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Supporting Information

A Simple Approach to Design Proteins for the Sustainable Synthesis of Metal Nanoclusters

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Experimental Procedures

Chemicals

All chemicals were purchased from Sigma Aldrich and used without further purification. Milli-Q water was used in all experiments.

Protein Design, Cloning and Molecular Biology

Consensus tetratricopeptide repeat (CTPR) proteins were created and characterized as previously described.[1] The proteins were composed of three repeats, W or H, with an extra sequence at the N-terminal end due to the cloning strategy (GAMGS), and an additional helix at the C-terminal end for improved solubility (AEAKQNLGNAKQKQG). H motif (AEAW*H*NLG*H*AYYKQGDYDEAIEYY QKALELDPRS) was created from the previously described W motif (AEAWY NLGNAYYKQGDYDEAIEYYQKALELDPRS) by QuickChange Site-Directed mutagenesis. By combinations of the modules H and W three CTPR proteins were generated CTPR3-WWW, CTPR3-HWW, and CTPR3-WHW. CTPR3-WWW composed of three identical W CTPR modules. CTPR3-HWW with a module H placed at the first repeat, and CTPR3-WHW with a module H placed at the middle repeat. For this purpose, DNA encoding the W and/or H modules were fused using a modular cloning strategy based on BamHI and BglII digestion.[2] The genes encoding the three CTPR3 proteins (WWW, HWW, and WHW) were cloned into the pProEx-HTA vector and their identities verified by DNA sequencing (Stab Vida).

Protein Expression and Purification

Synthetic genes for the desired protein in pPROEx-HTA vector, coding for N-terminal hexahistidine tag and ampicillin resistance, were expressed in *Escherichia coli* C41 (DE3) after induction with 1 mM isopropyl β-d-thiogalactoside (IPTG) at an optical density of 0.6-0.8 followed by 5 h growth at 30°C. The cell pellets were resuspended in lysis buffer (300 mM sodium chloride, 50 mM Tris pH 8.0) and lysed by sonication. The proteins were purified from the supernatant using standard Ni-NTA affinity purification protocol. The N-terminal hexahistidine tag was then cleaved from the CTPR proteins using Tobacco Etch Virus (TEV) protease. As a final step, the aqueous solutions of CTPRs were dialyzed against 10 mM phosphate buffer three times at 4°C using a dialysis membrane with molecular weight cutoff of 6-8 kDa. Protein concentration was measured by UV absorbance at 280 nm, using extinction coefficients at 280 nm calculated from amino acid composition.

Synthesis of protein-stabilized metal nanoclusters

The protein-stabilized metal NCs were synthesized using a modified protocol based on a previously reported procedure[3], using sodium ascorbate instead of ascorbic acid as reducing agent. Briefly, 1000

µL of protein at 10 µM were mixed with $HAuCl_4$, $AgNO_3$, or $CuSO_4$ (5 µL 10 mM, 5 eq. respect to protein) for at least 30 minutes to allow the binding of the metal ions to the protein's coordination sites. Then, the reduction of the metal ions to metal NCs was achieved by adding 5 µL of sodium ascorbate at 100 mM (10 eq. respect to metal ions). The reaction was incubated at 37°C for 24 h. The samples were concentrated to 500µL using Amicon ultrafiltration tubes with a 10-kDa membrane. Finally, the unreacted salts were removed via gel filtration using a Sephadex G-25M column and the protein stabilized metal NCs were purified by fast protein liquid chromatography (FPLC). The purified samples were stored at 4°C. The absorption and fluorescence spectra of protein stabilized metal NCs were collected using a Varioskan microplate reader (Thermo Scientific).

Then, the influence of the protein:metal ratio; metal:reducing-agent ratio; and reaction time was evaluated in the synthesis and stabilization of metal NCs using the CTPR3 protein WHW as template. Three different metal: protein ratios (5:1, 20:1, and 50:1); two different metal: reducing agent ratios (1:10 and 1:100); and three different reaction times (24, 48, and 72 h) were tested.

Steady-state optical spectroscopy

UV-visible absorption spectra were measured in a Jasco V-630 Bio spectrophotometer. Fluorescence spectra were recorded in a LS-55 PerkinElmer spectrofluorometer with a slit of 1 nm band-pass. All the absorption and emission measurements were performed using a standard quartz cuvette of 1 cm path length. The fluorescence quantum yield (Φ_x) was calculated using anthracene in ethanol as a reference (Φ_{ref} = 0.27, λ_{exc} = 370 nm and λ_{em} = 423 nm) and the following formula:

$$\phi_x = \phi_{ref} \ \frac{Grad_x}{Grad_{ref}} \left(\frac{\eta_x^2}{\eta_{ref}^2}\right)$$

where $Grad_x$ and $Grad_{ref}$ are the gradient from the plot of integrated fluorescence intensity versus absorbance at excitation wavelength, for the sample and the reference, respectively, and η_x and η_{ref} are the refractive indexes of the solvents, PBS (150 mM NaCl, 50 mM phosphate pH 7.4), and ethanol, respectively.

Time–resolved fluorescence spectroscopy

Fluorescence lifetime measurements were carried out exciting with a 405 nm PDL 828 Picoquant Sepia laser, with a 50 ps pulse duration. The fluorescence was dispersed by a spectrometer (SP2500, Acton Research), and detected with a Picoquant PMA Hybrid-Photomultiplier Assembly with a transit time spread of less than 50 ps and a Picoquant Hydraharp time-correlated single photon counting (TCSPC) electronics. The resulting time resolution after de-convolution of the instrumental response function was better than 50 ps. Measurements were carried out in solutions placed in 2 mm-optical path quartz cuvettes.

Matrix assisted laser desorption ionization (MALDI) mass spectrometry

Mass spectra were acquired on an Applied Biosystems Voyager Elite MALDI-TOF mass spectrometer with delayed extraction (Applied Biosystems, Framingham, MA, USA) equipped with a pulsed N_2 laser (λ= 337 nm). Sinapic acid was used as matrix. An extraction voltage of 20 kV was used. All mass spectra were acquired in positive reflection mode using delayed extraction with an average of 50−100 laser shots. MALDI-TOF sample preparation included 1 µL of the sample mixed with 3 µL of sinapic acid in 50:50 water/acetonitrile with 0.01% TFA. Then, 1 µL of the mixture was deposited onto the MALDI plate and allowed to air-dry. The instrument was externally calibrated using monoisotopic peaks from the sinapic acid matrix (MH^+ at m/z 225.071).

Liquid chromatography coupled to a time-of-flight mass spectrometry detector with electrospray ionization source (LC-ESI-TOF)

Liquid chromatography was performed on an UPLC Acquity (Waters) equipped with a Acquity C4, 150x2.1mm,1.7µm column (Waters) eluting in a gradient using as mobile phase H_2O-TFA (0.1%)/ACN-TFA (0.1%) and 10 µL of injection volume at a column temperature of 70ºC. Mass spectra were acquired on a LCT Premier XE (Waters) equipped with an electrospray positive mode (W mode m/z range 100-2000), using a capilar volt = 2000 and a cone volt = 50. Molecular weight was calculated from multiply charged positive ions by deconvolution using maximum entrophy algorithm (MaxEnt).

Inductively coupled plasma mass spectrometry (ICP-MS)

100 µL of each FPLC purified metal NCs at approximately at 100 µM concentration were mixed with 300 µL of 37% HCl and the resultant suspension was sonicated for 30 minutes at 40ºC. Finally, 2700 mL of bi-distilled water was added. The Cu, Au and Ag concentration was determined by measuring the sample using an iCAP-Q ICPMS (Thermo Scientific, Bremen, Germany) equipped with an autosampler ASX-520 (Cetac Technologies Inc., NE, USA) (n = 3) and QtegraTM v2.6 (Themo Scientific).

SDS-PAGE Gel Electrophoresis

The samples were run on a 10-20% acrylamide gradient gel at 150V and the gel was visualized under a UV transilluminator and after Coomassie blue staining.

Circular Dichroism (CD)

The protein secondary structure was examined by CD using a Jasco J-815 spectrometer (JASCO Corporation, Tokyo, Japan). CD spectra were acquired at 10 µM protein concentration in a 1 mm path length cell at 25ºC using a 1 nm bandwidth with 1 nm increments and 10 s average time.

X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy measurements were performed with a SPECS SAGE HR 100 spectrometer equipped with a 100 mm mean radius PHOIBOS analyzer and a nonmonochromatic X-ray source (Mg Kα line of 1253.6 eV energy and 250 W), placed perpendicular to the analyzer axis and calibrated using the 3d5/2 line of Ag, with a full width at half maximum of 1.1 eV. All measurements were made in an ultrahigh vacuum chamber at a pressure of around 8×10^{-8} mbar. An electron flood gun was used to neutralize for charging. Measurements were conducted directly on dry deposited films, which were previously washed with absolute ethanol and cut into samples of 1 cm × 1 cm. The analysis of spectra was done with CasaXPS 2.3.15dev87 software.

Transmission Electron Microscopy (TEM)

Ultrathin carbon films on holey carbon support film, 400 mesh copper grids (TED PELLA INC.) were exposed to glow-discharge treatment before sample deposition. TEM samples were prepared by depositing 5 µL of the sample solution on the grid. After 3 min, the excess solution was removed from the grid using filter paper. To remove the deposited salt, the grid was then washed with a drop of water, and the excess water was dried using filter paper. Micrographs were recorded using a JEOL JEM 2100F electron microscope operating at 200 kV with a Field Emission Gun equipped with an INCA x-sight energy dispersive X-ray radiation (EDX) detector (Oxford Instruments) to analyze the elemental composition of the material. HAADF STEM images were acquired on a JEOL JEM-2100F UHR microscope at 200 kV in scanning mode, with a probe size of 1.5 nm and a choice of the camera length that ensures an inner detector angle of 75 mrad (HAADF) to enhance the contrasts of the metal atoms on the carbon support film.

