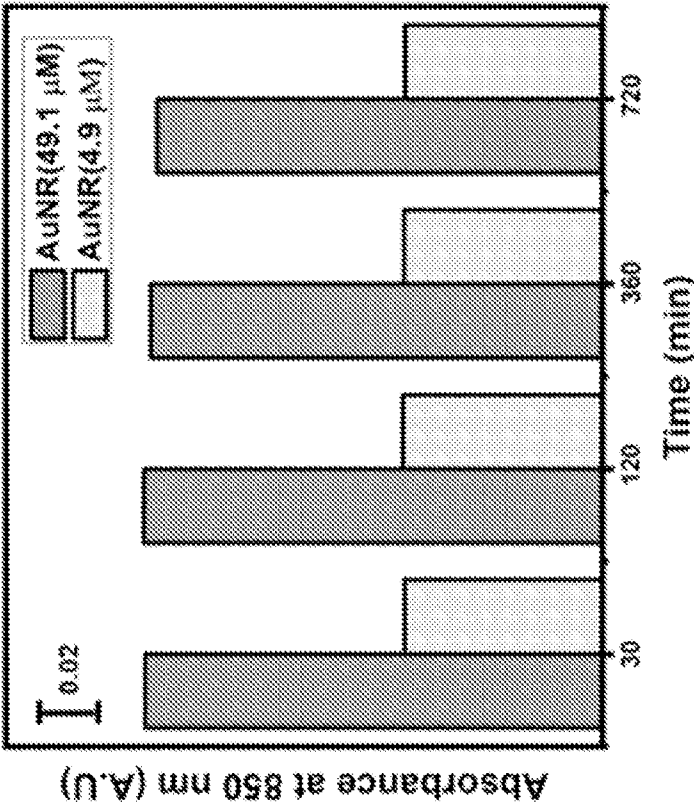


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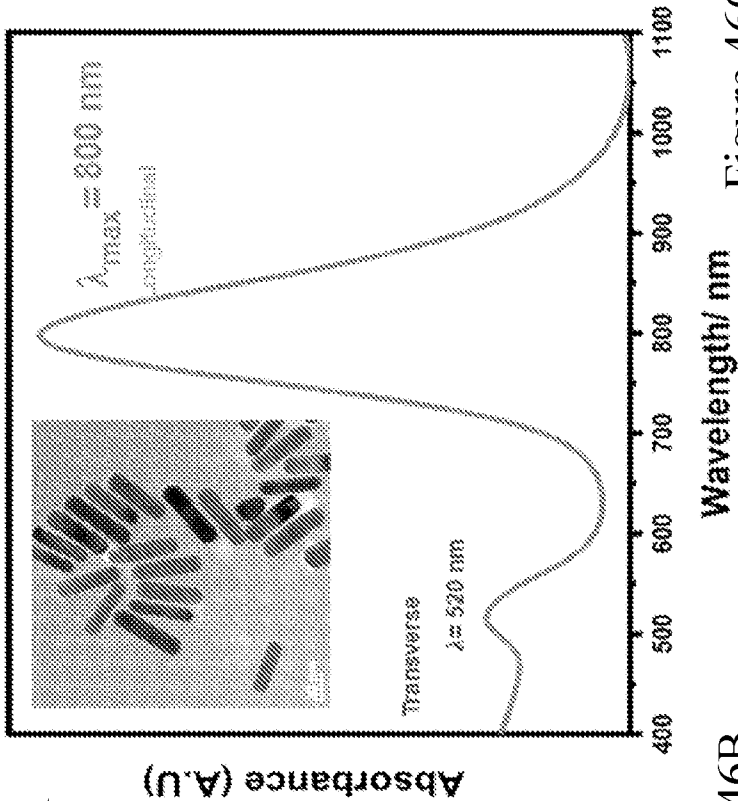


Figure 46C

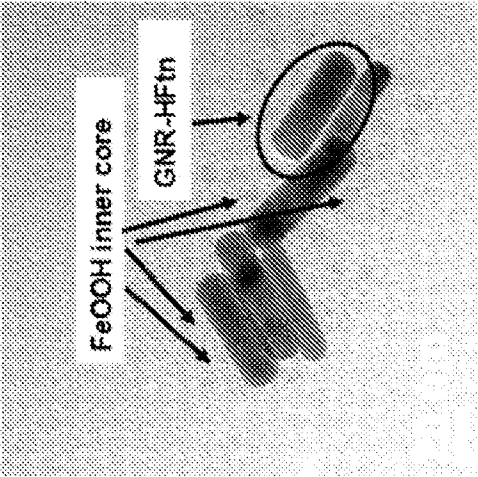


Figure 46B

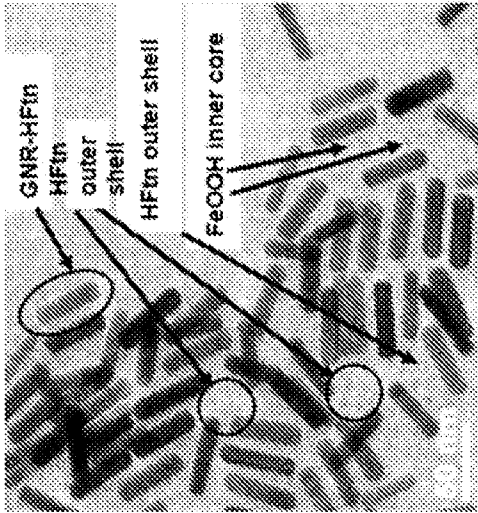


Figure 46

Figure 47A

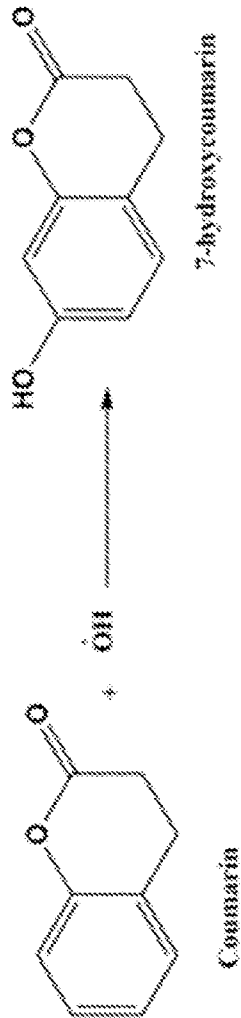


Figure 47B

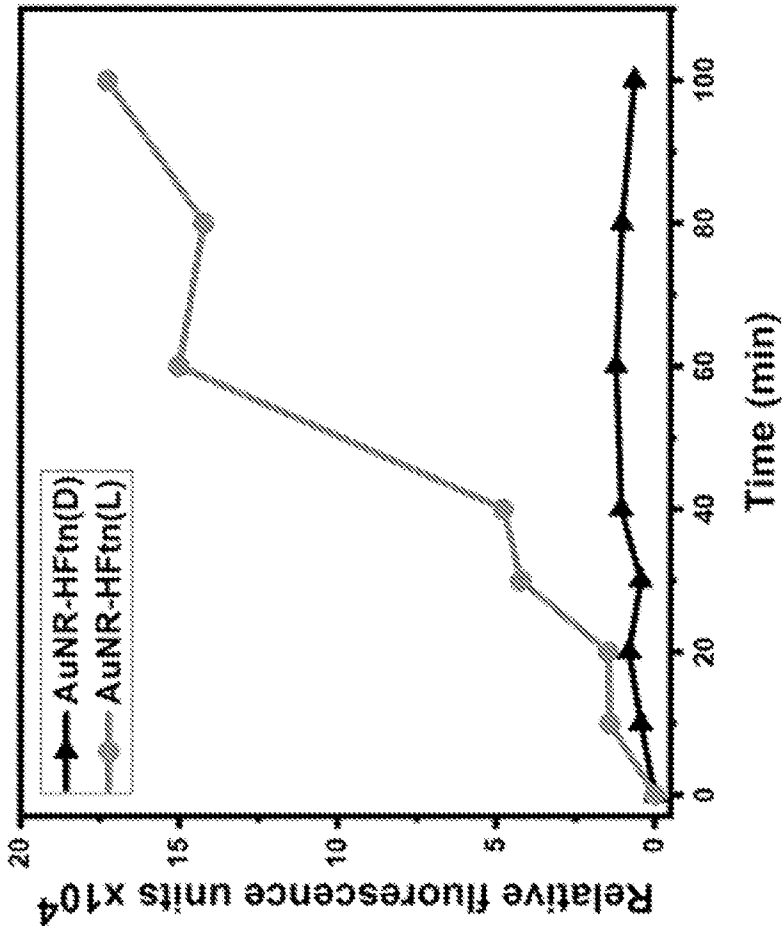


Figure 47

Figure 48B

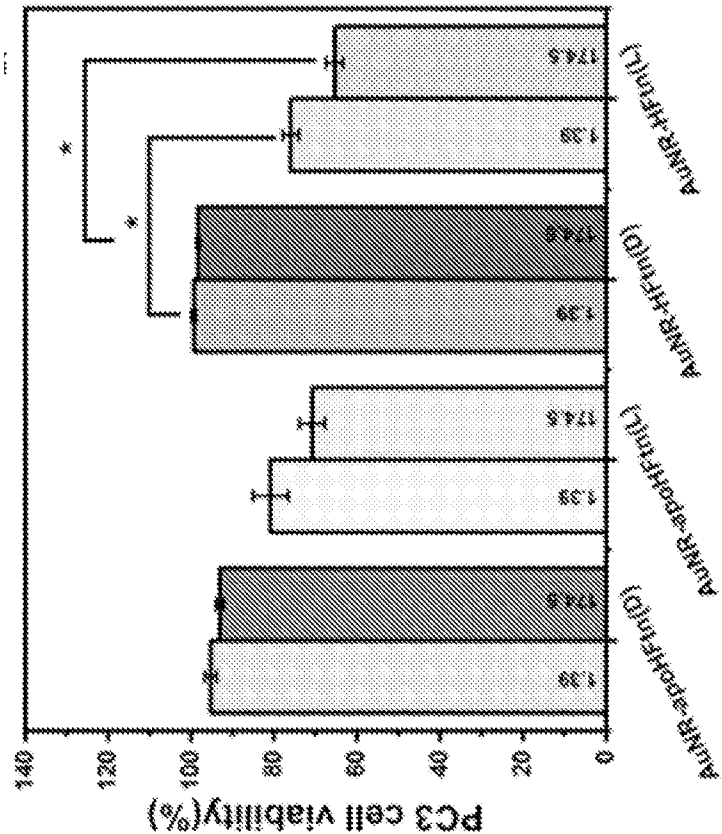


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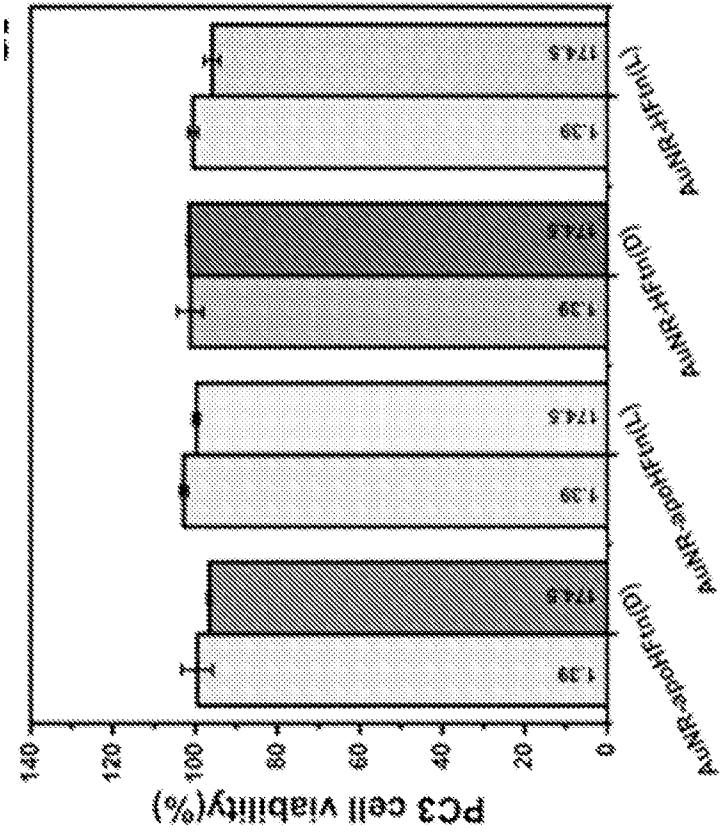


Figure 48

Figure 48D

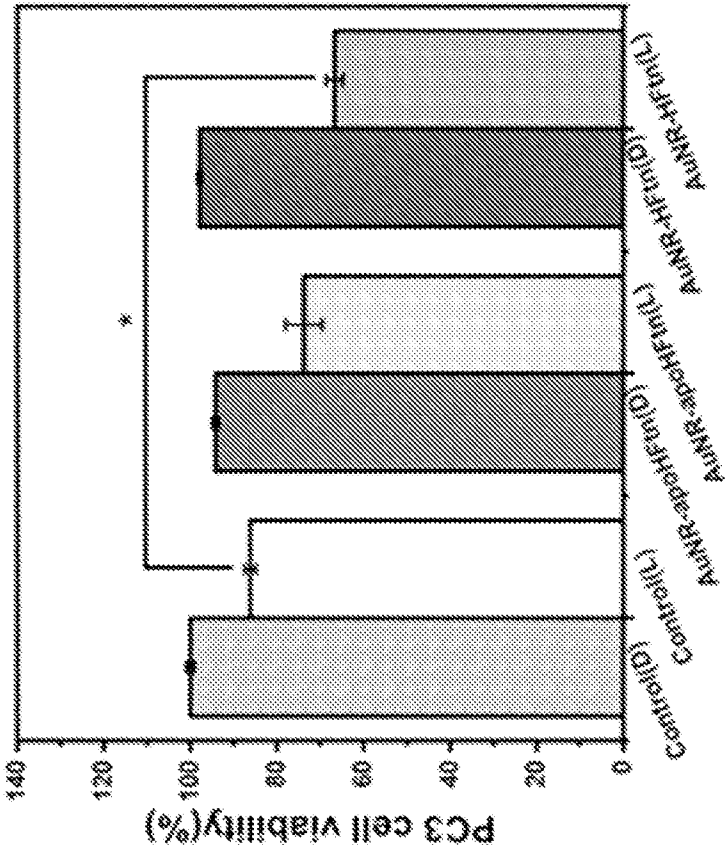


Figure 48C

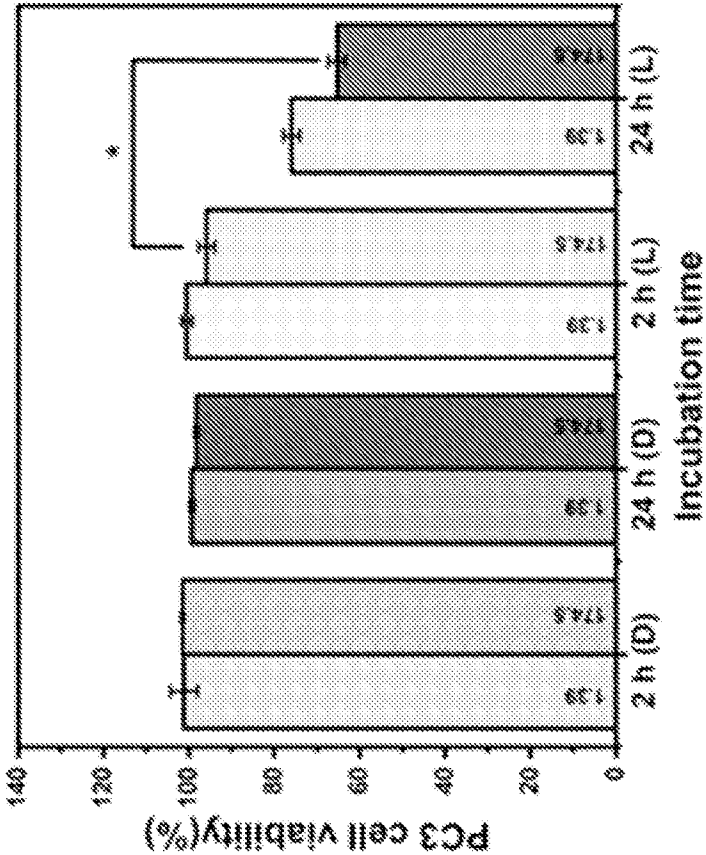


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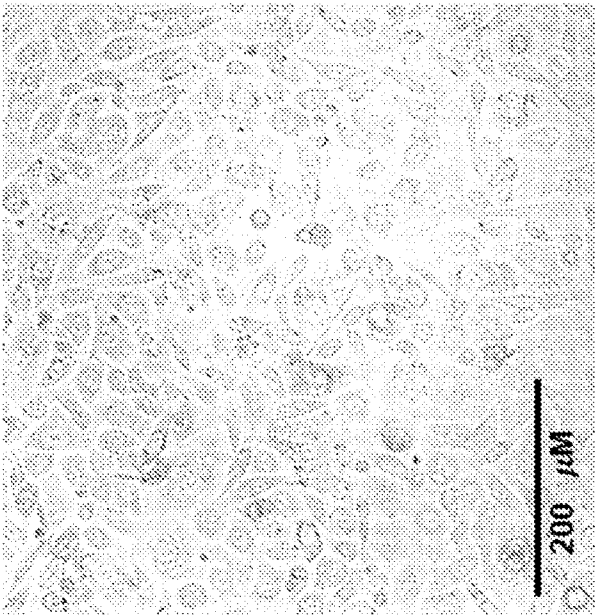


Figure 49A

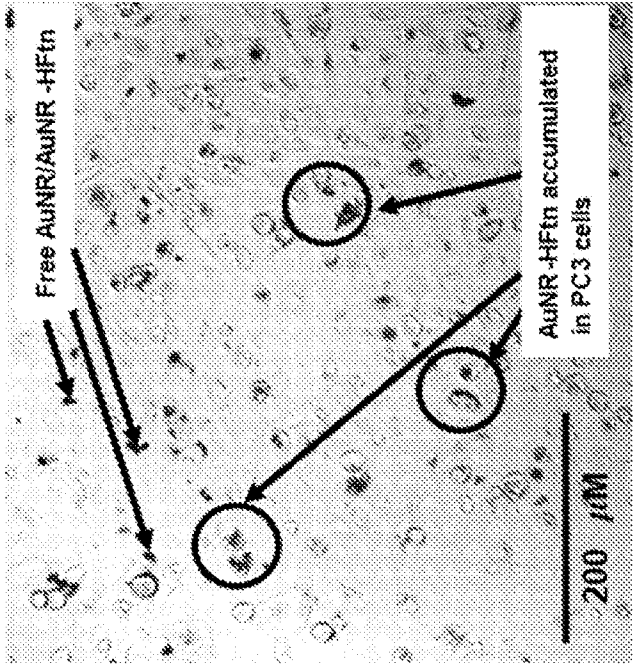


Figure 49C

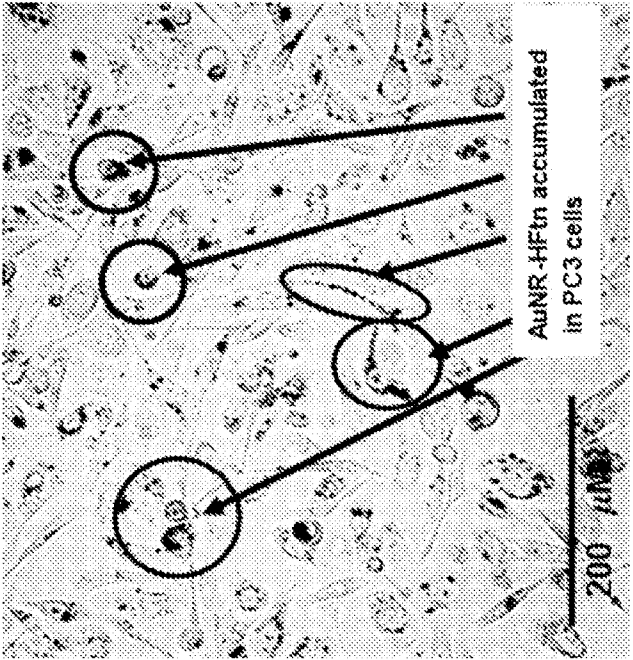


Figure 49B

Figure 49

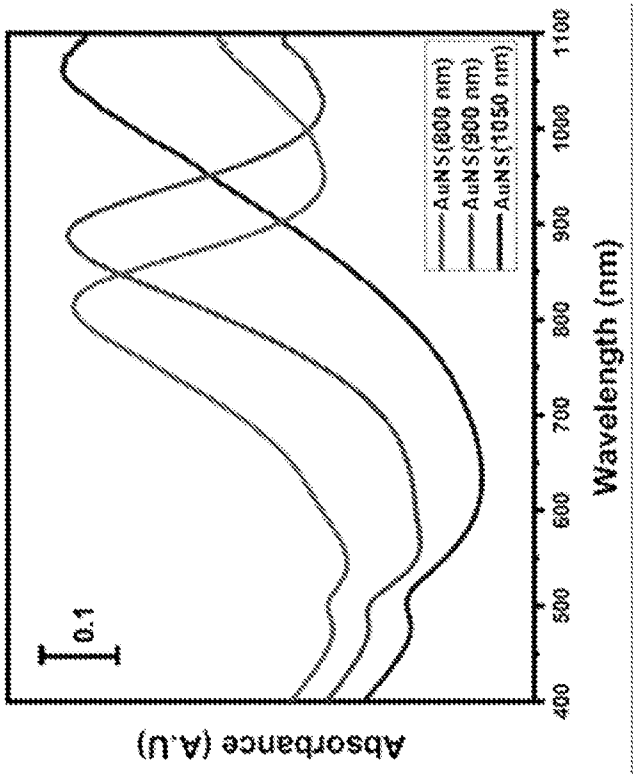


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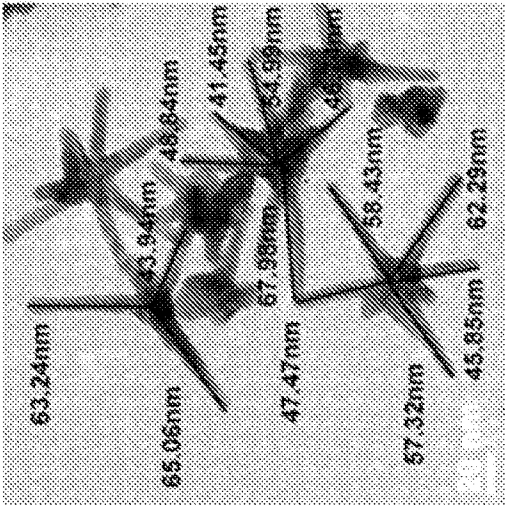


Figure 50C

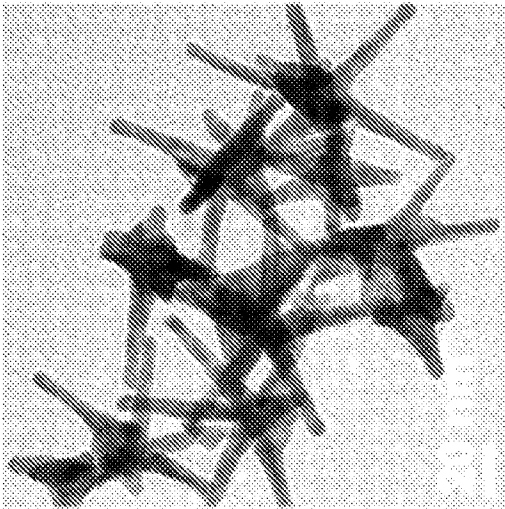


Figure 50B

Figure 50

Figure 51B

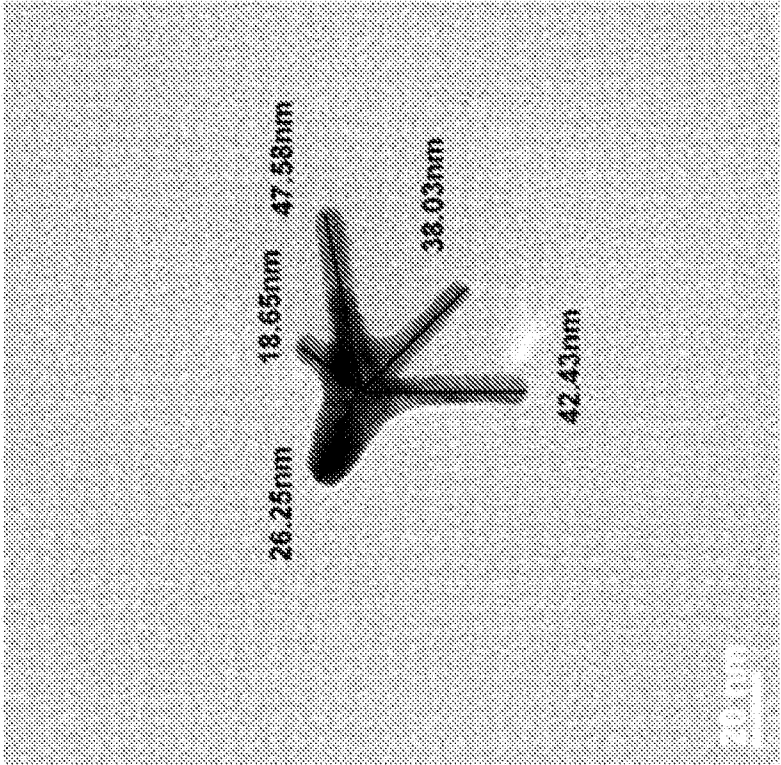


Figure 51A

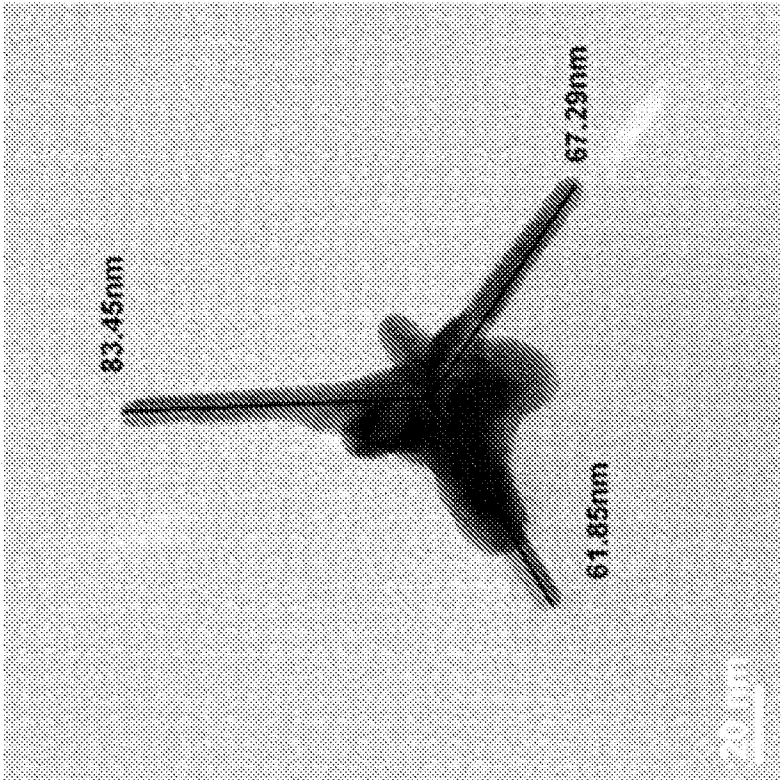


Figure 51

Figure 51E

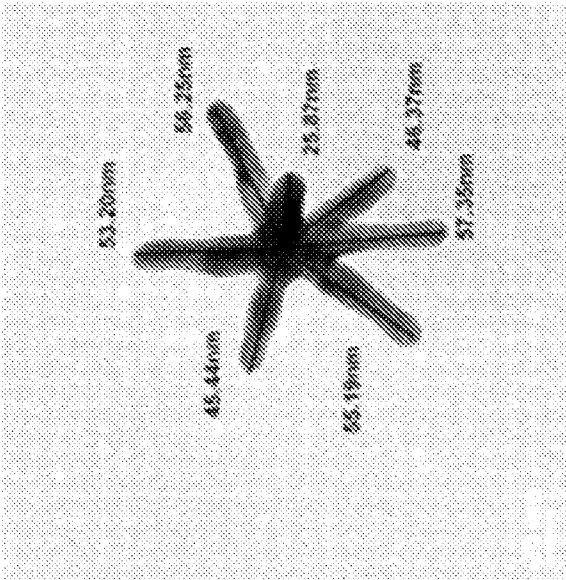


Figure 51D

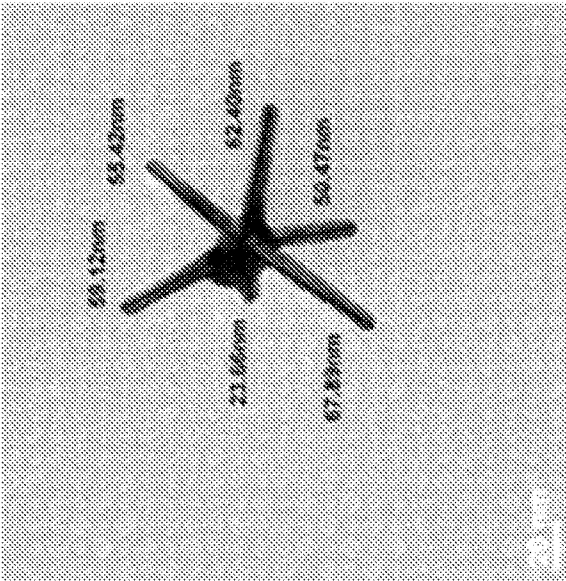


Figure 51C

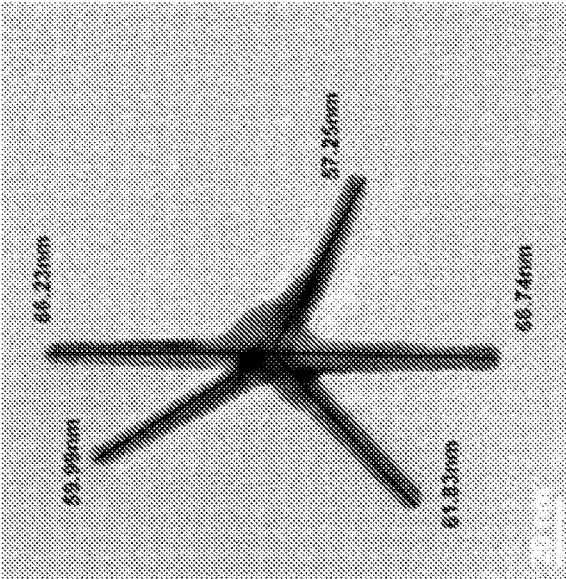


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Figure 52A

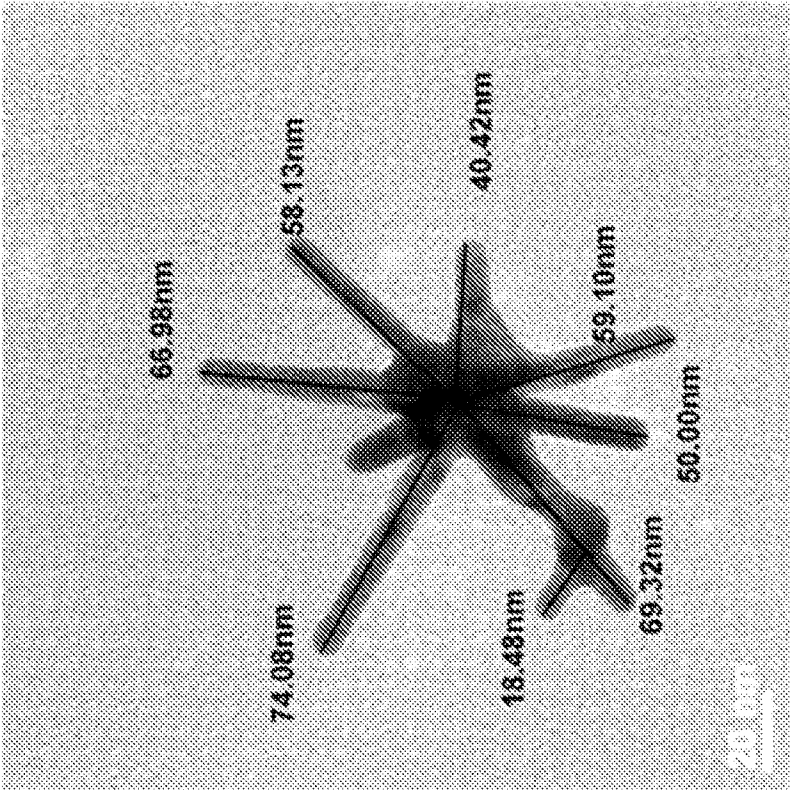


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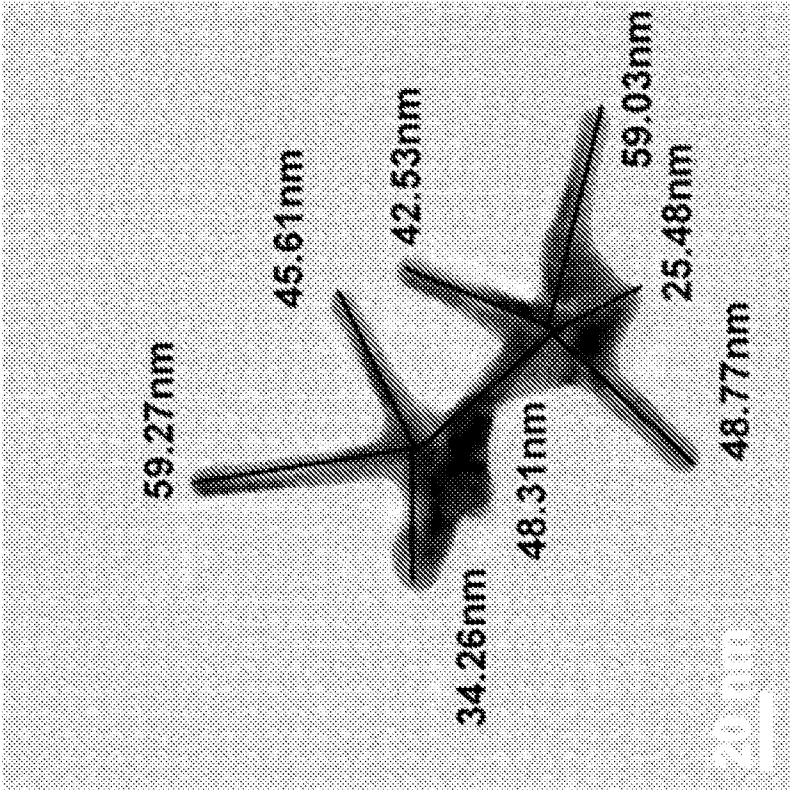