Nanocluster storage and working stability

The storage stability of the protein-stabilized metal NCs in physiological conditions was evaluated during one month at 4ºC by fluorescent spectrophotometry using a Varioskan microplate reader (Thermo Scientific). The stability under physiological conditions (PBS and human plasma (HP)) was examined during one week at 37ºC by fluorescent spectrophotometry using a Varioskan microplate reader (Thermo Scientific).

Temperature stability of Prot-NCs

To evaluate the thermal stability of the Prot-NCs, the fluorescence spectra of 500 µL of WHW-metal NCs suspension at 20 µM of protein concentration were measured at different temperatures ranging from 20ºC to 70ºC. The reversibility and the cycle stability of the metal NCs were tested repeating the process for 5 cycles.

pH stability of Prot-NCs

To evaluate the pH stability of the Prot-NCs, the fluorescence spectra of a WHW-metal NCs suspension at 20 μM of protein concentration were measured at different pH ranging from 5 to 12.

Ionic strength stability

To evaluate the stability of the Prot-NCs under an ionic strength range, the fluorescence spectra of a WHW-metal NCs suspension at 20 μM of protein concentration were measured at different NaCl concentration ranging from 0.15 to 1.0 M.

Stability in the presence of thiols

To evaluate the stability of the Prot-NCs in presence of biothiols, the fluorescence spectra of a WHW-metal NCs suspension at 20 μM of protein concentration were measured at different DL-cysteine (Cys) concentration from 0.005 to 30.0 mM.

Stability in the presence of different metal ions

To evaluate the chemical stability of the Prot-NCs towards different metal ions, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, Mg^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} at 50, 100 and 150 μM were incubated with the metal NCs. Briefly, 500μL of the protein-stabilized metal NCs at 20 μM were mixed with 5 μL of the different metal ion solutions at 5, 10 and 15 mM. After 30 min of reaction, 200 μL of the reactant solution was transferred into a quartz cuvette for fluorescence spectra recording at room temperature.

Fluorescence photobleaching experiments

The photostability of the protein stabilized metal NCs was evaluated measuring the fluorescence intensities of 20 μM solutions of protein stabilized metal NCs, Green Fluorescent Protein (GFP) and 4',6-diamidino-2-phenylindole (DAPI) in PBS. These samples in aqueous solutions were deposited onto quartz slides. The fluorescent intensities of the different solutions were recorded at different times under continuous mercury arc lamp illumination using a Leica DMI-6000 fluorescence microscope. The fluorescence intensities were quantified using Image J software.

Protein and Prot-NCs labeling with fluorescein (FITC)

500μL of WHW protein or Prot-metal NCs at 20μM were mixed with 50μL of FITC at 1 mM. After 2 hours at 37ºC, the unreacted FITC were removed via gel filtration using a Sephadex G-25M column.

Cell culture and NCs incubation

MDA-MB breast cancer cell line was purchased from American Type Culture Collections (Manassas, VA, USA). This cell line was grown as monolayer in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum (FBS) at a final concentration of 10%, 2 mM L-glutamine, 0.25 μg mL^{-1} fungizone and 100 Units of penicillin and 100μg mL^{-1} streptomycin. All the media, serum, L-

glutamine, fungizone, and antibiotics were purchased from GIBCO. Cell lines were maintained at 37°C in a humidified atmosphere consisting of 75% air and 5% CO_2 in an incubator. 500 µL of metal NCs dispersed in PBS were diluted in medium containing 10% FBS at the desired concentration. The resulting sample was filtered through a 0.22 µm Millex-GP filter (Merck-Millipore Darmstadt, Germany). Cells were incubated with metal NCs for 4-24h. Then, cell media with metal NCs was removed and cells were washed with phosphate buffered saline (PBS) for complete removal of metal NCs from cell medium. Then fresh cell media was added to continue further viability and internalization studies.

***In vitro* cytotoxicity assays**

Resazurin dye (Sigma-Aldrich) was used as indicator of cell viability in the proliferation and cytotoxicity assays. To assess cell viability, MDA-MB-231 cells were cultured on a 24-well plate at a density of $2.5x10^4$ cells per well in 500 µl of complete medium. After 24 h, the growth medium was removed and cells were then incubated 24 h at 37°C in the presence of different concentrations of protein stabilized metal NCs (1, 2.5, 5, 10 and 20 µM). After incubation, cells were washed three times with PBS, then DMEM supplemented with 10% FBS was added to cell culture, and incubated at 37°C and 5% CO_2. After 5 days, the medium was replaced with DMEM supplemented with 10% FBS, and 10% of resazurin dye (1mg ml^{-1} PBS). Cells were maintained in the incubator at 37°C and 5% CO_2 for 3 hours and then, a Varioskan microplate reader (Thermo Scientific) was used to determine the amount of resazurin reduced by measuring the absorbance of the reaction mixture (λ_{exc}=570 nm, λ_{em}=600 nm). 500 µl of 10% of resazurin dye was added to empty wells as a negative control. The viability of the cells was expressed as the percentage of absorption of treated cells in comparison with control cells (without metal NCs). All experiments were carried out in triplicate.

Confocal fluorescence microscopy imaging of the nanoclusters in cells

MDA-MB-231 cells were cultured on a µ-Slide 8-well plate at a density of $1x10^4$ cells per well in 250 µl of complete medium. After 24 h, the growth medium was removed and cells were then incubated 16 h at 37°C in the presence of the protein-stabilized metal NCs (5 µM). Then, the cells were incubated 30 min with NucRed™ Live 647 (ThermoFisher) for nuclear staining, washed three times with PBS to remove free unbound protein stabilized metal NCs and finally 250 µL of Opti-MEM medium was added to each well. Cellular uptake of the nanoclusters was detected using a high-content imaging fluorescent microscope (LSM 510 Meta, Carl Zeiss Jena, German). Excitation wavelengths of 405 nm to image the nanoclusters and of 633nm to image the NucRed were used for all the confocal fluorescent microscopy experiments.

MDA-MB-231 cells with fluorescein-labeled WHW-CuNCs, and fluorescein-labeled WHW protein as control at 5 µM. Cellular internalization of the Prot-NCs was detected using a Confocal Fluorescence Microscope (Zeiss LSM 880). Z-stack images of MDA-MB-231 cells incubated with WHW-CuNCs were acquired. Excitation wavelengths of 405 nm to image the Prot-NCs, 488 nm to image the fluorescein

labeled protein, and of 633nm to image the NucRed were used for all the confocal fluorescent microscopy experiments. The confocal pinhole was set to 1 AU to optimize z-sectioning. Images and z-stacks were recorded sequentially. A step size of 300 nm was chosen.

Results and Discussion

Influence of the protein:metal ratio, metal:reducing agent ratio, and the reaction time in the synthesis and stabilization of metal NCs

To investigate the influence of ratio protein: metal, ratio metal: reducing agent and reaction time in the synthesis and stabilization of metal NCs using the CTPR3 protein WHW as template a similar protocol has been developed. Three different ratios metal: protein (5:1, 20:1 and 50:1), two different ratios metal: reducing agent (1:10 and 1:100) and three different reaction times (24, 48 and 72 h) were tested (Figure S1-S3). Briefly, 1000 µL of protein at 10 µM were mixed with $HAuCl_4$, $AgNO_3$ or $CuSO_4$ (5, 20 or 50 eq. respect to protein) for at least 30 minutes to allow the adsorption of metal ions to the protein's stabilizing sites. Then, the reduction of the metal ions to metallic NCs was achieved by sodium ascorbate (10 or 100 eq. respect to metal ions). The reaction was incubated at 37°C for 24, 48 and 72 h. The samples were concentrated to 500µL using Amicon ultrafiltration tubes with a 10-kDa membrane. Finally the protein stabilized metal NCs were purified via FPLC and stored at 4°C.

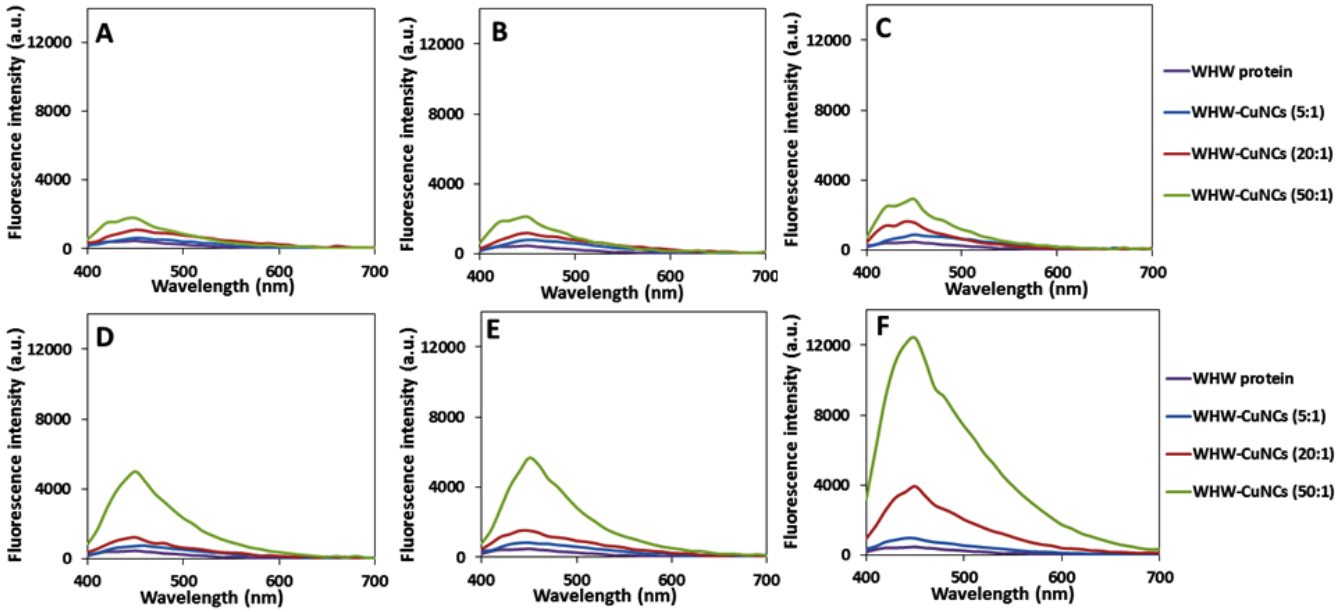


Figure S1. Fluorescence spectrum of WHW-CuNCs synthetized using different reaction conditions. Metal:protein ratio (5:1, blue; 20:1, red and 50:1, green). Reducing-agent: metal ratio (10:1, A, B and C; 100:1, D, E and F). Reaction time (A and D, 24 h; B and E, 48 h; C and F, 72 h).