Figure 52

Figure 53B

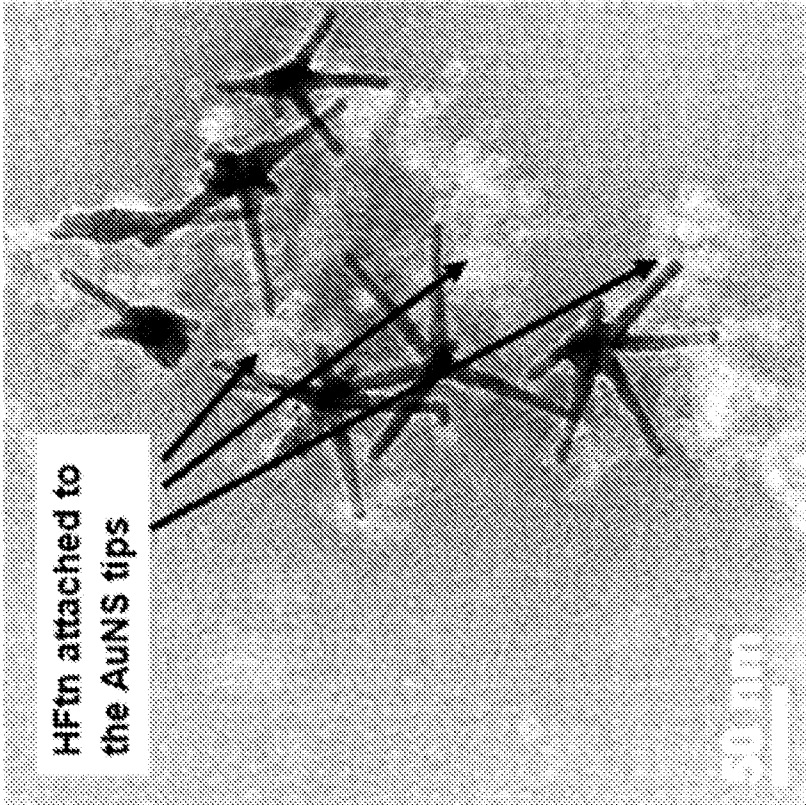


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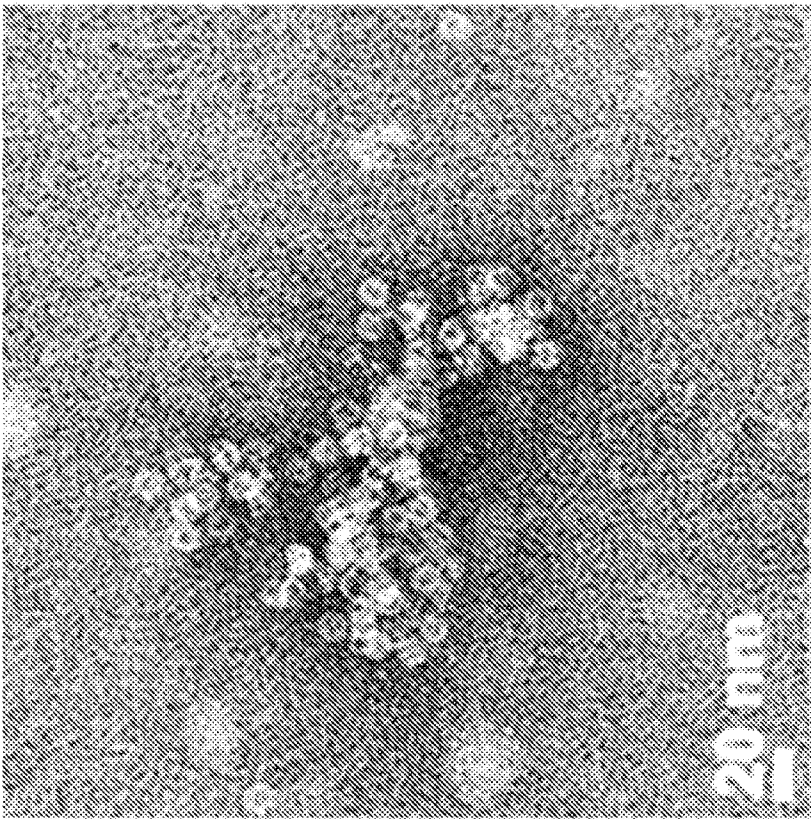


Figure 53

Figure 53D

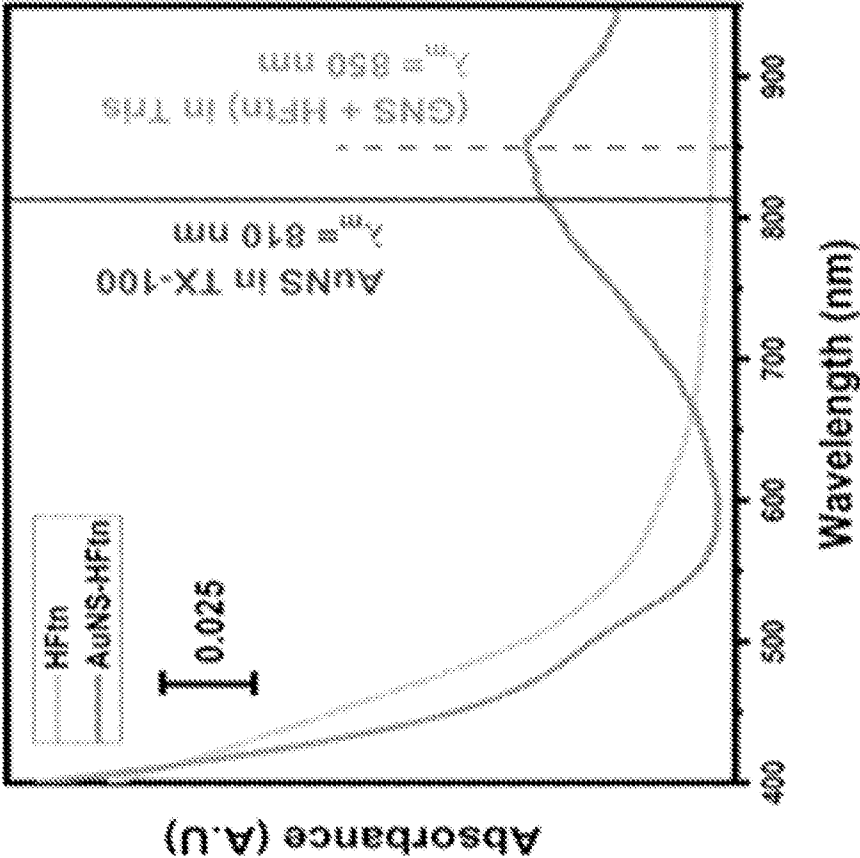


Figure 53C

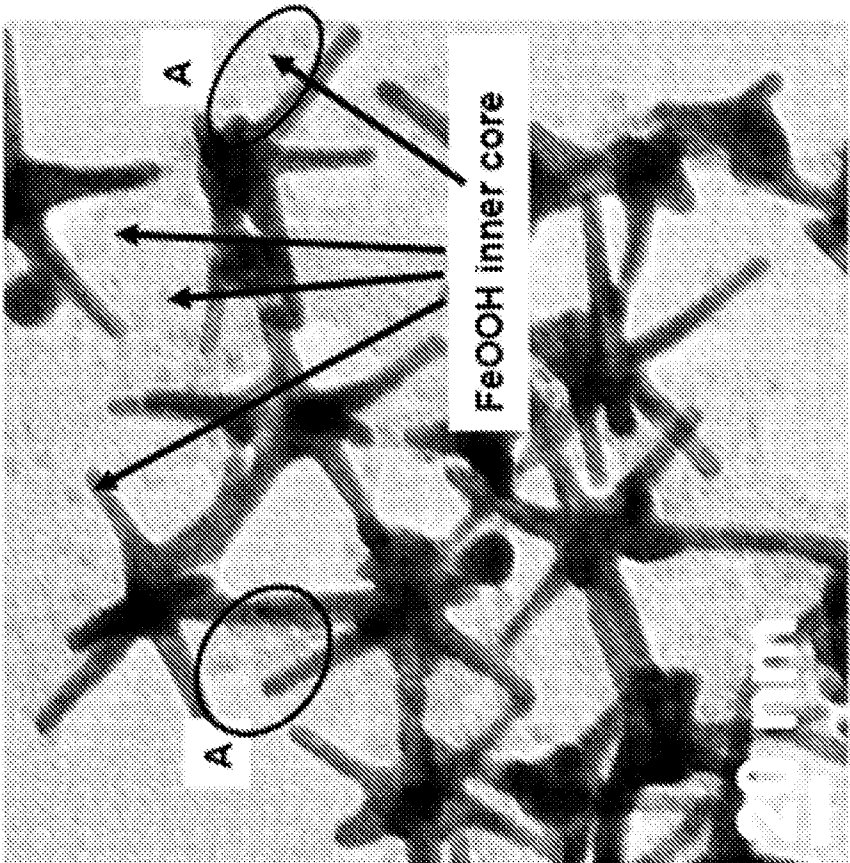


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Figure 54A

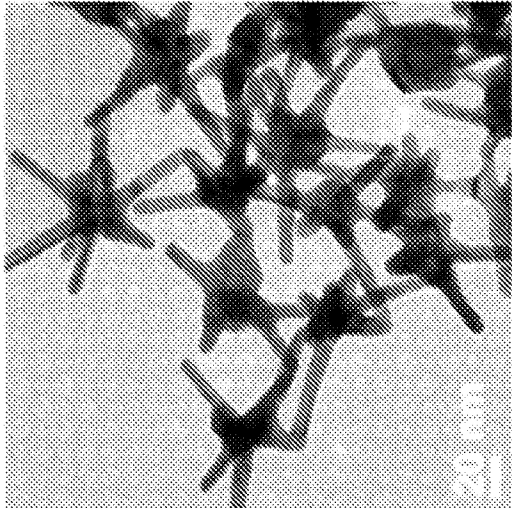


Figure 54B

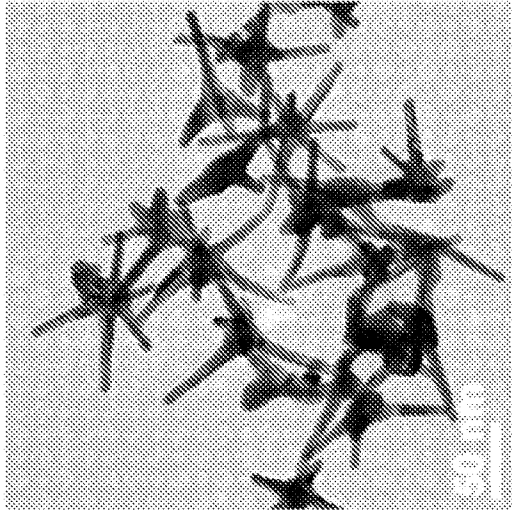


Figure 54C

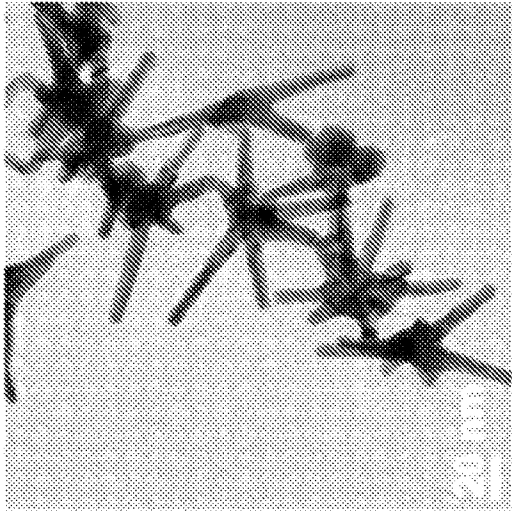


Figure 54D

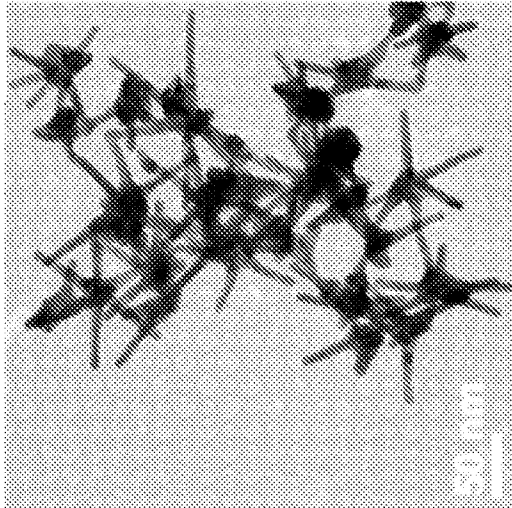


Figure 54E

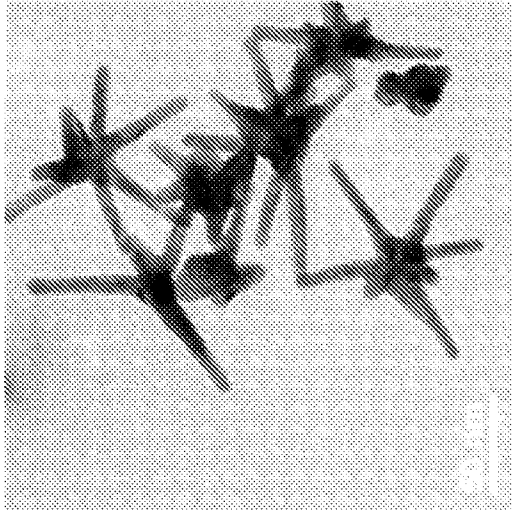


Figure 54F

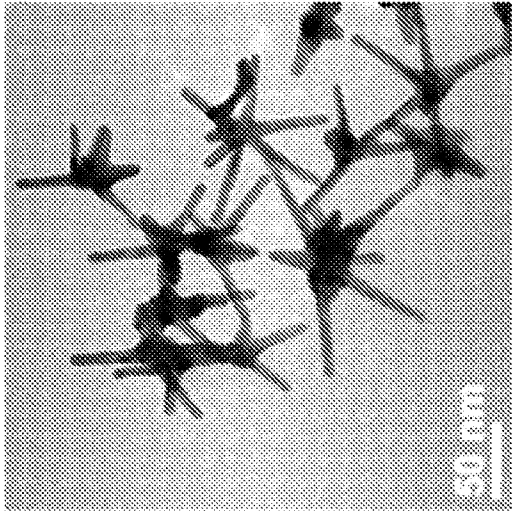


Figure 54

Figure 55A

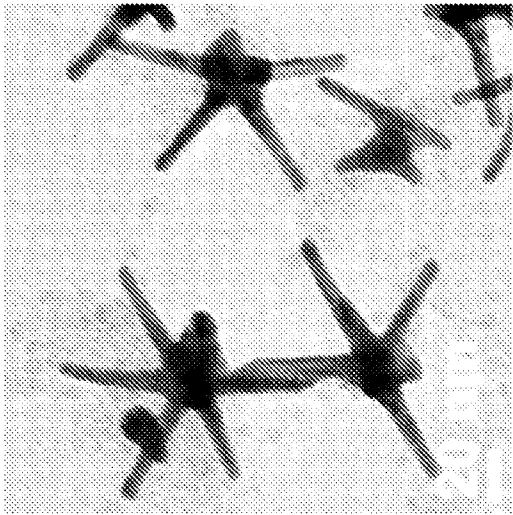


Figure 55B

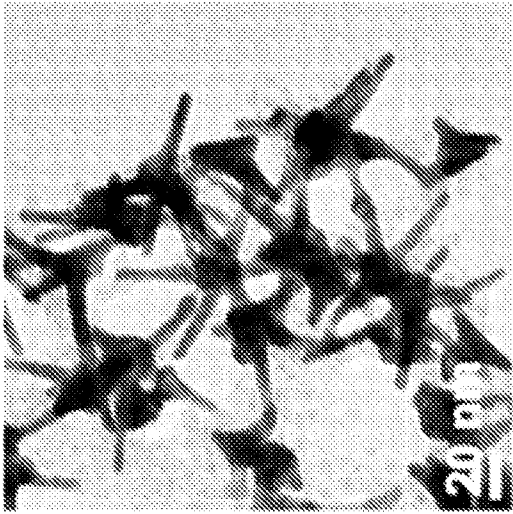


Figure 55C

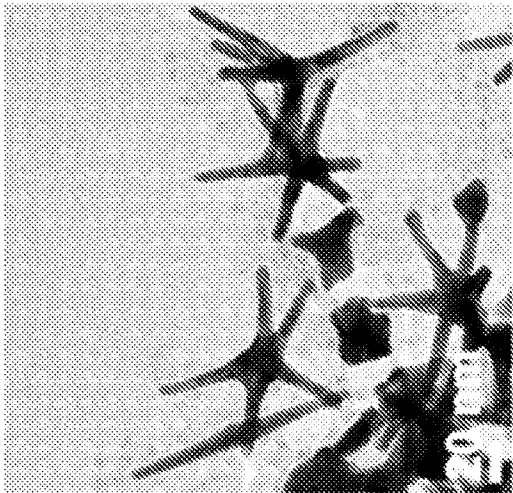


Figure 55D

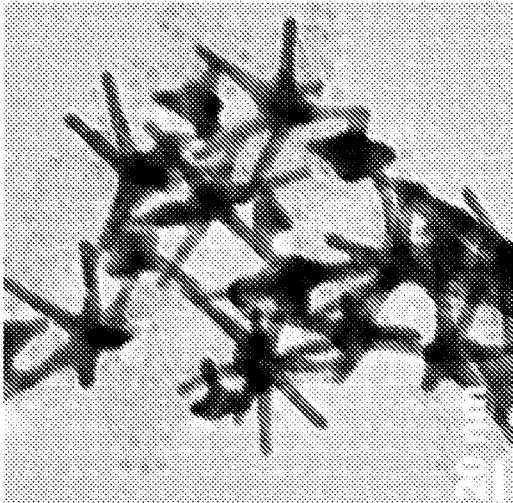


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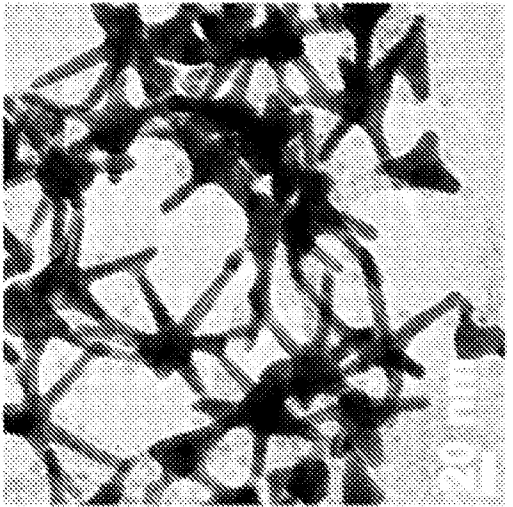


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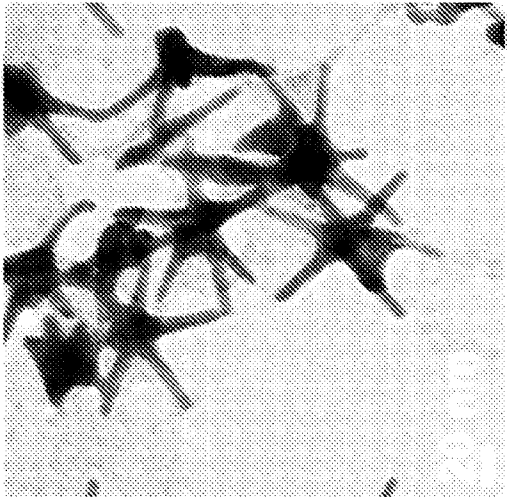


Figure 55

Figure 56A

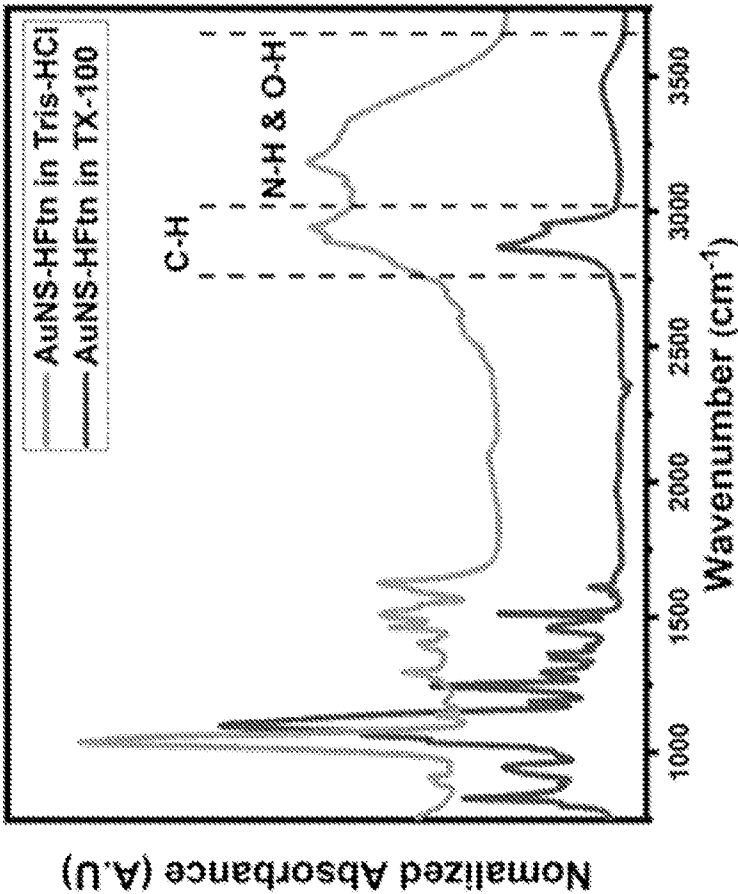


Figure 56B

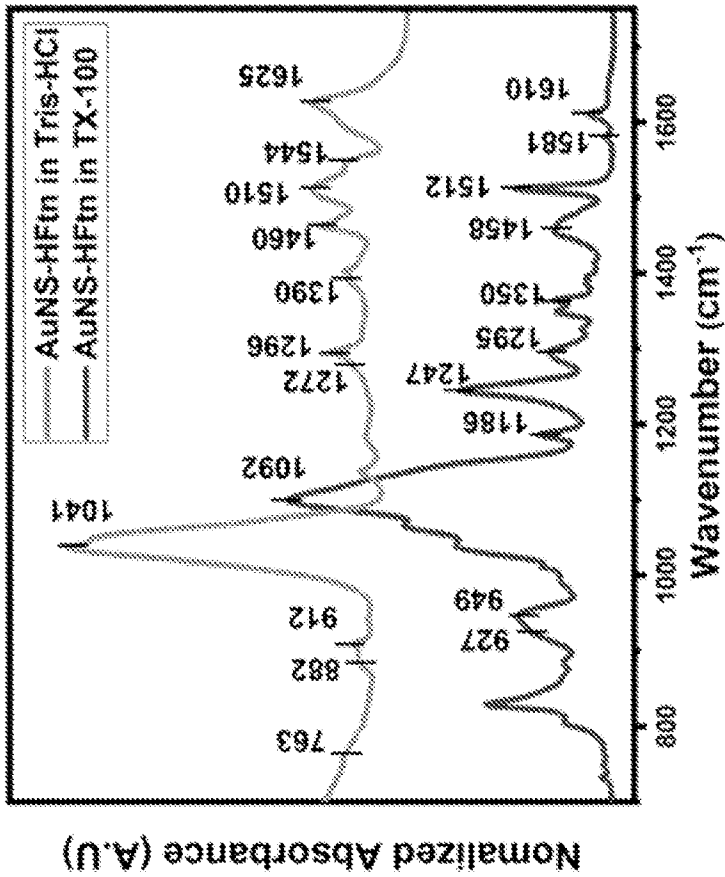


Figure 56

Figure 57A

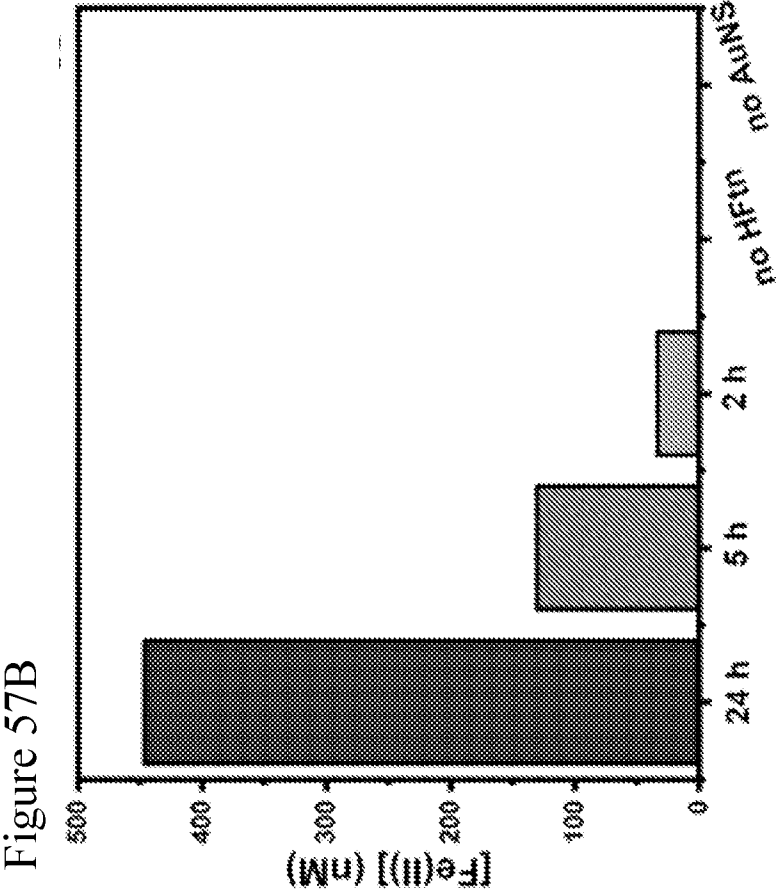
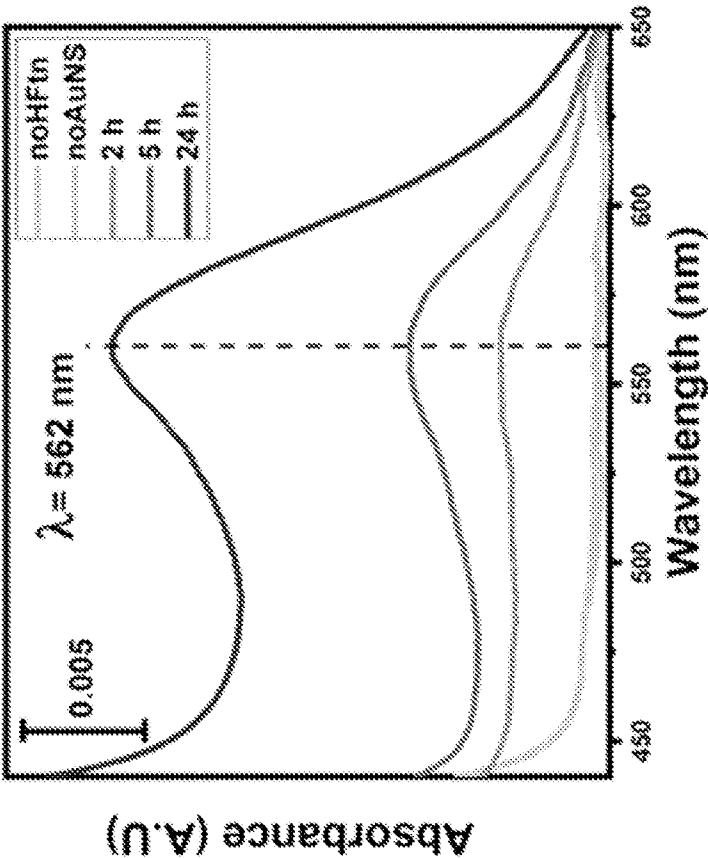