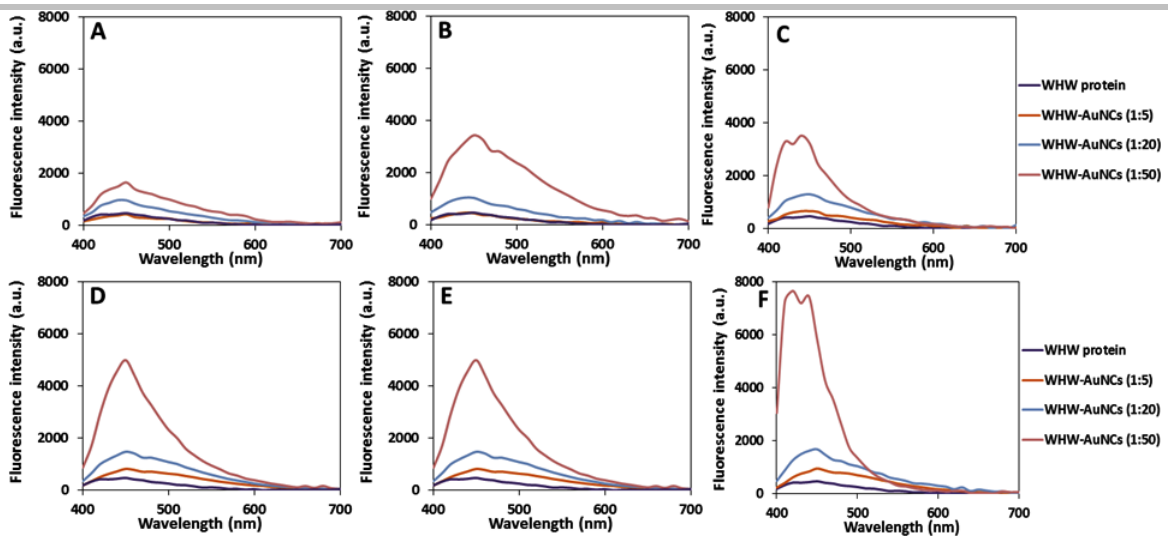


Figure S2. Fluorescence spectrum of WHW-AuNCs synthetized using different reaction conditions. Metal:protein ratio (5:1, orange; 20:1, blue and 50:1, pink). Reducing-agent:metal ratio (10:1, A, B and C; 100:1, D, E and F). Reaction time (A and D, 24 h; B and E, 48 h; C and F, 72 h).

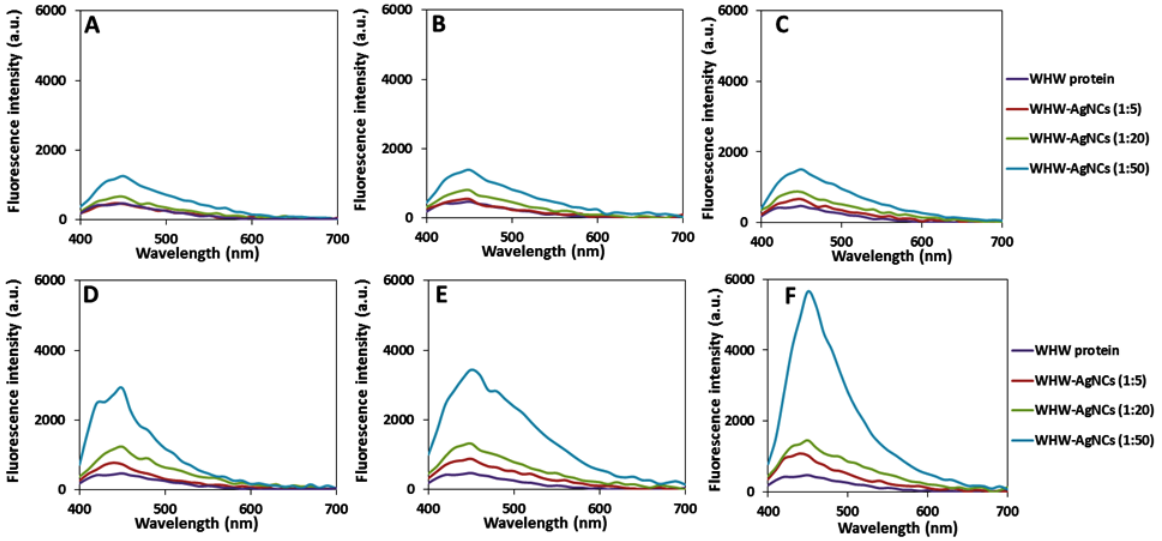


Figure S3. Fluorescence spectra of WHW-AgNCs synthetized using different reaction conditions. Metal:protein ratio (5:1, red; 20:1, green and 50:1, blue). Reducing-agent:metal ratio (10:1, A, B and C; 100:1, D, E and F). Reaction time (A and D, 24 h; B and E, 48 h; C and F, 72 h).

Control experiments for the synthesis and stabilization of metal NCs

In order to ensure that the fluorescence emitters were WHW-metal NCs, the cluster-forming reaction was carried out removing the protein, the metal salt, or the reducing agent. It is clear that the presence of the WHW protein is essential for the stabilization of metal NCs and also that the sodium ascorbate is needed for the reduction of metal salts given that fluorescence emission were observed only when all the reagents were present (Figure S4).

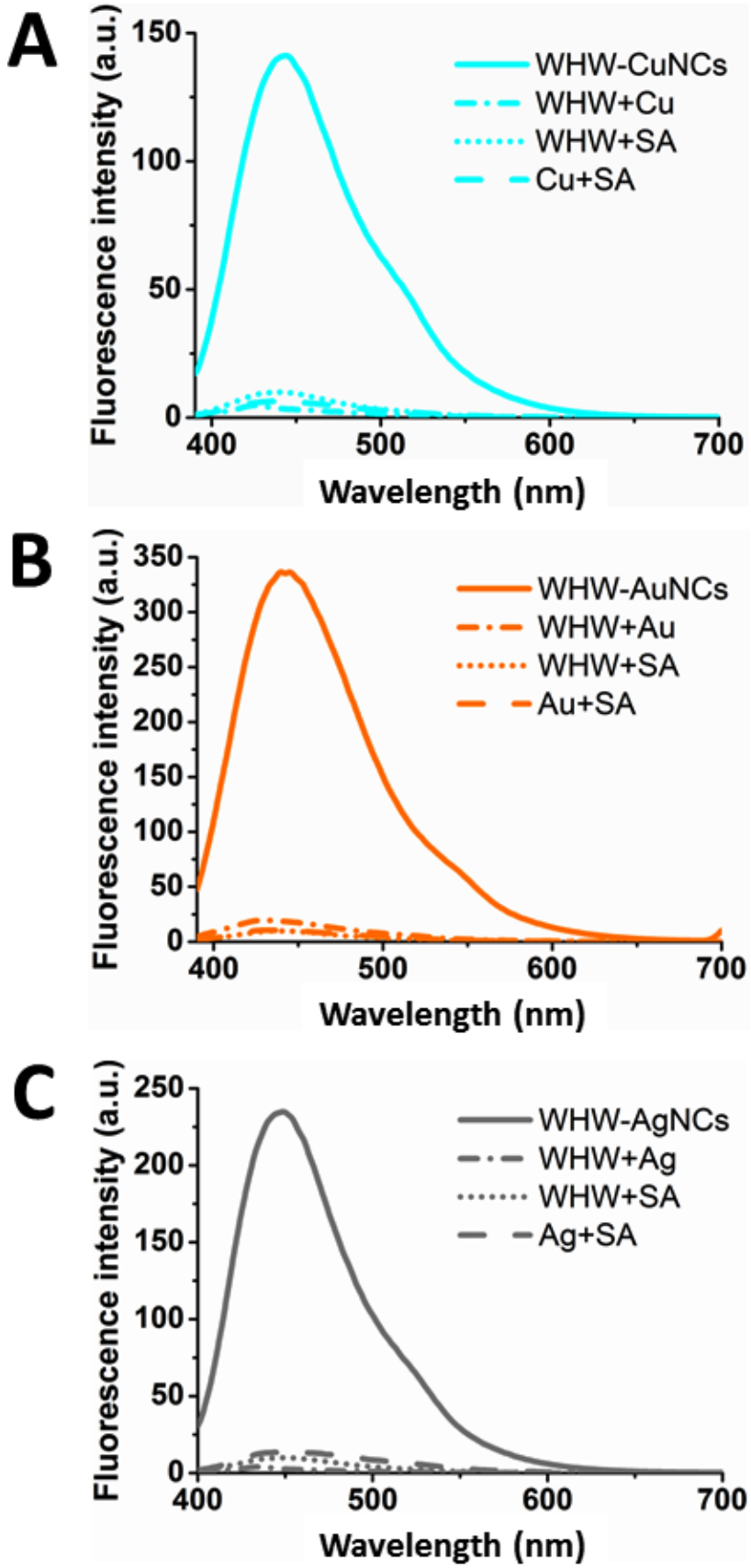
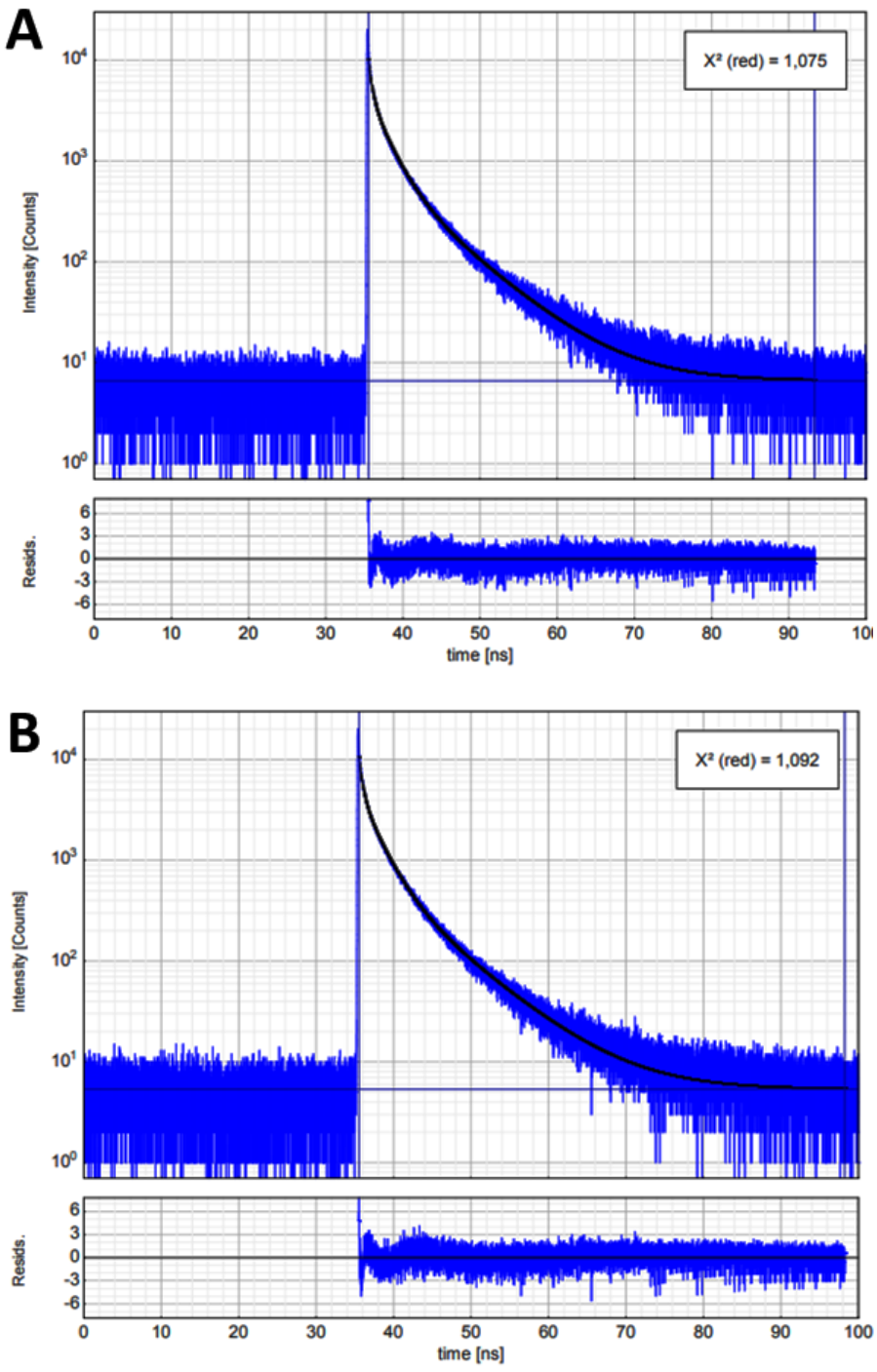