Figure 57

Figure 58A

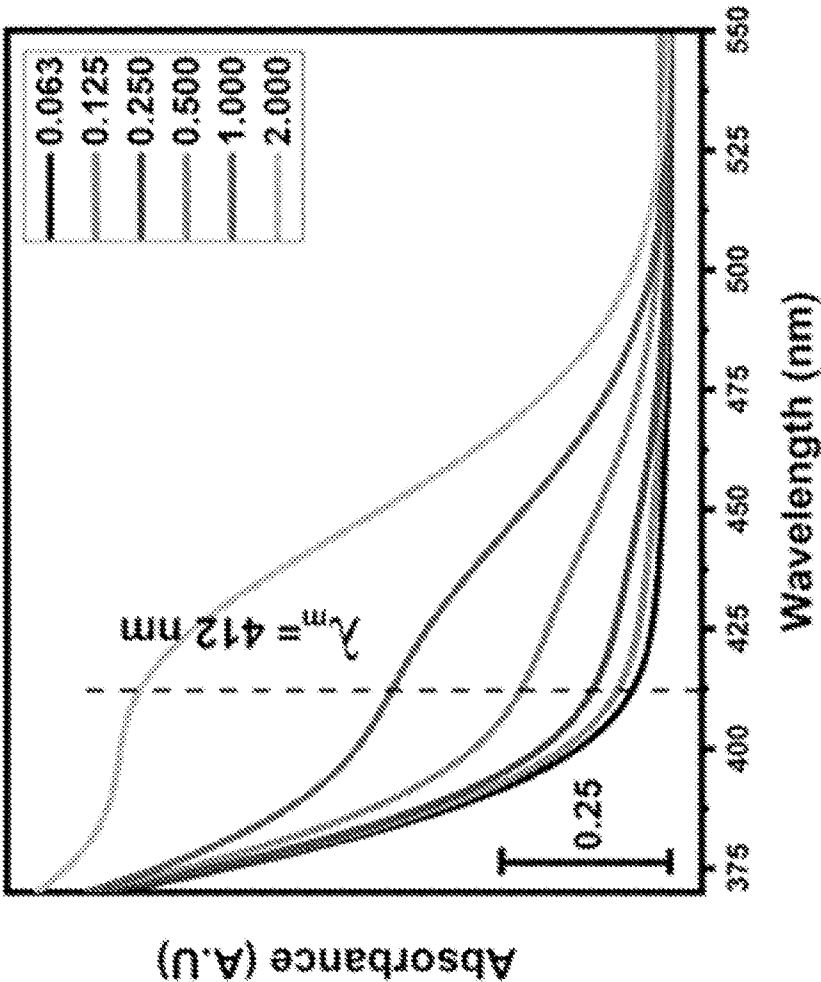


Figure 58

Figure 58B

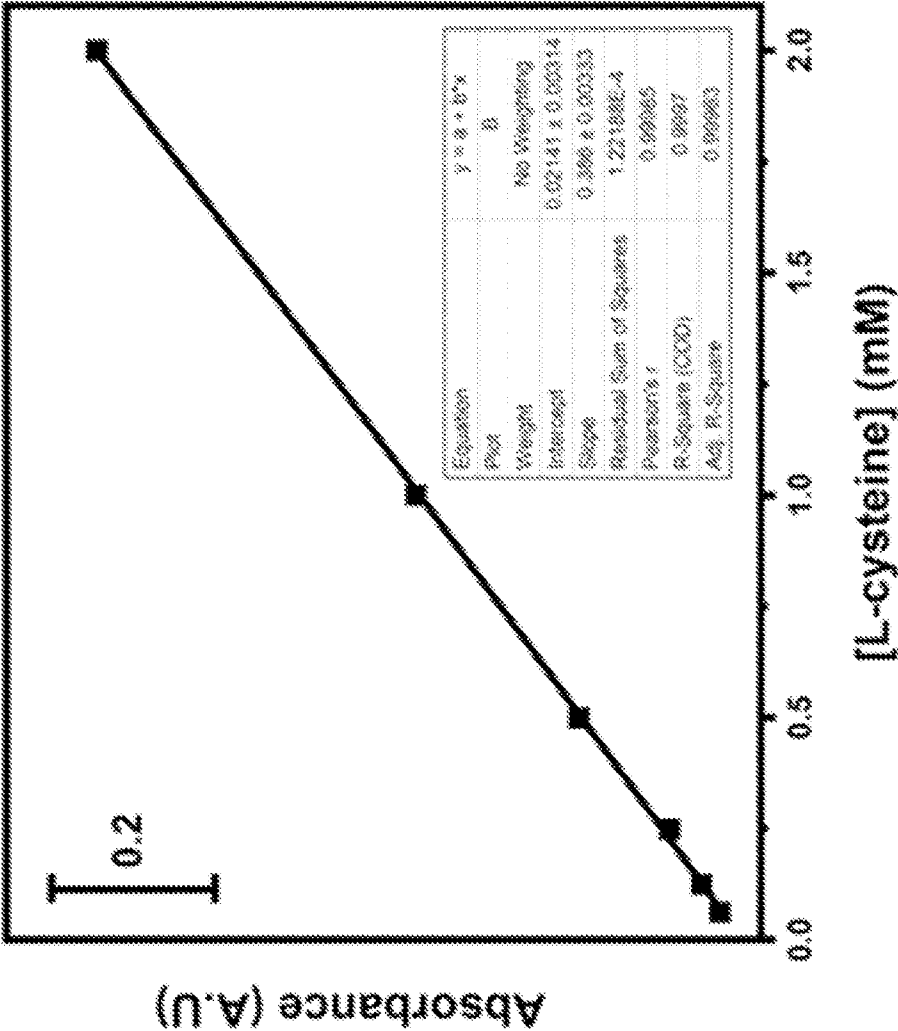


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Figure 59B

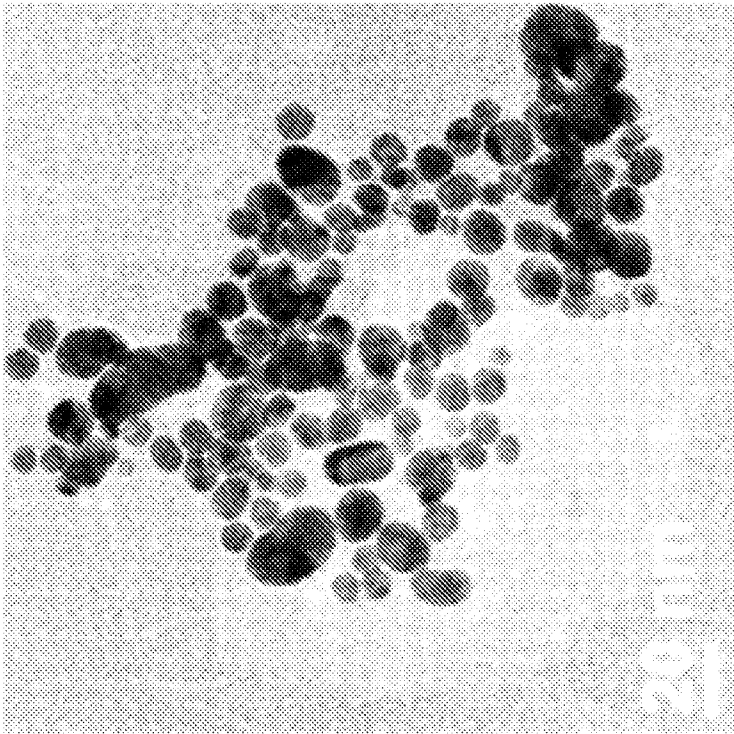


Figure 59A

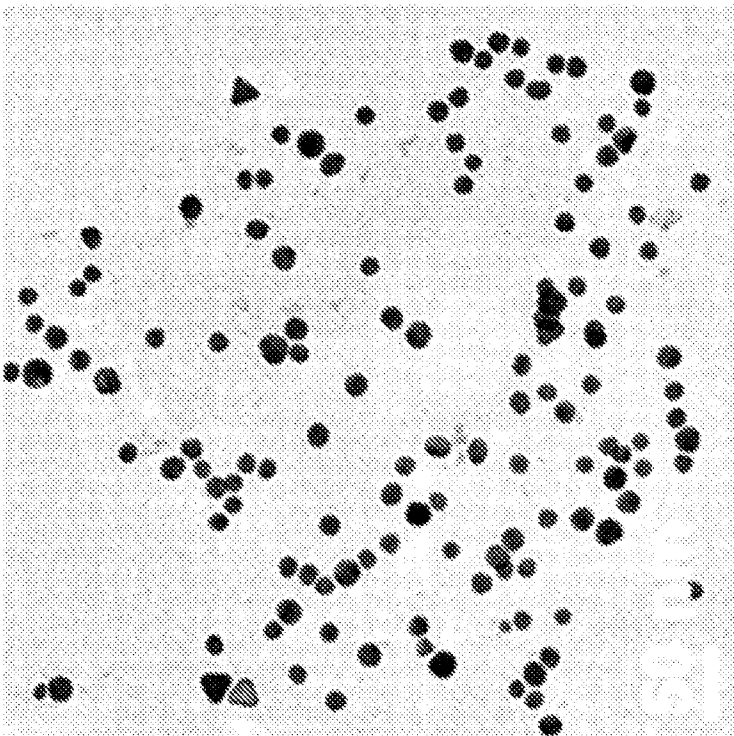


Figure 59

Figure 59E

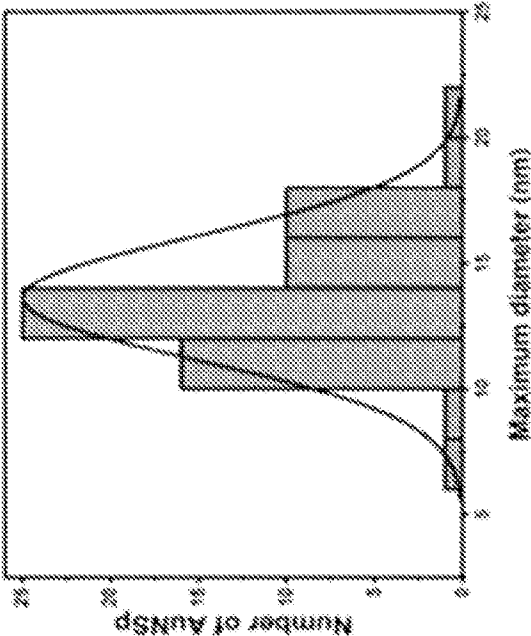


Figure 59D

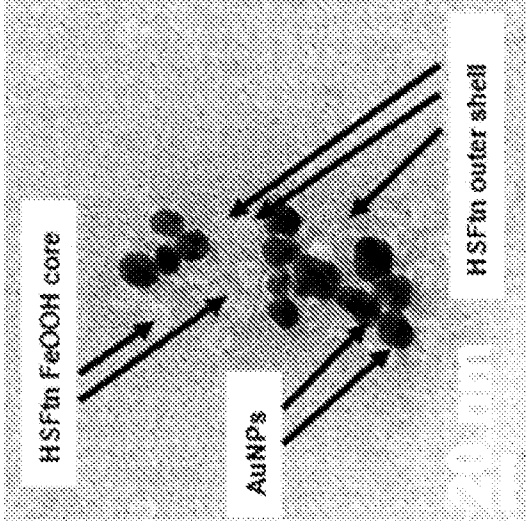


Figure 59C

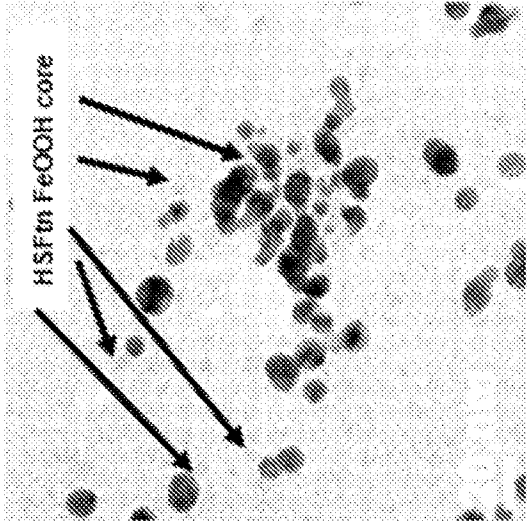


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Figure 60A

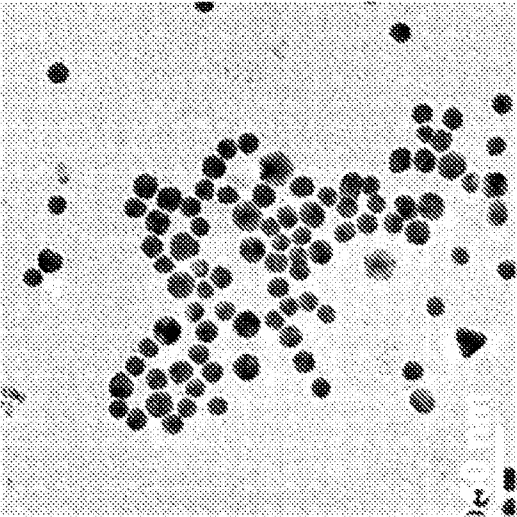


Figure 60B

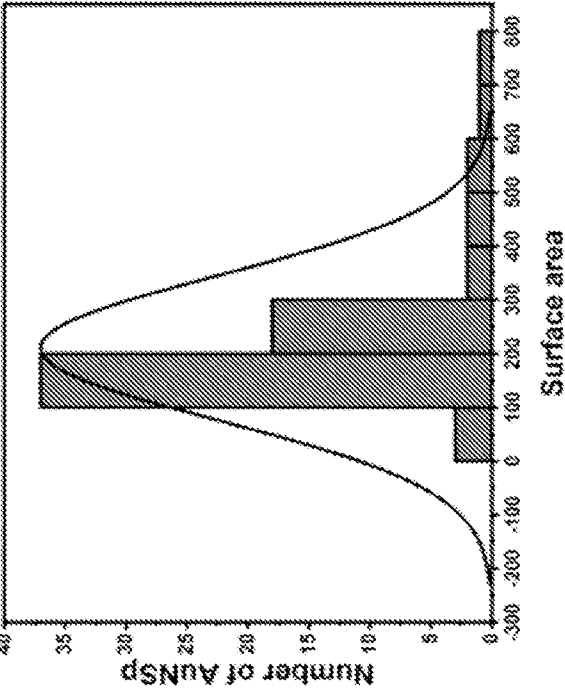


Figure 60C

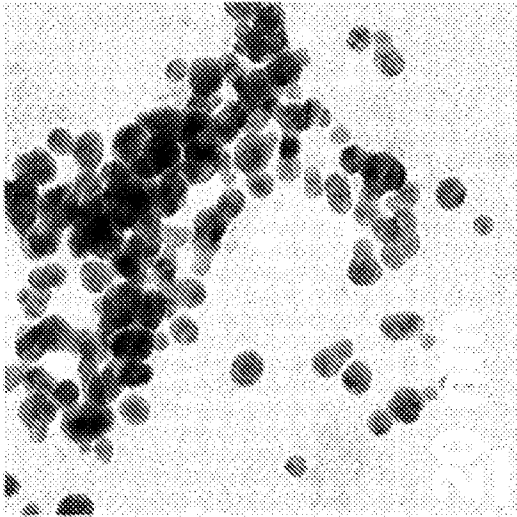


Figure 60D

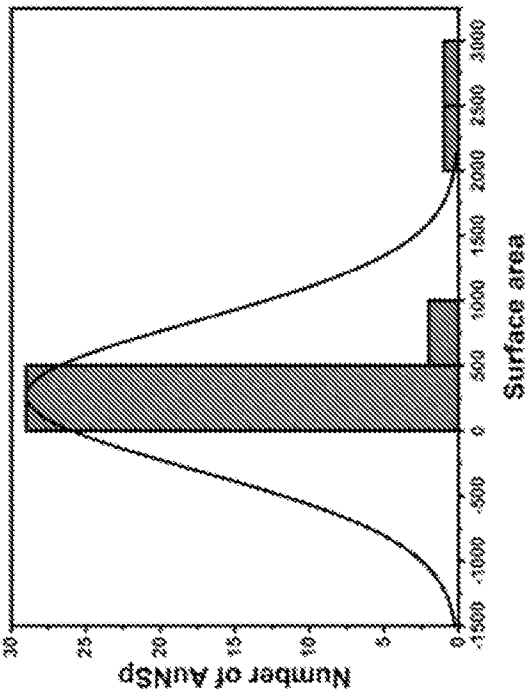


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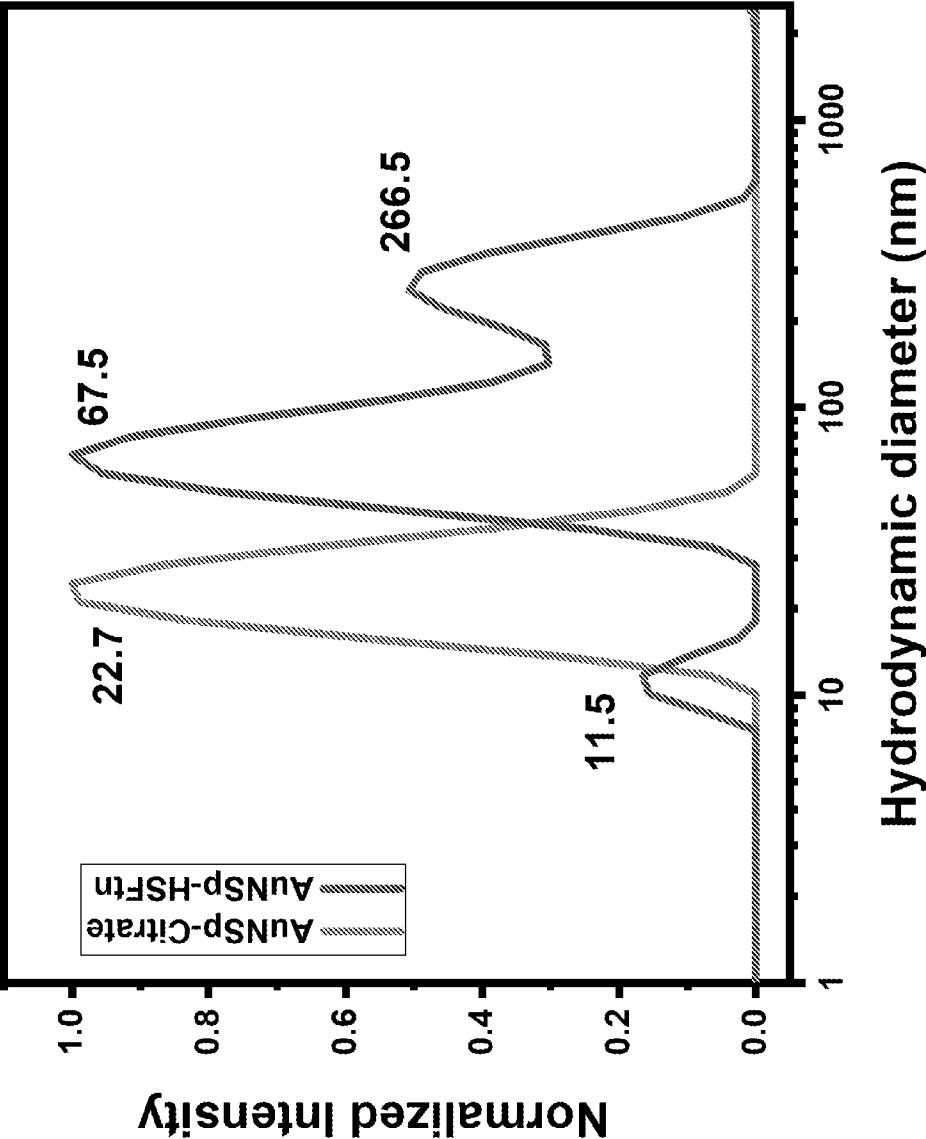


Figure 61

Figure 62B

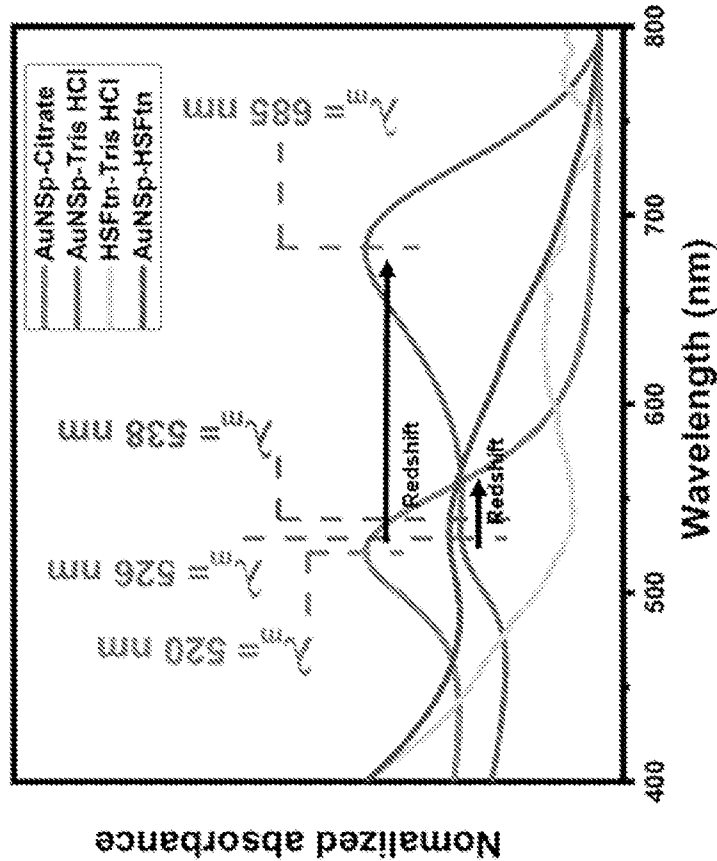


Figure 62A

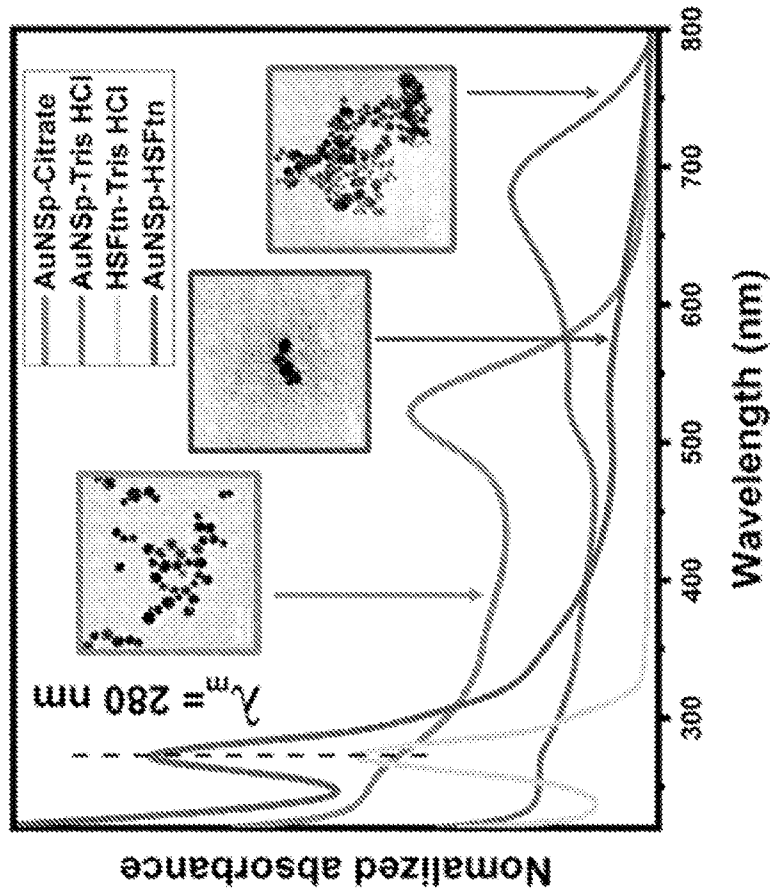


Figure 62

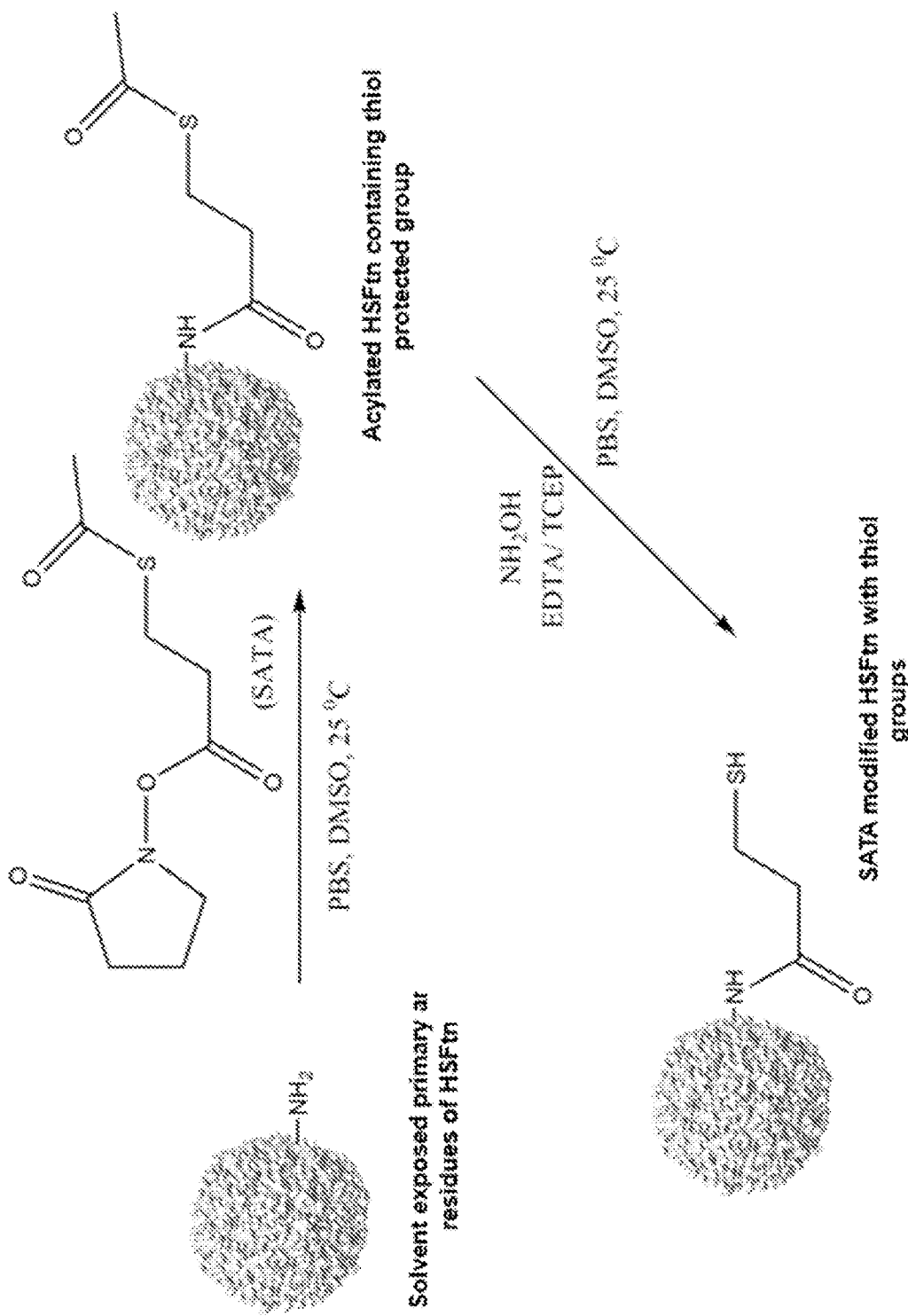


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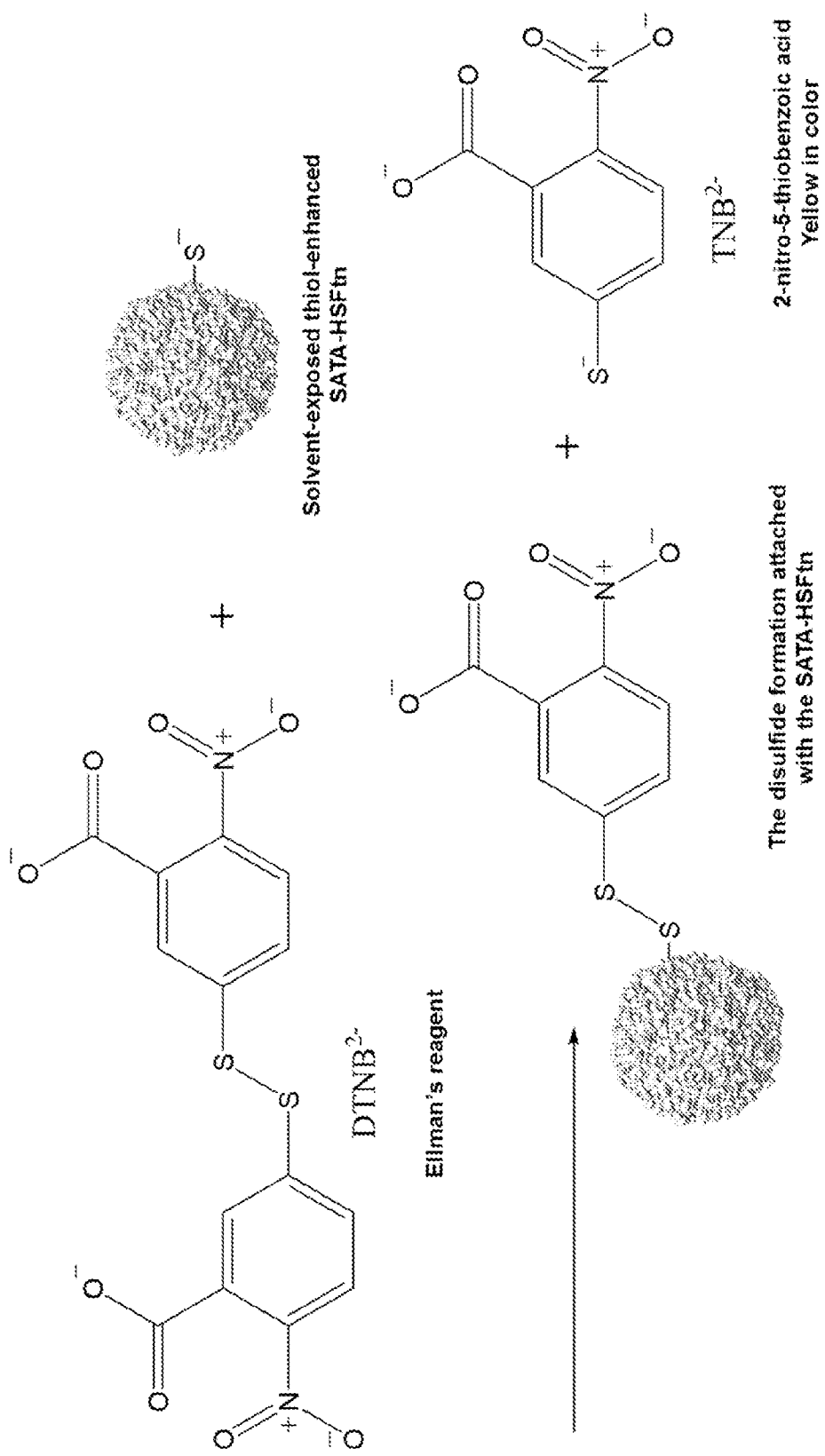