Figure S4. Fluorescence emission spectra of the Cu (A), Au (B) and Ag (C) NC-forming reactions (solid line) and the reactions lacking sodium ascorbate (WHW + metal) (short dashed-dotted line), metal salt (WHW + SA) (dotted line), and the protein (metal + SA)(dashed line).

Time-resolved Fluorescence Measurements

Analysis of PL decay data was carried out with Fluofit (Picoquant). The emission intensities (I) were best fit to a tri-exponential decay over time (t): $I(t) = A1 \exp(t/\tau1) + A2 \exp(t/\tau2) + A3 \exp(t/\tau3)$ in which A1, A2, A3, τ1, τ2 and τ3 are respectively the amplitudes and lifetimes of the three emission components. Reduced chi-square values ($\chi2$) of 1.075, 1.092, and 1.028 were obtained for WHW-CuNCs, WHW-AuNCs, and WHW-AgNCs, respectively. The results are shown in Figure S5.



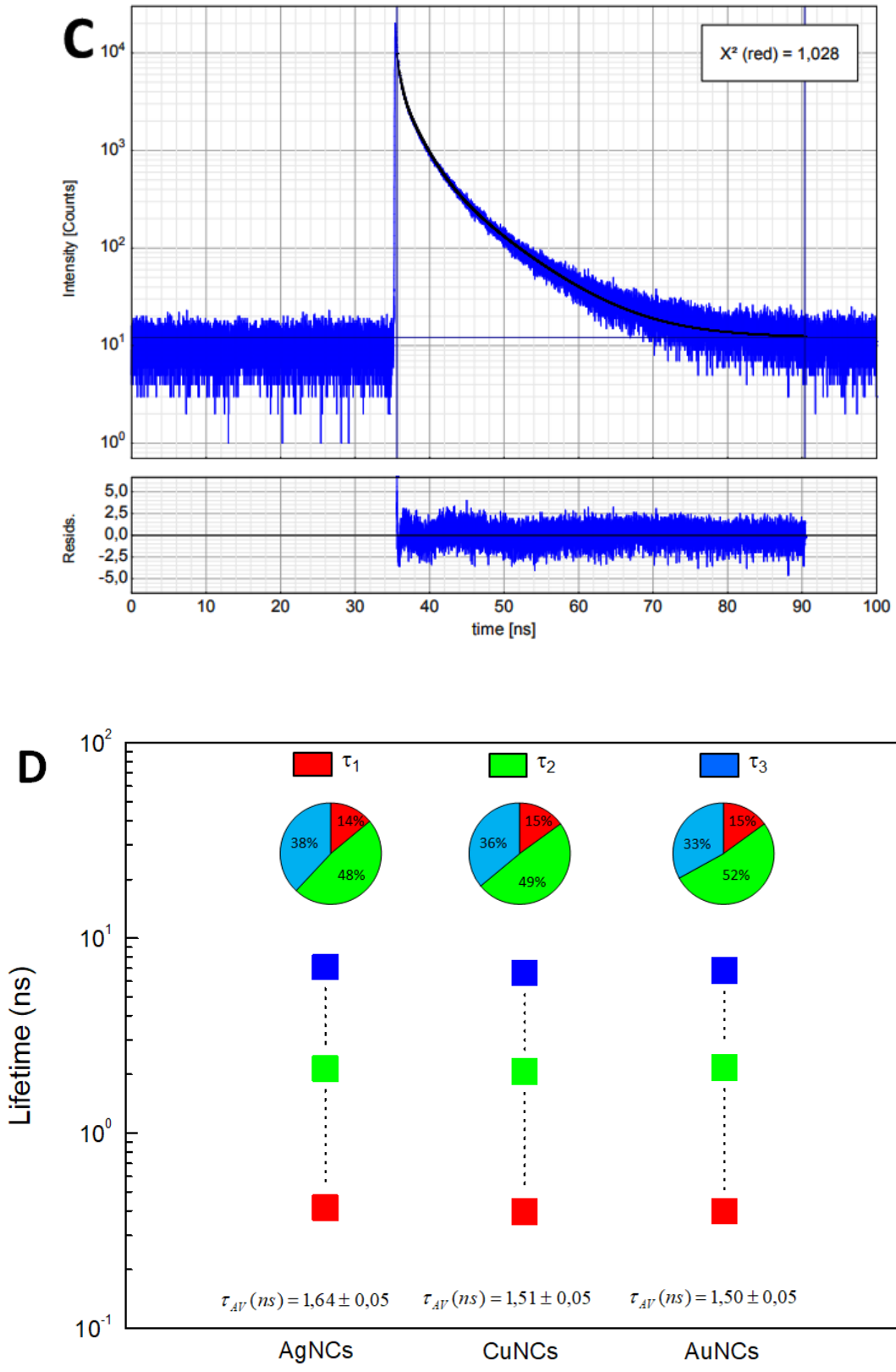
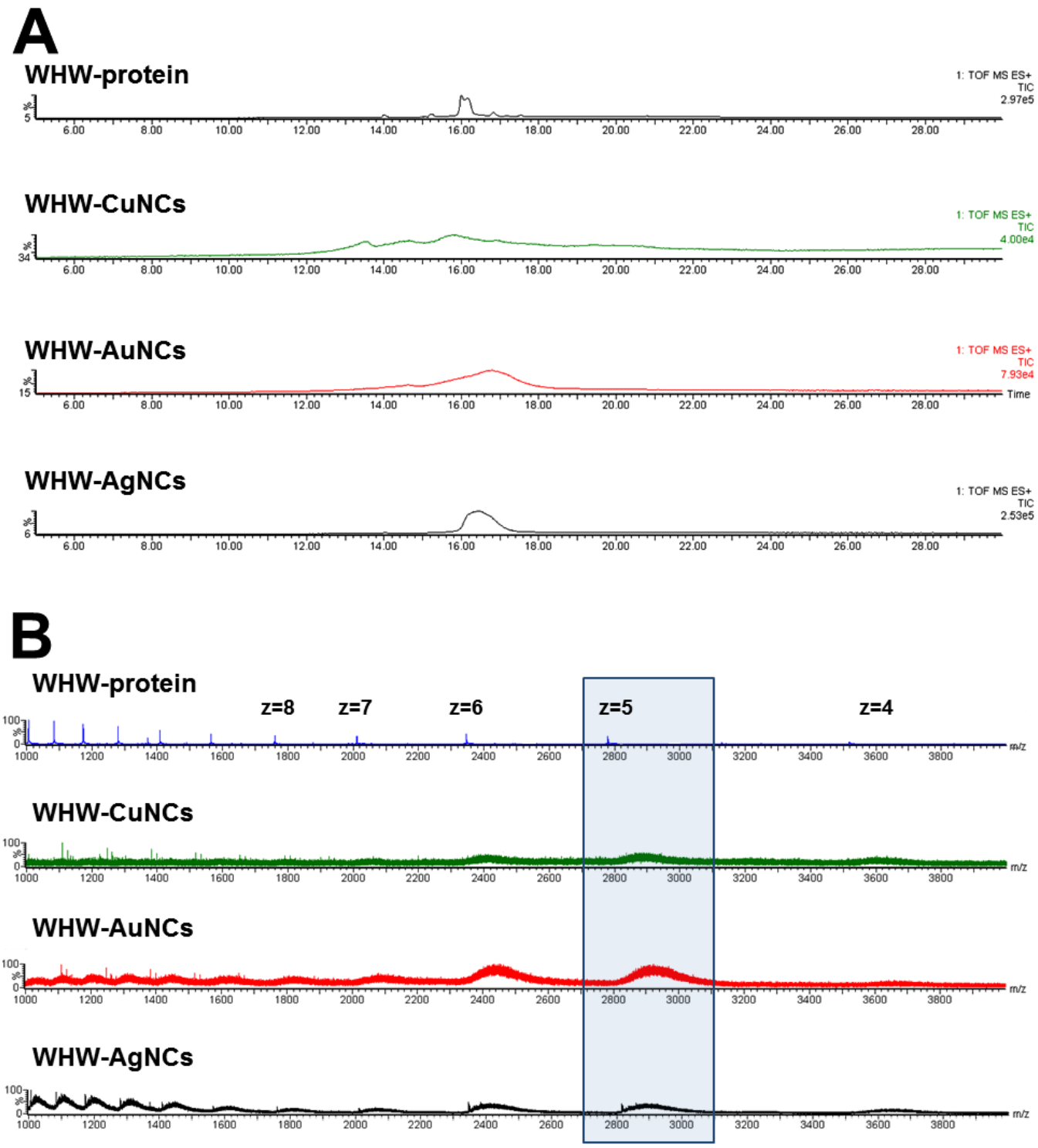