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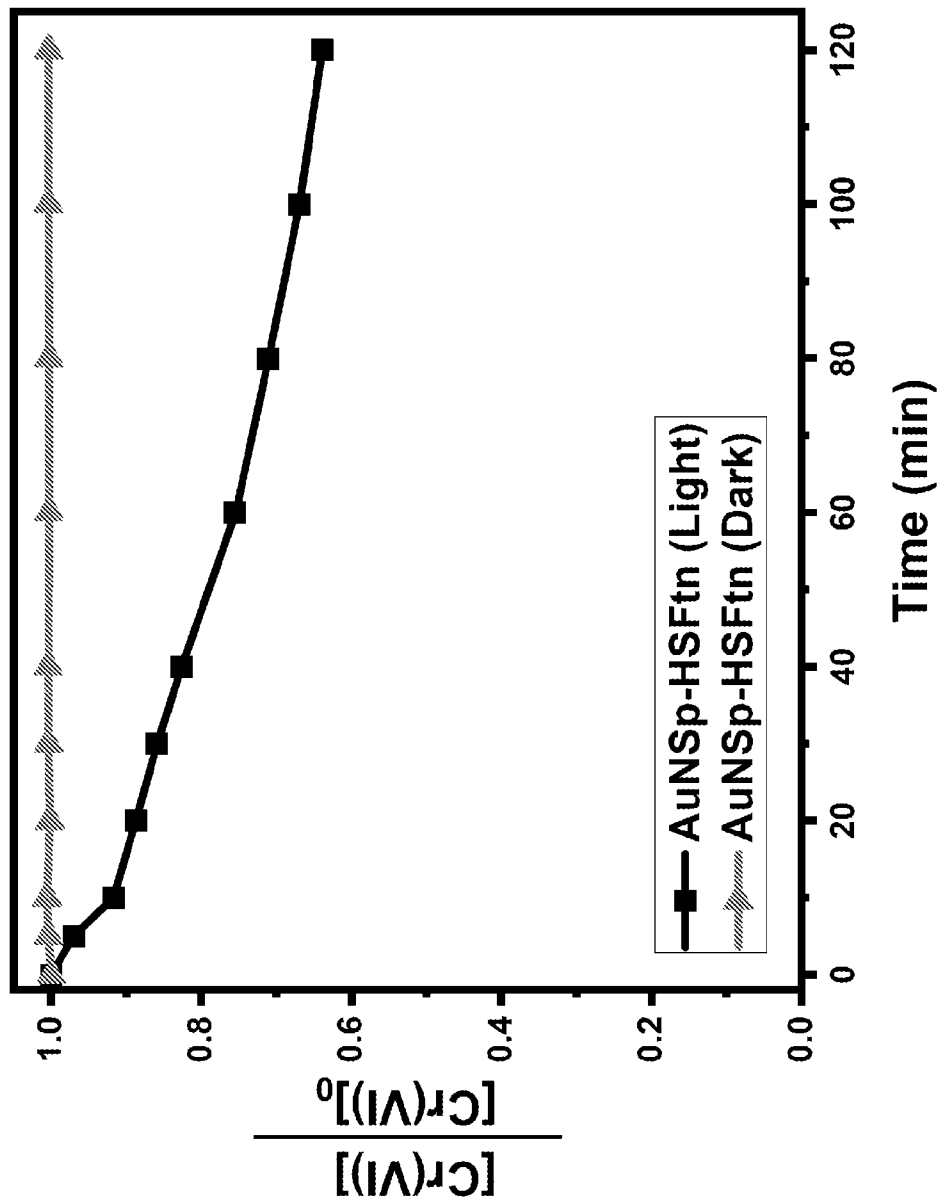


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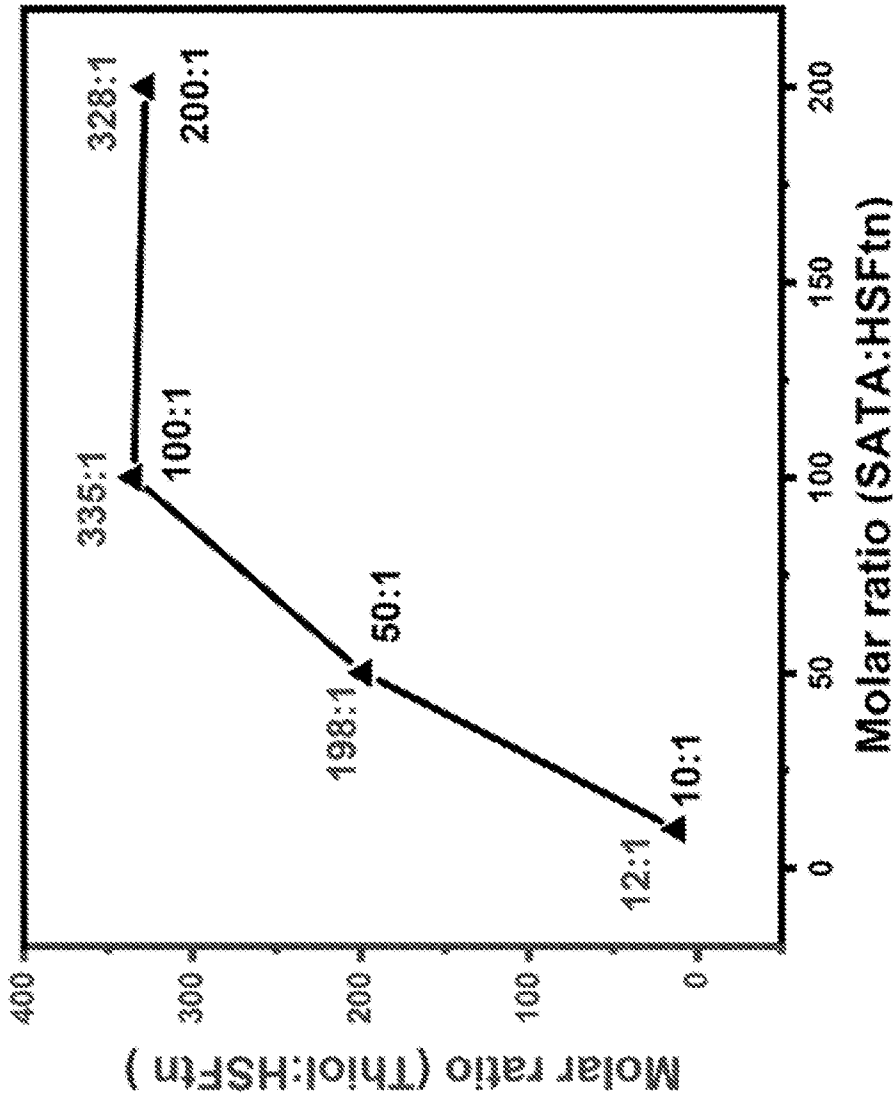


Figure 66

Figure 67A

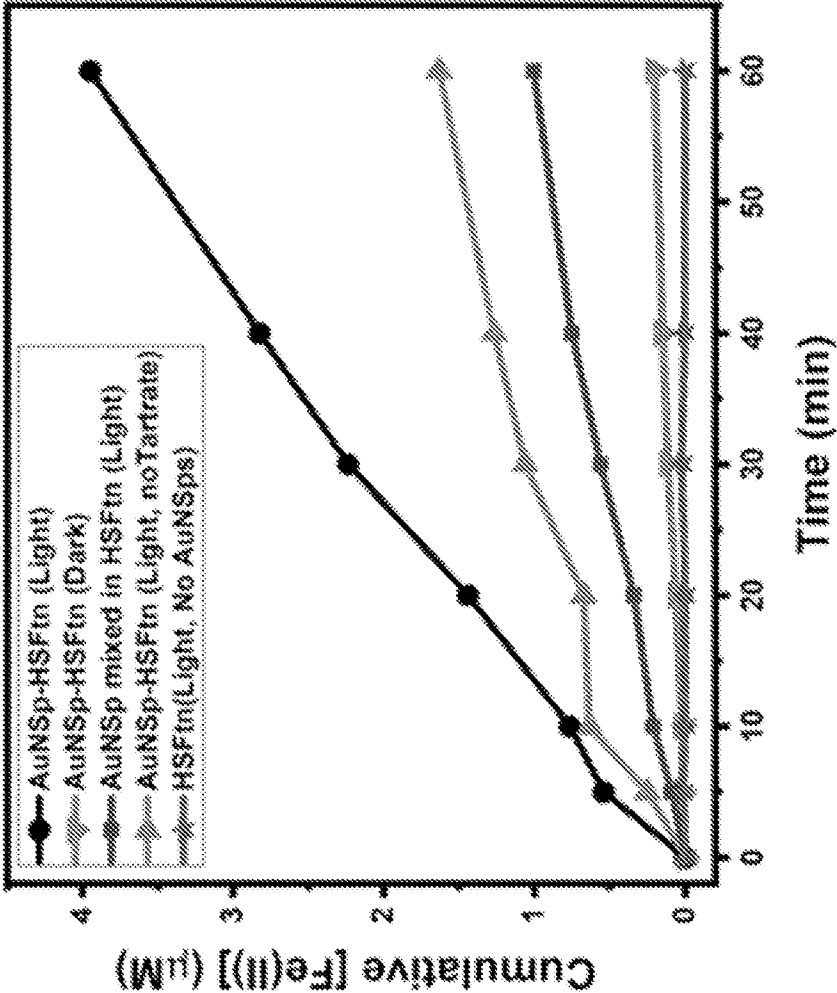


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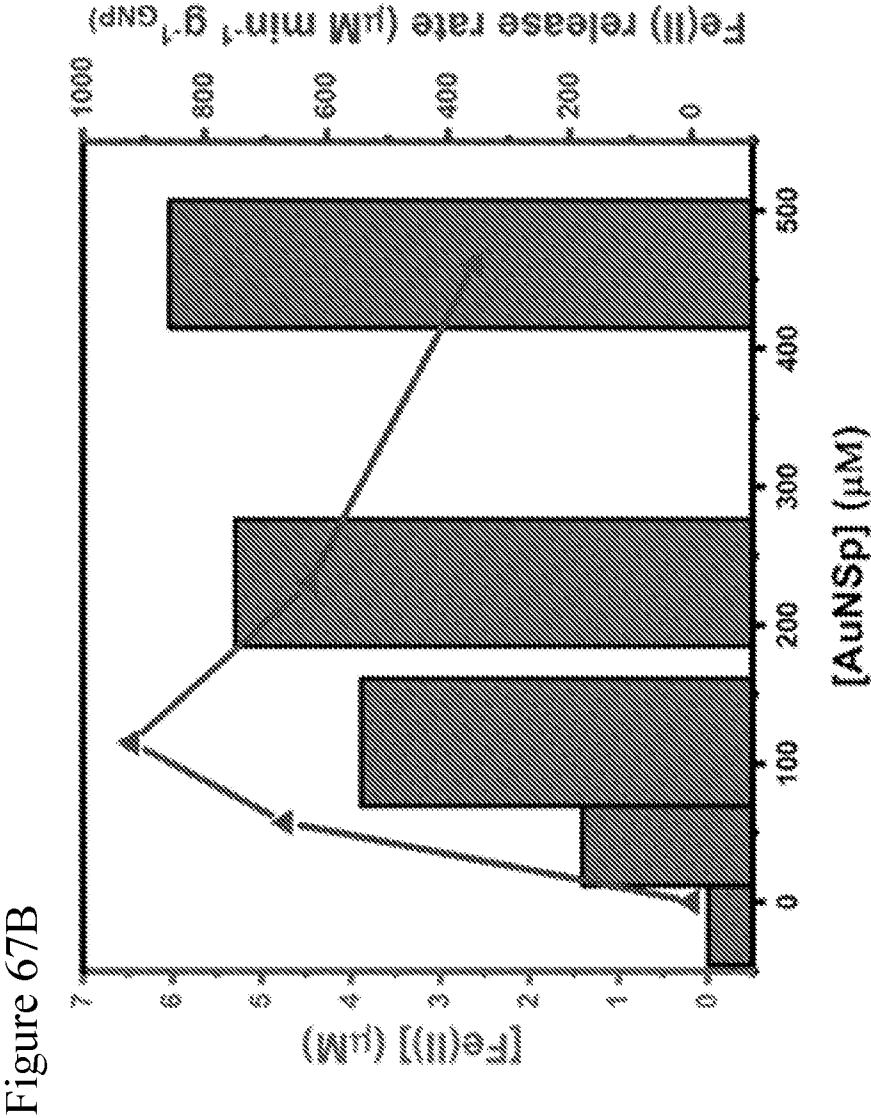


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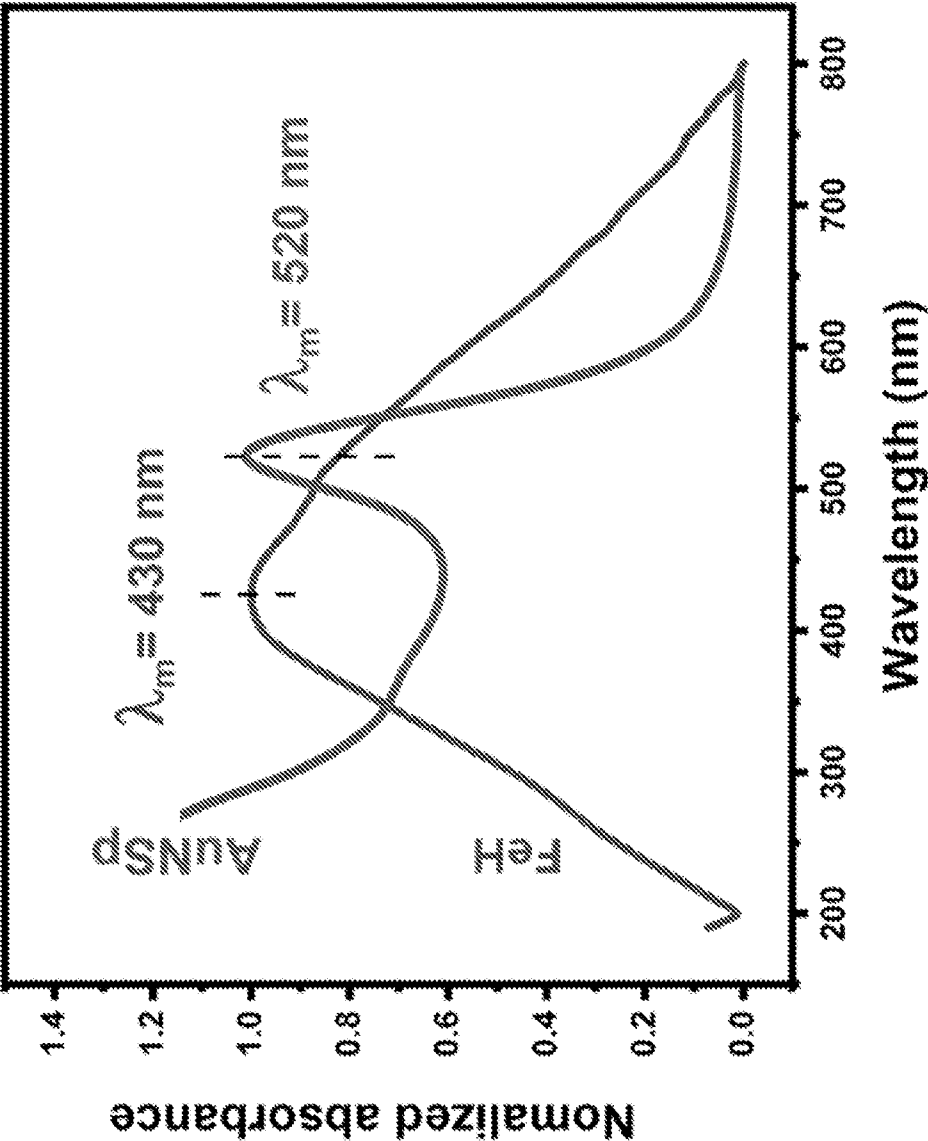