Figure S5. The fluorescent lifetime analysis of WHW-CuNCs (A), WHW-AuNCs (B) and WHW-AgNCs (C) PL decay curves with corresponding three-exponential fits depicted by a solid black line. (D) Corresponding lifetime components and their statistical weights for the three types of protein assemblies. Amplitude-weighted average lifetimes are displayed for each sample.

Liquid chromatography coupled to a time-of-flight mass spectrometry detector with electrospray ionization source (LC-ESI-TOF)

In order to confirm the MALDI-TOF dataset obtained for the protein stabilized metal NCs, liquid chromatography coupled to a time-of-flight mass spectrometry detector with electrospray ionization source (LC-ESI-TOF) was performed, providing very similar results to those obtained by MALDI-TOF, thus confirming the size polydispersity of metal NCs.



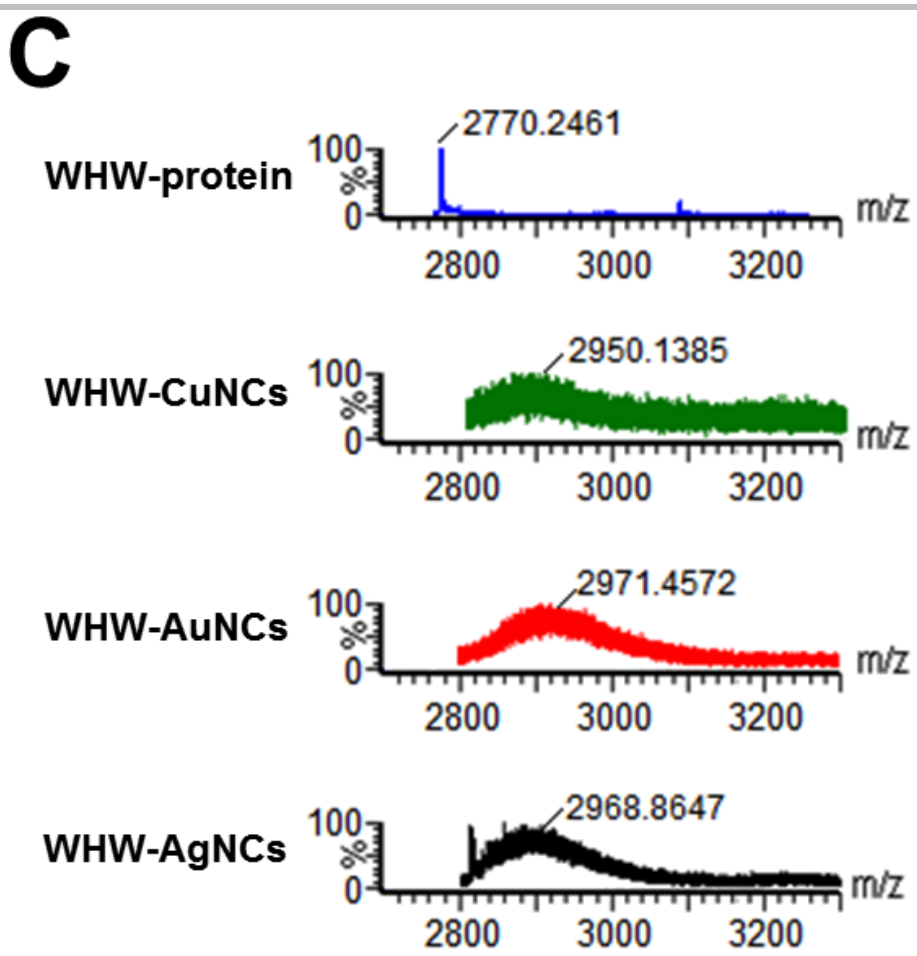


Figure S6. A. Chromatograms obtained for WHW protein (blue), WHW-CuNCs (green), WHW-AuNCs (red) and WHW-AgNCs (black) in the liquid chromatography. B Complete mass spectra obtained for WHW protein (blue), WHW-CuNCs (green), WHW-AuNCs (red) and WHW-AgNCs (black).C Extended mass spectra for WHW protein (blue), WHW-CuNCs (green), WHW-AuNCs (red) and WHW-AgNCs (black) with z=5.

Determination of the number of metal atoms in the Prot-NCs

MALDI-TOF and LC-ESI-TOF were used to determine the number of metal atoms in the Prot-NCs. Table S1 shows that the metal NCs present certain polydispersity in size.

Table S1. Determination of the number of metal atoms in the Prot-NCs by MALDI-TOF, ESI-TOF, and ICP-MS.

Sample	MALDI-TOF			ESI-TOF
	MSC[a]	FWHM[b]	NMA[c]	NMA[c]
WHW-Protein	13228.07	10.23	--	--
WHW-CuNCs	14092.54	581.67	6-21	6-20
WHW-AuNCs	14153.88	585.14	2-7	2-8
WHW-AgNCs	14167.47	586.76	4-13	4-12

[a]Center of mass peak in Daltons (MSC). [b] Full width at half maximum in Daltons (FWHM). [c] Number of metal atoms per cluster (NMA).

Inductively coupled plasma mass spectrometry (ICP-MS)

100 µL of each FPLC purified metal NC at approximately 100 µM concentration were mixed with 300 µL of 37% HCl and the resultant suspension was sonicated for 30 minutes at 40ºC. Finally, 2700 mL of bi-distilled water was added. The Cu, Au and Ag concentration of the FPLC purified metal NC was determined by measuring the sample ICPMS (Table S2). For this purpose 100 µL of each FPLC purified metal NC were previously digested using 37% HCl. The protein concentration of each FPLC purified metal NC was measured by Bradford assay (Table S2).

Table S2. Quantification of metal atoms per protein by ICP-MS

Sample	Protein concentration [µM]	Metal concentration [µg/L] / [µM]	Number of metal atoms per protein
WHW-CuNCs	98	91660±3356 / 1444±53	15±1
WHW-AuNCs	91	91905±5378 / 467±27	5±1
WHW-AgNCs	107	108689±780 / 1007±71	9±1

SDS-PAGE Gel Electrophoresis

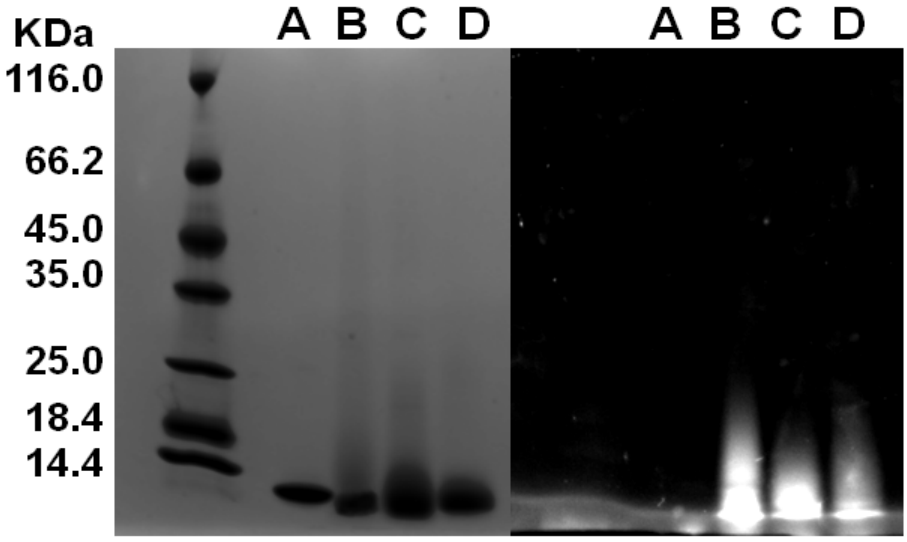


Figure S7. SDS-PAGE gel electrophoresis of WHW protein (A), WHW-CuNCs (B), WHW-AuNCs (C) and WHW-AgNCs (D) visualized under a UV transilluminator (right) and after Coomassie blue staining (left).

Transmission Electron Microscopy (TEM)

The particle size distribution was determined from STEM micrographs using ImageJ software to measure the diameter of a total of 100 NCs (Figure S8).

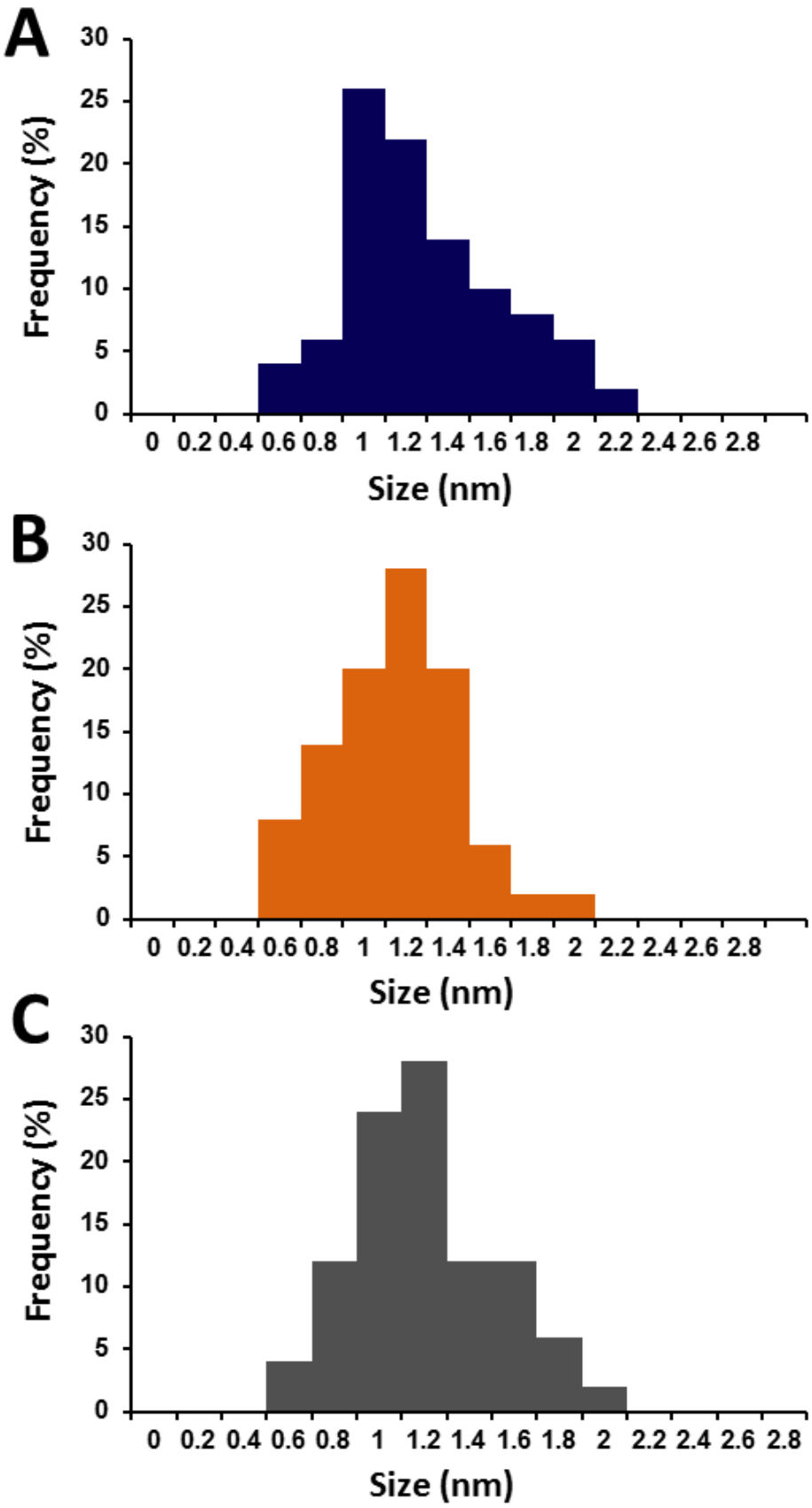


Figure S8. Size distribution histograms of WHW-CuNCs (A), WHW-AuNCs (B) and WHW-AgNCs (C) measured from STEM micrographs.

Storage stability

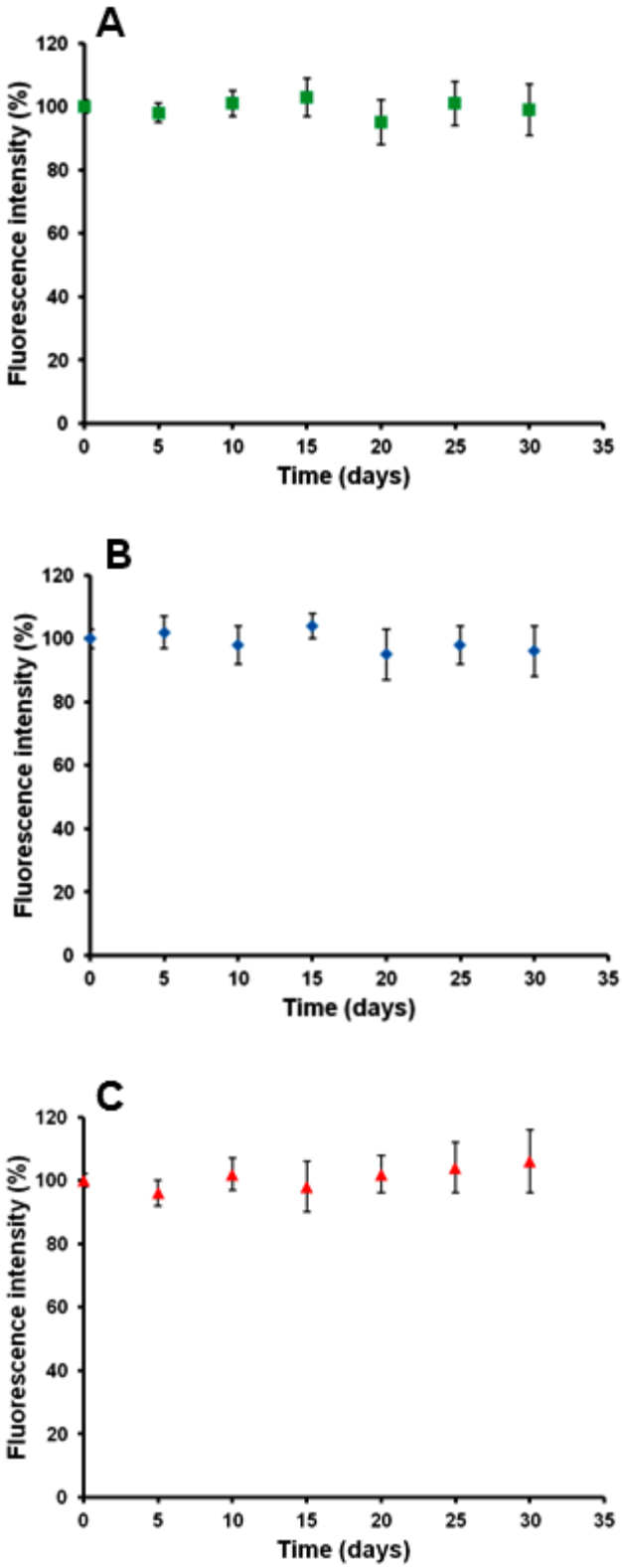


Figure S9. Storage stability of WHW-CuNCs (A), WHW-AuNCs (B) and WHW-AgNCs (C) in PBS at 4°C.