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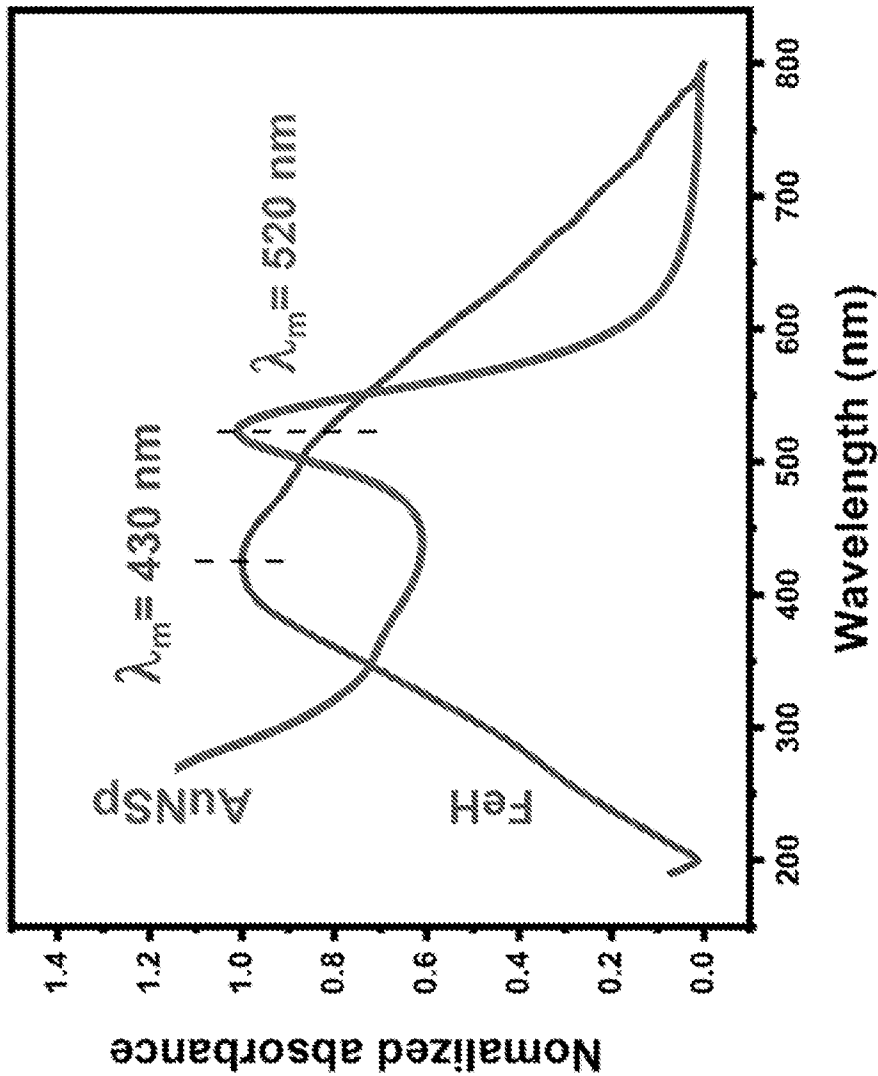


Figure 69

Figure 70B

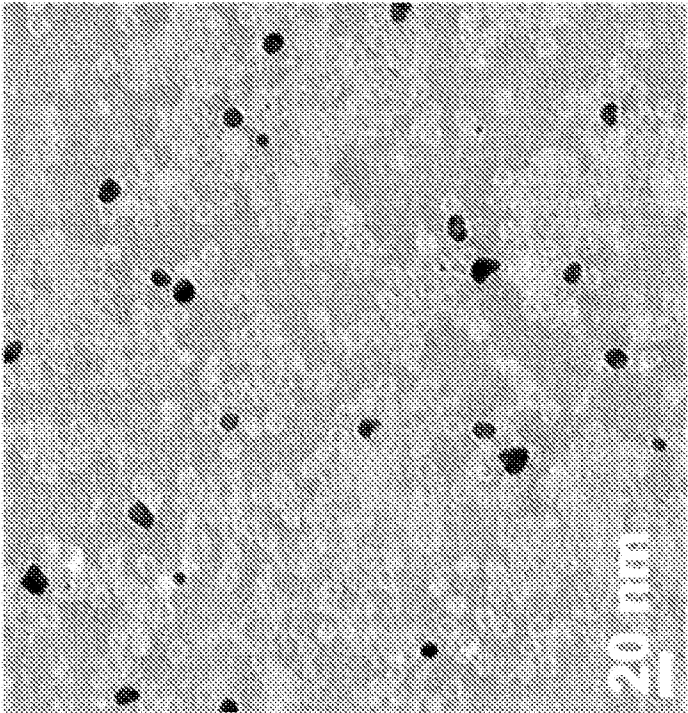


Figure 70A

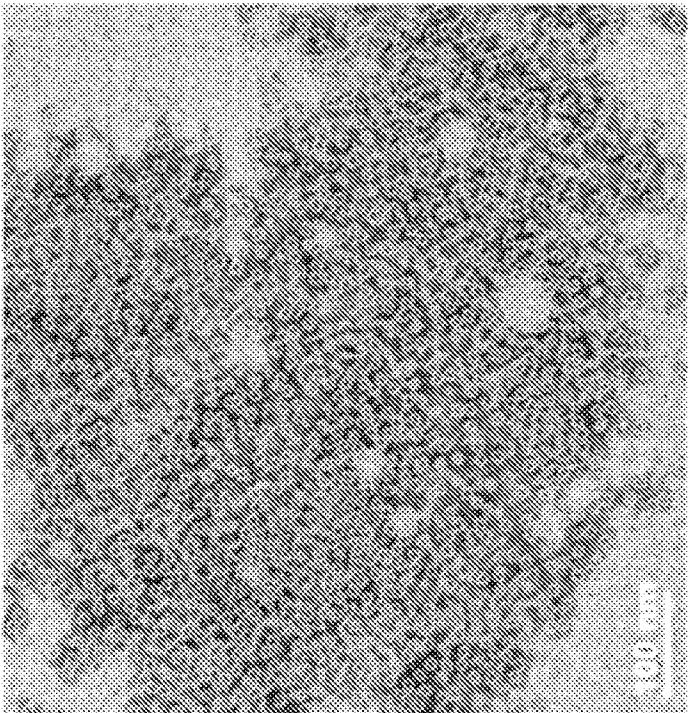


Figure 70

Figure 70D

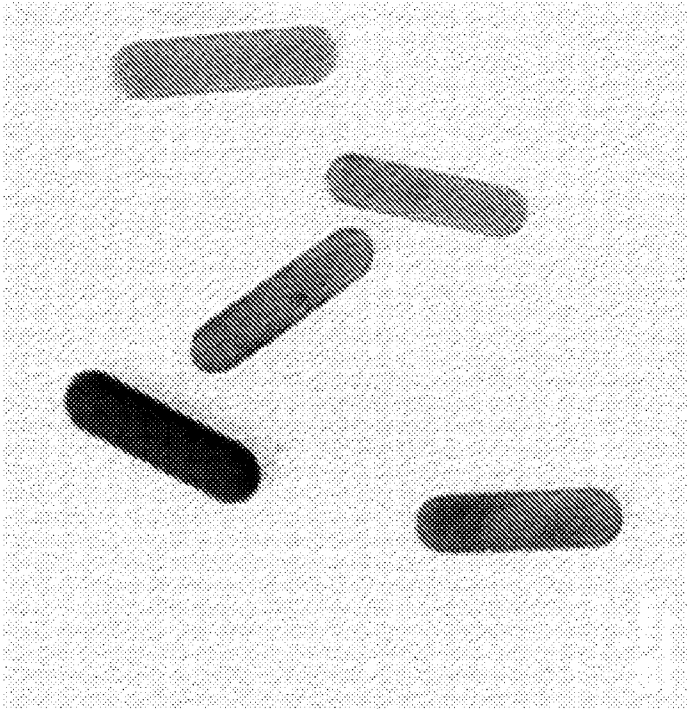


Figure 70C

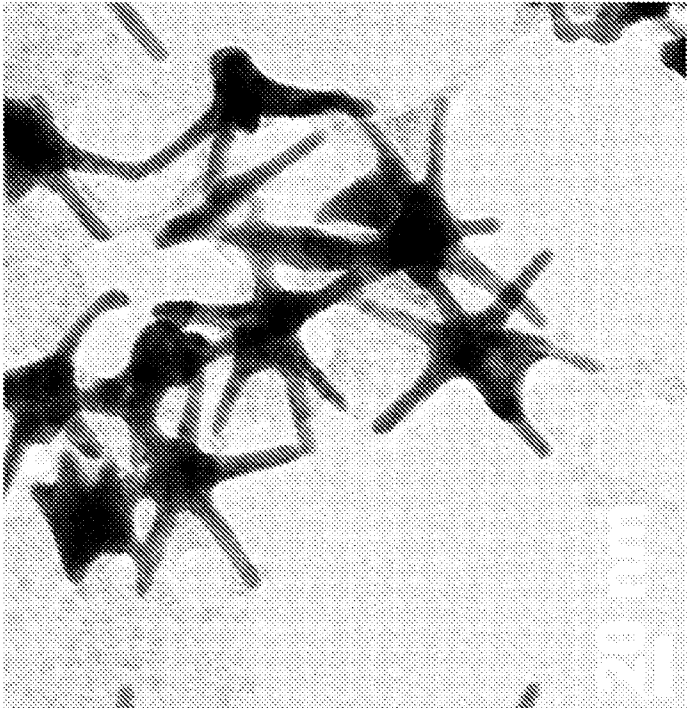


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Figure 70F

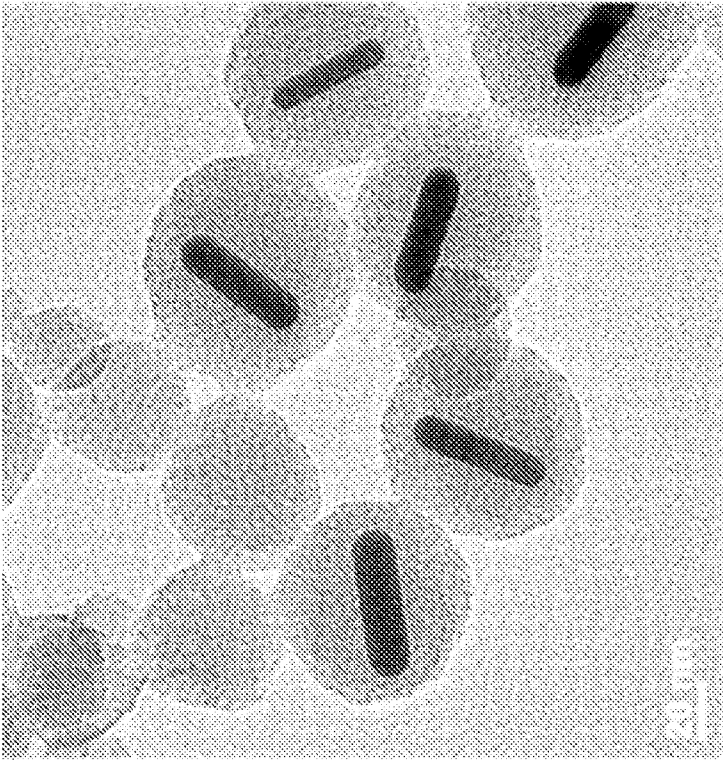


Figure 70E

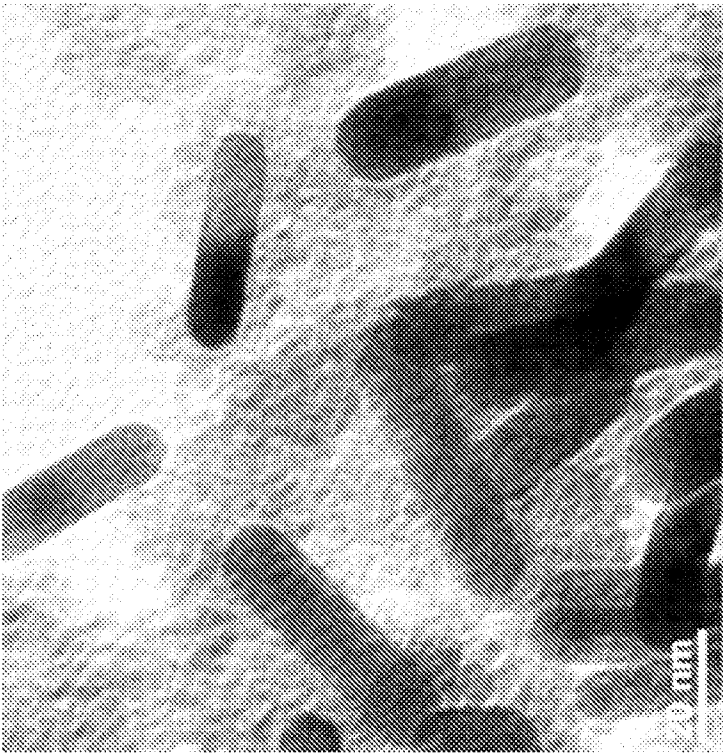


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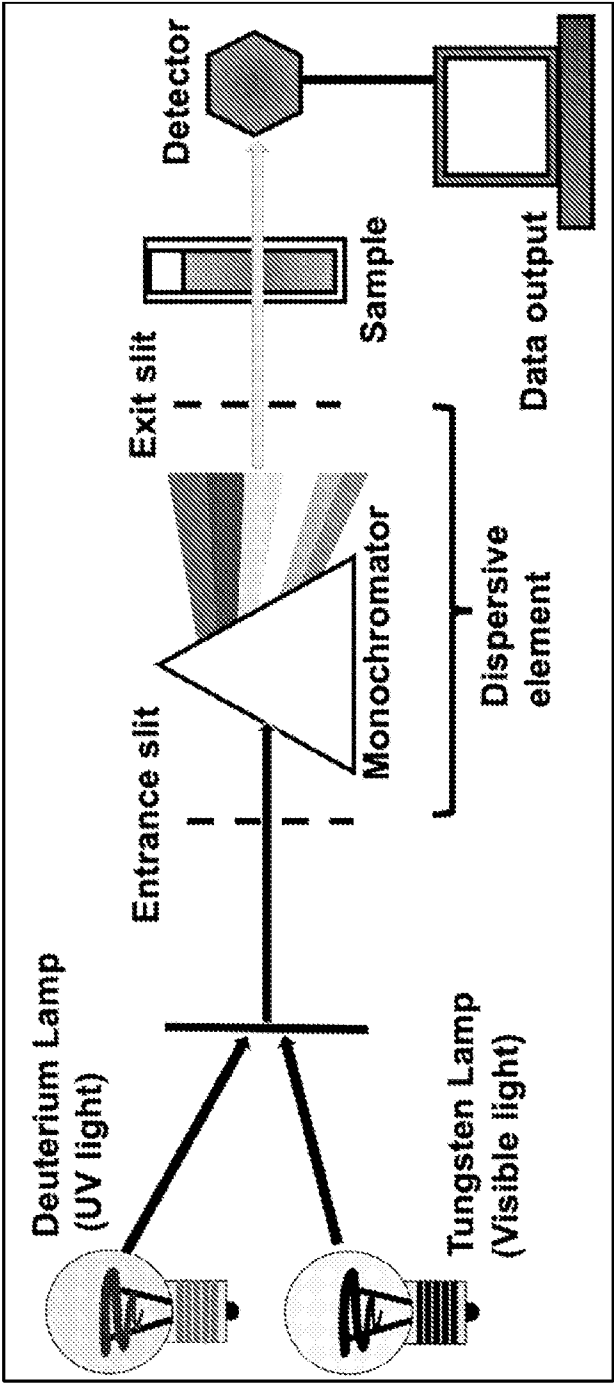


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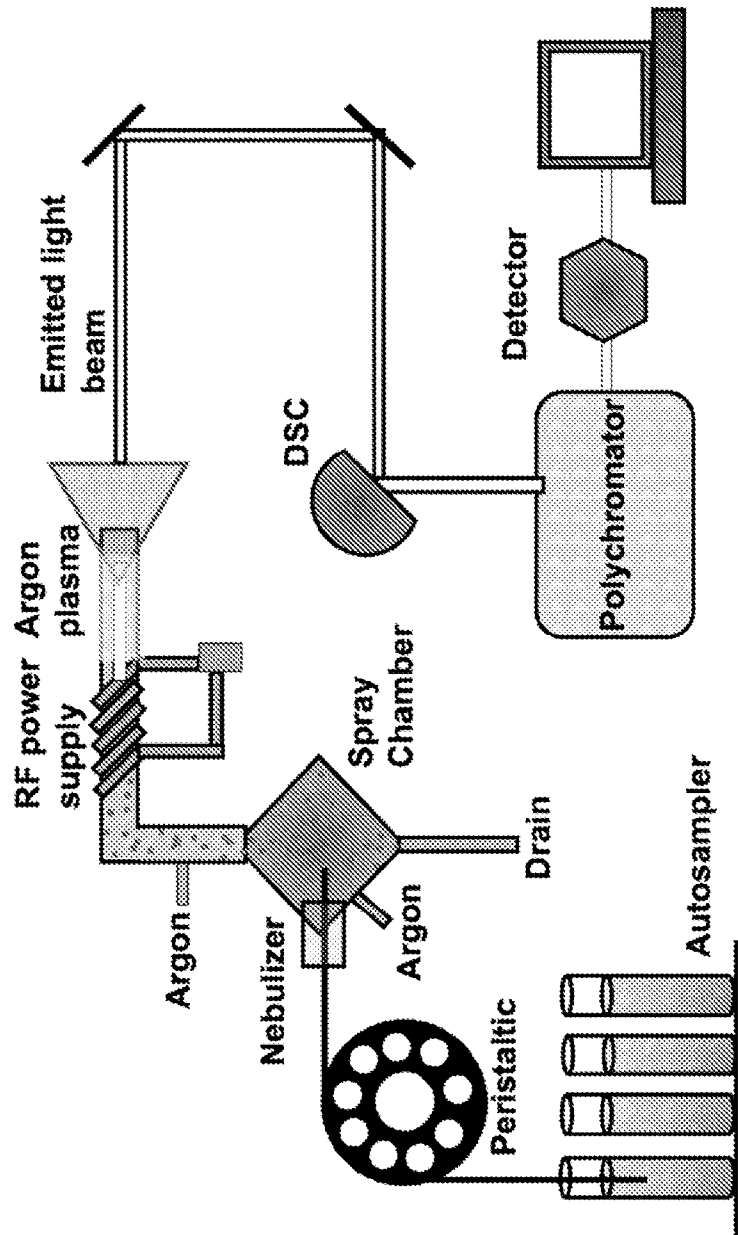