Stability under working conditions

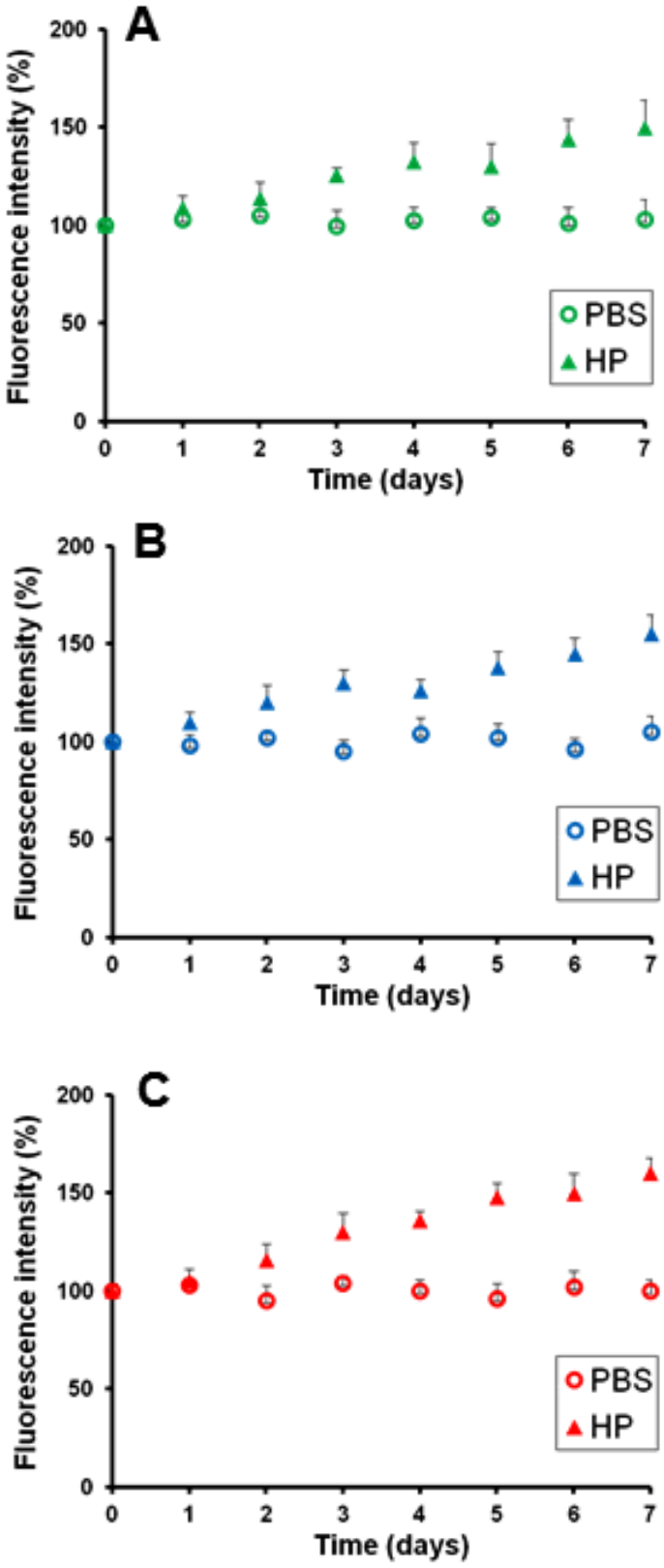


Figure S10. Stability under working conditions of WHW-CuNCs (A), WHW-AuNCs (B) and WHW-AgNCs (C) evaluated in phosphate buffered saline (PBS) and human plasma (HP) at 37ºC.

Temperature stability of Prot-NCs

The thermal stability of the Prot-NCs was tested under a temperature range from 20ºC to 70ºC (Figure S11). The fluorescence decays down to 45.2% in the case of CuNCs, 76.3% for AuNCs, and 49.9% in the case of AgNCs (Figure S11A-C respectively).

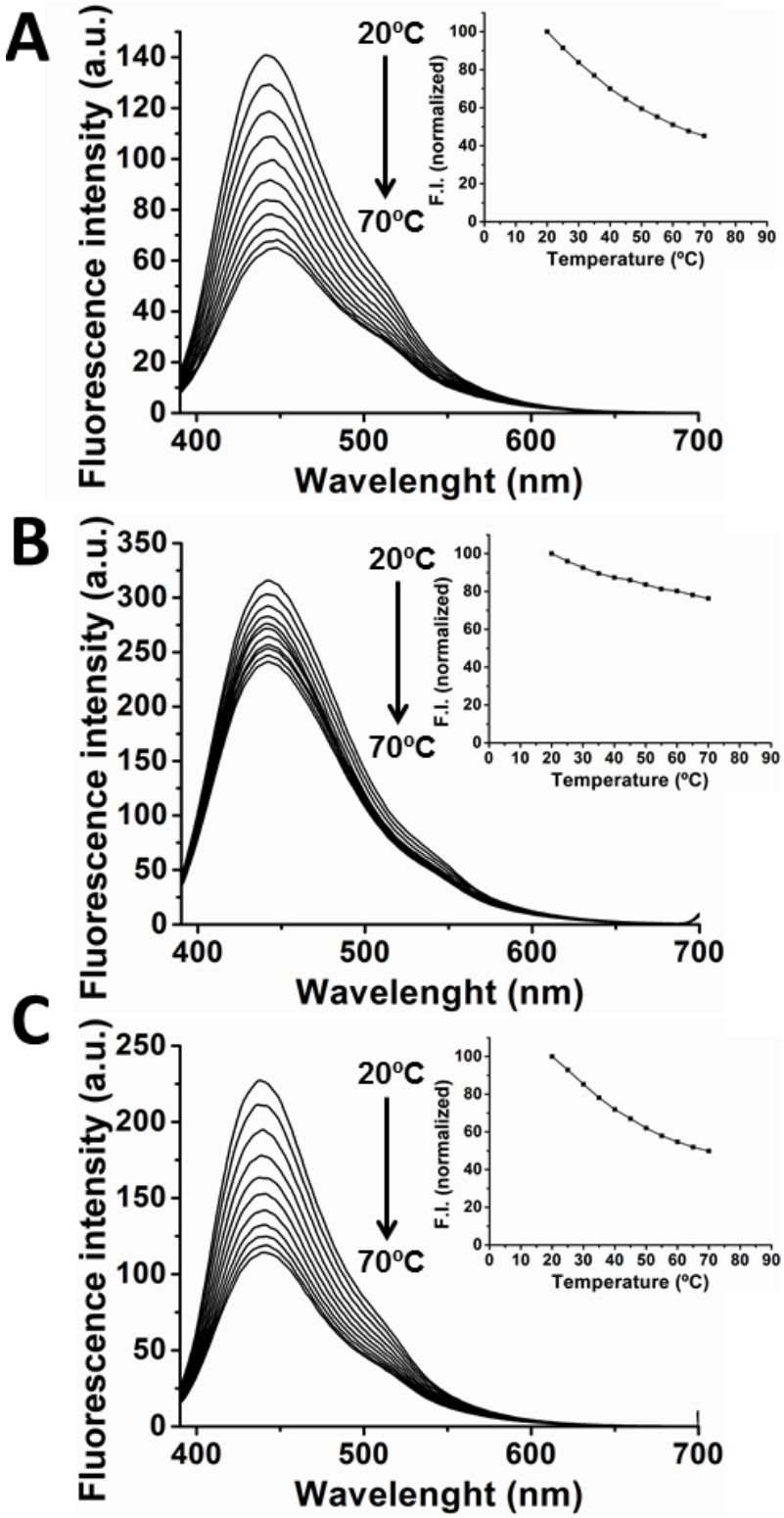


Figure S11. Protein stabilized metal NCs emission spectra under a temperature gradient from 20°C to 70°C for WHW-CuNCs (A); WHW-AuNCs (B); and WHW-AgNCs (C). Inset, the normalized fluorescence intensity vs. temperature.

pH stability of Prot-NCs

The pH stability of the Prot-NCs was tested under a pH range from 5 to 12 (Figure S12). Figure S12 shows that the Prot-NCs are stable under a broad pH range with a maximum emission intensity at pH 7-8.

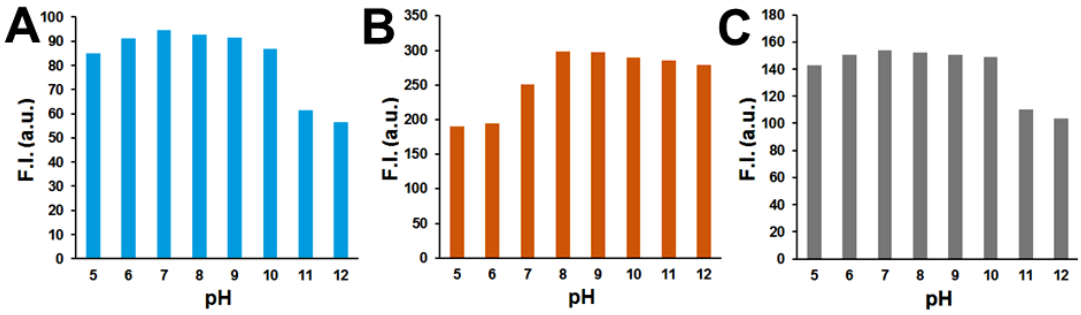


Figure S12. Protein stabilized metal NCs normalized emission intensity under a pH range from 5 to 12 for WHW-CuNCs (A); WHW-AuNCs (B); and WHW-AgNCs (C).

Ionic strength stability

The stability of the Prot-NCs under an ionic strength range was evaluated measuring the fluorescence spectra of the metal NCs at different NaCl concentration ranging from 0.15 to 1.0 M. Figure S13 shows that the Prot-NCs are very stable under a broad ionic strength range.

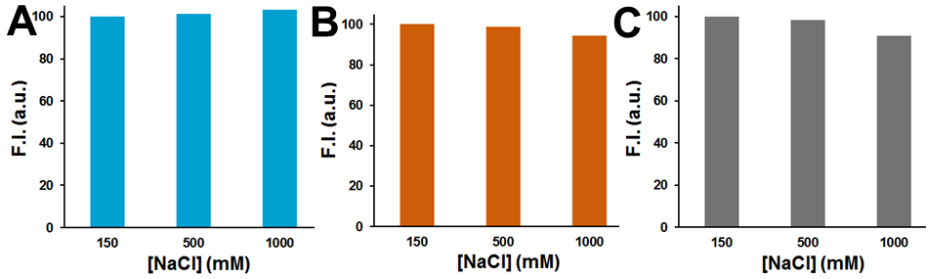


Figure S13. Protein stabilized metal NCs normalized emission intensity under an ionic strength range from 0.15 to 1 M of NaCl for WHW-CuNCs (A); WHW-AuNCs (B); and WHW-AgNCs (C).

Stability in presence of thiols

The stability of the Prot-NCs in presence of biothiols was evaluated at different DL-cysteine (Cys) concentration ranging 0.005 to 30.0 mM (Figure S14). Figure S14 shows that the Prot-NCs are very stable up to 1 mM of Cys and then their fluorescence emission is quenched in a dose-dependent manner.