Figure 72

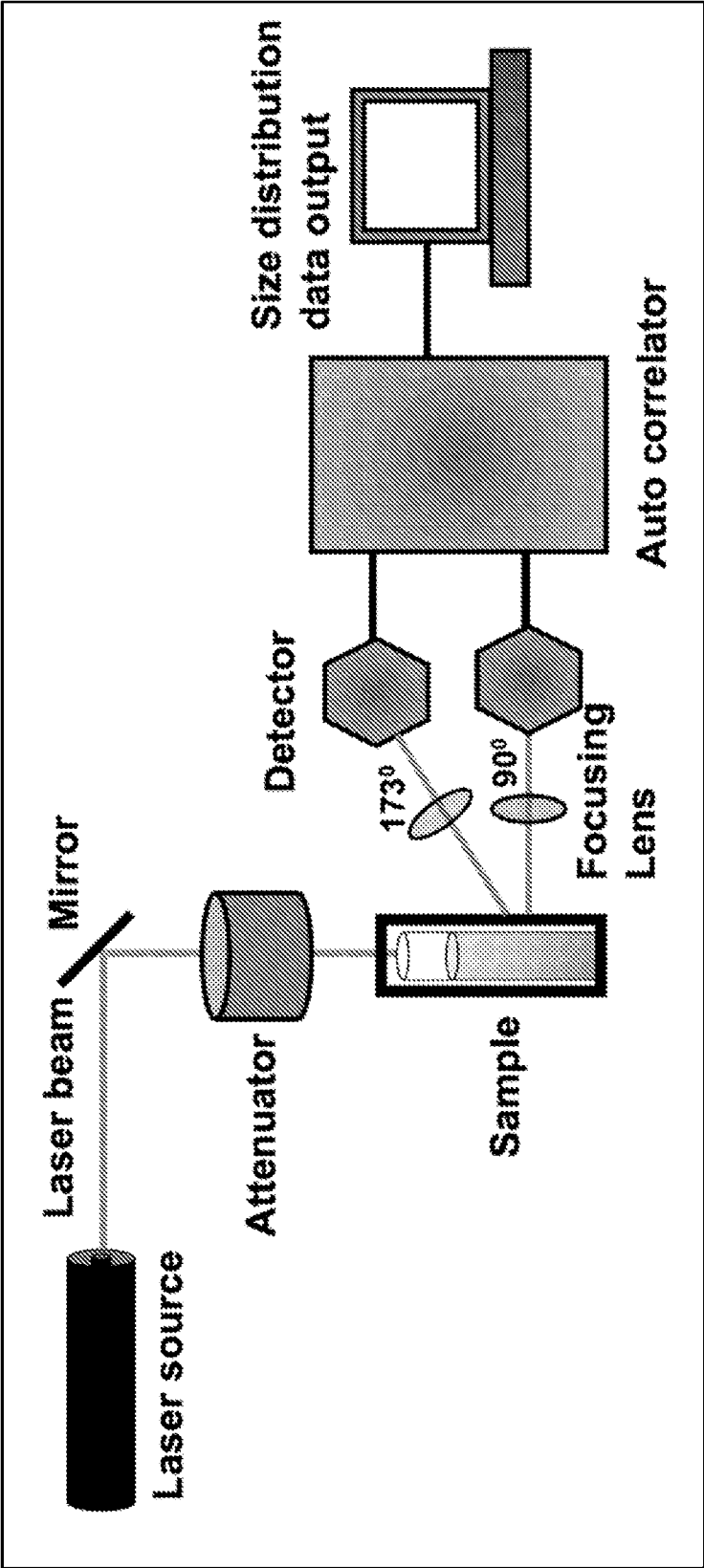


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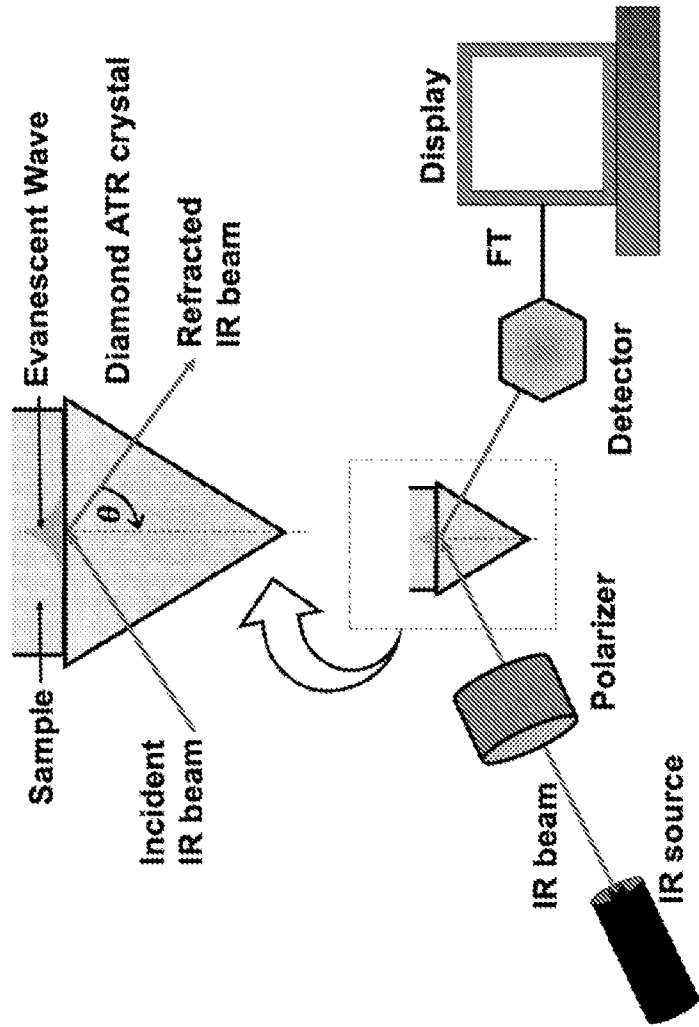


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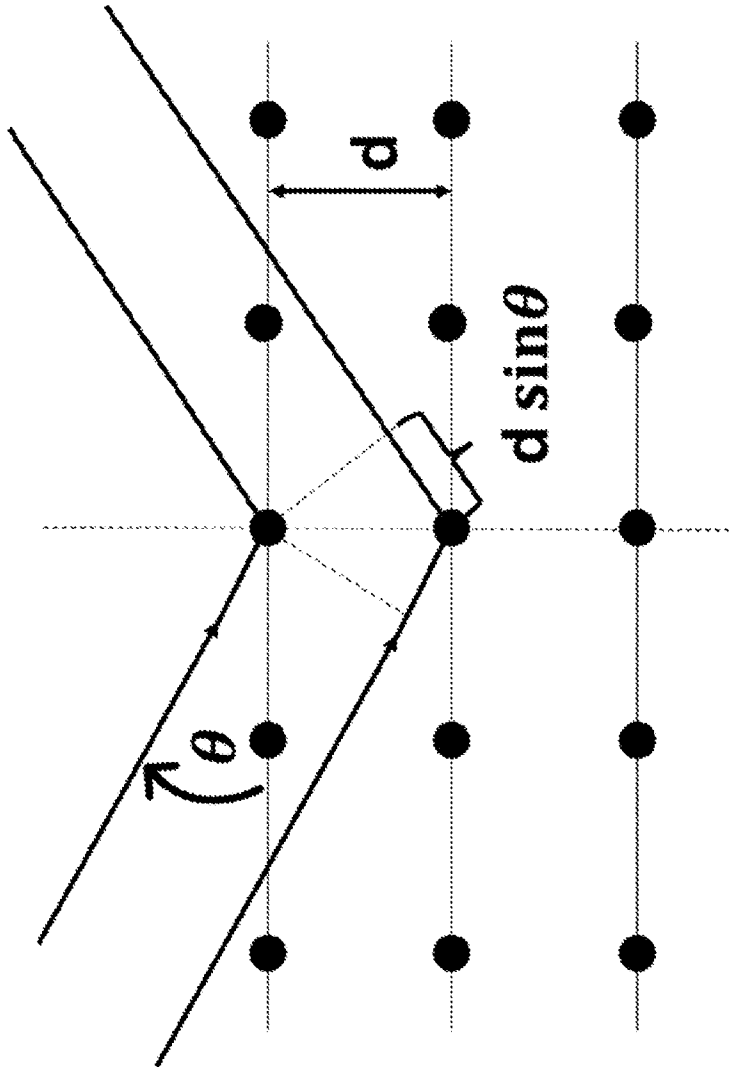


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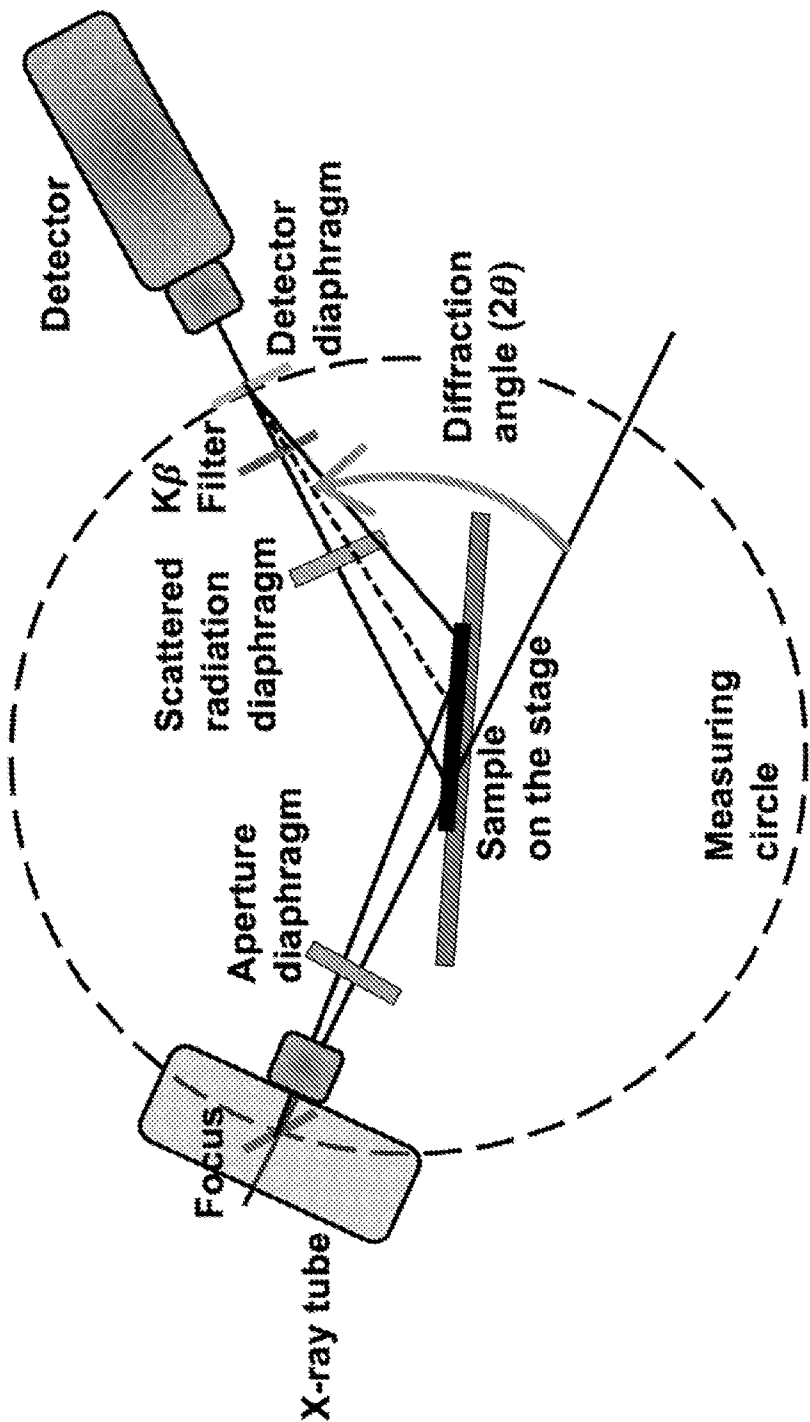


Figure 76

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/036054

A. CLASSIFICATION OF SUBJECT MATTERIPC: **A61K 33/26** (2024.01); **A61K 47/62** (2024.01); **A61K 47/69** (2024.01); **A61N 5/06** (2024.01); **A61P 35/00** (2024.01); **G01N 33/574** (2024.01); **B82Y 30/00** (2024.01); **B82Y 5/00** (2024.01)CPC: **A61K 33/26**; **A61K 47/629**; **G01N 33/574**; **A61P 35/00**; **A61N 5/062**; **A61K 47/62**; **A61K 2121/00**; **B82Y 30/00**; **B82Y 5/00**

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)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2020/0390908 A1 (SONA NANOTEC) 17 December 2020 (17.12.2020) entire document	3, 13
Y	US 2023/0149546 A1 (BIOVENTURES LLC) 18 May 2023 (18.05.2023) entire document	5, 6
Y	US 2016/0123967 A1 (GU et al.) 05 May 2016 (05.05.2016) entire document	14
Y	US 2011/0318415 A1 (LI et al.) 29 December 2011 (29.12.2011) entire document	15

☒ 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

“D” document cited by the applicant in the international application

“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

21 August 2024 (21.08.2024)

Date of mailing of the international search report

23 October 2024 (23.10.2024)

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/036054

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	MOGLIA et al., Enhanced Cellular Uptake of H-Chain Human Ferritin Containing Gold Nanoparticles, <i>Pharmaceutics</i> , Vol. 13, 1966, 19 November 2021, Pgs. 1-15, [retrieved on 16.08.2024]. Retrieved from the internet: <URL: https://doi.org/10.3390/pharmaceutics13111966 >. entire document	1, 2, 4, 9, 10, 16, 18, 19 3, 5-8, 11-15, 17
Y	LUO et al., Targeted Chemoradiotherapy of Prostate Cancer Using Gold Nanoclusters with Protease Activatable Monomethyl Auristatin E, <i>ACS Applied Materials & Interfaces</i> , Vol. 14, Iss. 13, 6 April 2022, Pgs. 14916-14927, [retrieved on 20.08.2024]. Retrieved from internet: <URL: https://pubs.acs.org/doi/10.1021/acsami.1c23780 >. entire document	7, 8
Y	CERKEZ et al., Photochemistry of Ferritin Decorated with Plasmonic Gold Nanoparticles, <i>Environmental Science: Nano</i> , Vol. 9, 27.11.20188, Pgs. 85-93, [retrieved on 20.08.2024]. Retrieved from internet: <URL: https://pubs.rsc.org/en/content/articlelanding/2019/en/c8en01000e >. entire document	11, 12
Y	CRICH et al., Targeting ferritin receptors for the selective delivery of imaging and therapeutic agents to breast cancer cells, <i>Nanoscale</i> , Vol. 7, 6 March 2015, Pgs. 6527-6533, [retrieved on 20.08.2024]. Retrieved from internet: <URL: https://doi.org/10.1039/C5NR00352K >. entire document	17

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

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

Claim 22 is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because claim 22 is indefinite for the following reason:

Claim 22 is not present in the claims.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I: claims 1-19 are drawn to compositions comprising a Ferritin-metal nanoparticle bioconjugate.

Group II: claims 20, 21, and 23-28 are drawn to methods of inhibiting proliferation of at least one cell expressing a TfR1, methods of inducing ferroptosis of at least one cell expressing a TfR1, and methods of making a Ferritin-metal nanoparticle bioconjugate.

The inventions listed in Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The special technical features of Group I, compositions comprising a Ferritin-metal nanoparticle bioconjugate, are not present in Group II; and the special technical features of Group II, methods of inhibiting proliferation of at least one cell expressing a TfR1, methods of inducing ferroptosis of at least one cell expressing a TfR1, and methods of making a Ferritin-metal nanoparticle bioconjugate, are not present in Group I.

Additionally, even if Groups I and II were considered to share the technical features of a composition comprising a Ferritin-metal nanoparticle bioconjugate, wherein the Ferritin-metal nanoparticle bioconjugate comprises at least one metal nanoparticle and at least one Ferritin or a variant or a fragment thereof conjugated to the at least one metal nanoparticle; and wherein the Ferritin-metal nanoparticle bioconjugate specifically targets at least one cell expressing a transferrin receptor 1 (TfR1), these shared technical features do not represent a contribution over the prior art as disclosed by the publication entitled "Enhanced Cellular Uptake of H-Chain Human Ferritin Containing Gold Nanoparticles" to Moglia et al. (hereinafter, "Moglia").

Moglia teaches a composition (Gold nanoparticles (AuNP) capped with biocompatible layers... as theranostic agents in biomedicine, Abstract) comprising a Ferritin-metal nanoparticle bioconjugate wherein the Ferritin-metal nanoparticle bioconjugate comprises at least one metal nanoparticle and at least one Ferritin or a variant or a fragment thereof conjugated to the at least one metal nanoparticle (characterized each FT-AuNP homopolymer and analyzed their cytotoxic effect and ability to be taken up by different cell lines, Pg. 2, second full paragraph from bottom; ferritin surface potentials, including contributions from solvent and tightly bound metal. After AuNP synthesis, the Z-potential in both homopolymers decreased to -10.2 and -7.6 mV for FTH-AuNP and FTL-AuNP, respectively. The net charge decrease is caused by the interaction between the protein with Au ions, and the lowered electrostatic repulsion between ferritin monomers can trigger the formation of ferritin oligomers, Pg. 7, first full paragraph from top); and wherein the Ferritin-metal nanoparticle bioconjugate specifically targets at least one cell expressing a transferrin receptor 1 (TfR1) (Another relevant feature of ferritin for nanoparticle targeting is that it can be taken up by different cell types and tissues through a receptor-mediated endocytosis process. The primary receptor for ferritin is transferrin receptor-1 (TfR1), expressed in many cell lines, Pg. 2, second full paragraph from top; results are relevant for developing new imaging or drug-delivery systems based on gold-containing ferritin for brain targeting and cancer treatments where cells overexpress transferrin receptors, Pg. 11, first partial paragraph from top).

The inventions listed in Groups I and II therefore lack unity under Rule 13 because they do not share a same or corresponding special technical feature.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/036054

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: **1-19**

Remark on Protest

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