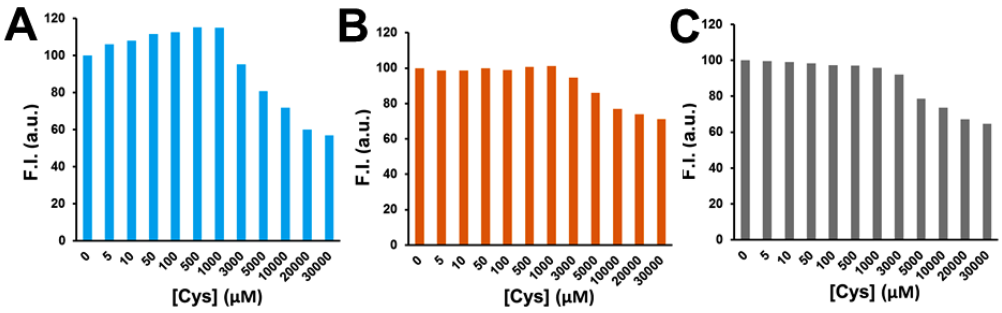


Figure S14. Protein stabilized metal NCs normalized emission intensity under a reducing environment range from 0.005 to 1 mM of DTT for WHW-CuNCs (A); WHW-AuNCs (B); and WHW-AgNCs (C).

Stability in the presence of different metal ions

The chemical stability of the Prot-NCs was evaluated recording the fluorescence emission spectra in presence of different metal ions at 50, 100 and 150 μM. Figure S15 shows that all the Prot-NCs are very stable in presence of different metal ions such us Ba^{2+}, Ca^{2+}, Cd^{2+}, Mn^{2+} and Mg^{2+}. The presence of different metal ions induced a dose-dependent decrease of the fluorescence emission of the CuNCs (Fe^{2+} >Ni^{2+}>Fe^{3+}>Co^{2+}>Hg^{2+}), AuNCs (Fe^{2+} >Co^{2+}>Hg^{2+}>Ni^{2+}>Fe^{3+}>Pb^{2+}>Zn^{2+}), and AgNCs (Fe^{2+} >Co^{2+}>Ni^{2+}>Fe^{3+}> Pb^{2+}>Hg^{2+}).

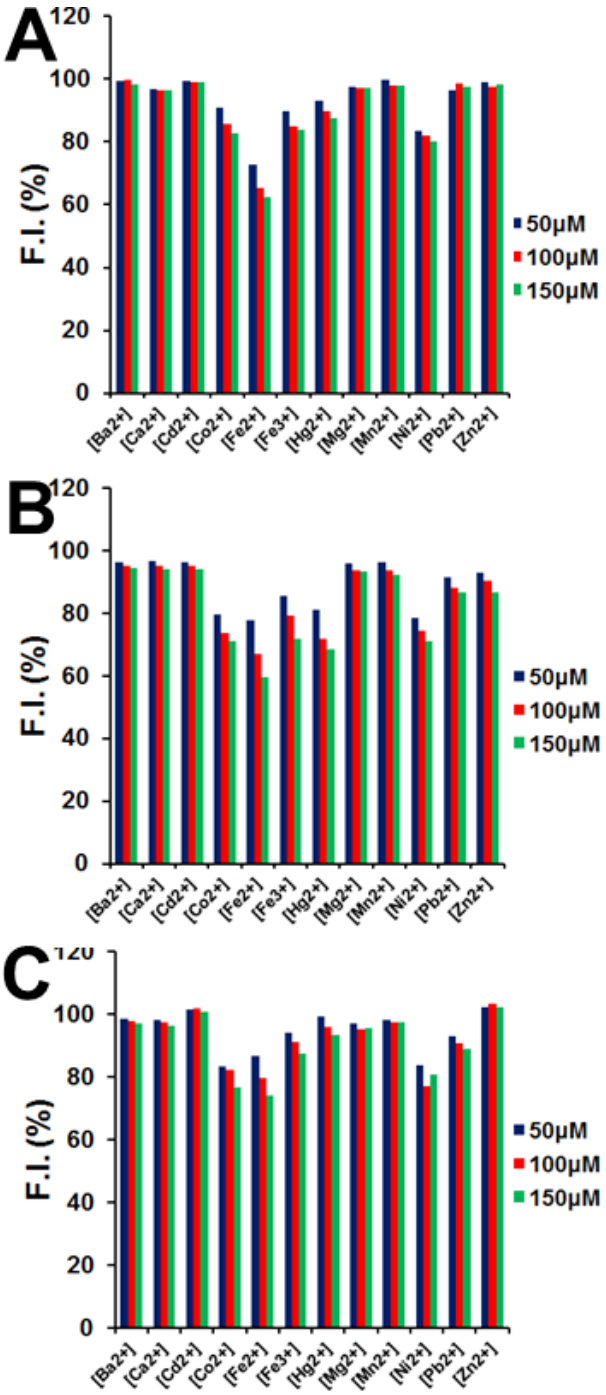


Figure S15. Protein stabilized metal NCs normalized emission intensity in presence of different metal ions at 50 μM (blue), 100 μM (red) and 150 μM (green) concentration for WHW-CuNCs (A); WHW-AuNCs (B); WHW-AgNCs (C).

Fluorescence photobleaching

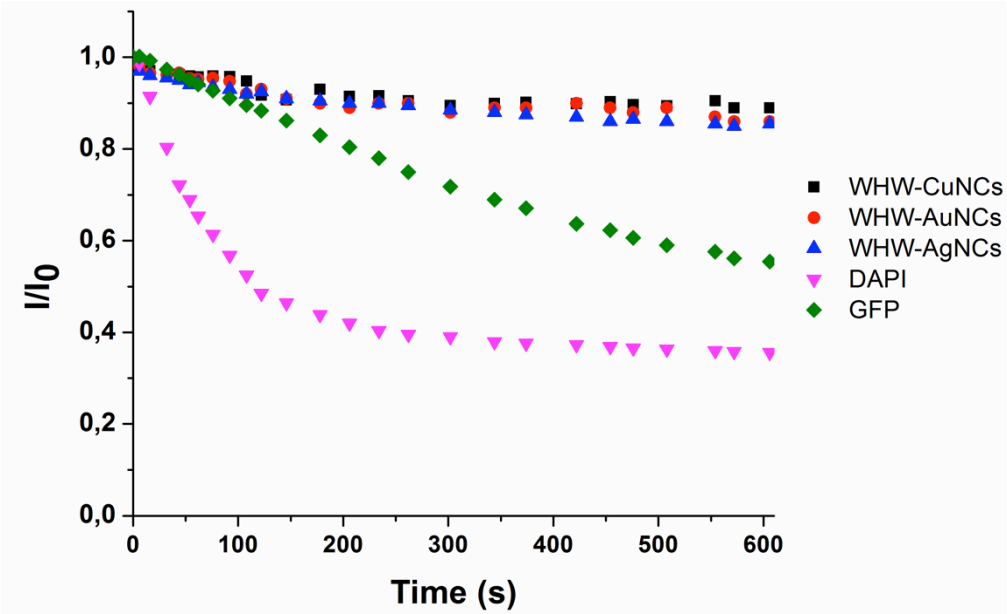


Figure S16. Fluorescence photobleaching of WHW-CuNCs, WHW-AuNCs, WHW-AgNCs, DAPI and GFP. These samples in aqueous solutions were deposited onto quartz slides and irradiated for 10 min (λ_{ex} = 360-380 nm). The fluorescence intensities before and after irradiation were normalized for comparison.

***In vitro* cytotoxicity assays**

The biocompatibility of the Prot-NCs was evaluated in MDA-MB-231 breast cancer at different concentrations of Prot-NCs (1-20 μM) under standard cell culture conditions. Following 5 days of incubation, none of the Prot-NCs complexes presented any cytotoxic effect even at the highest concentration tested (Figure S17).

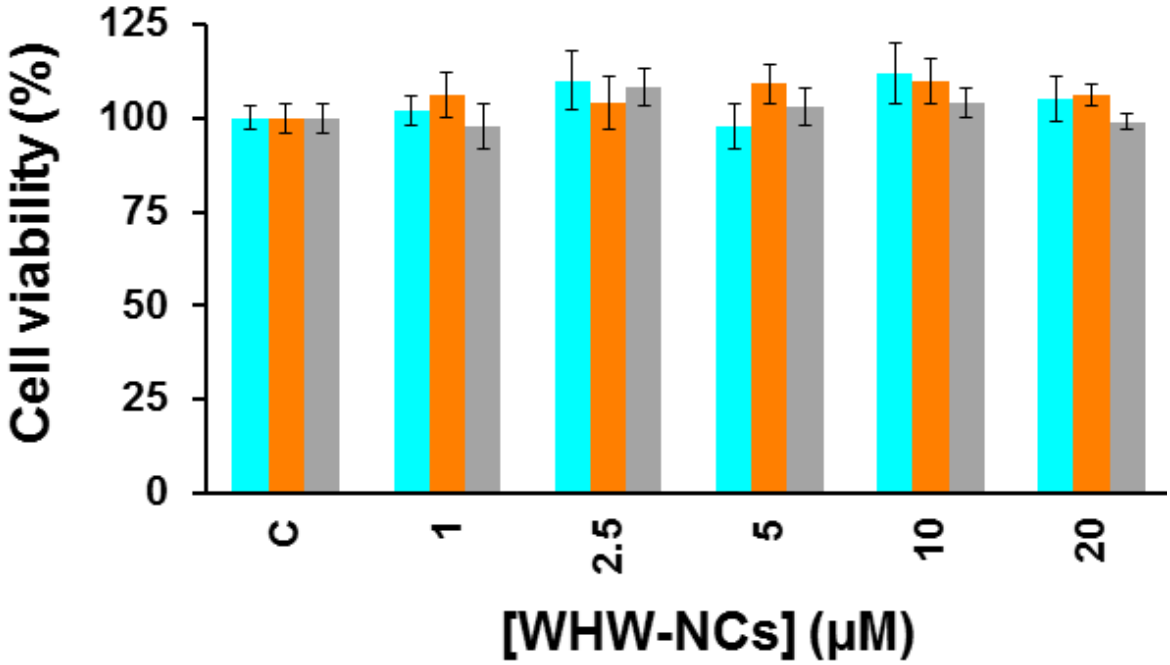


Figure S17. Cytotoxicity of WHW-CuNCs (blue), WHW-AuNCs (orange) and WHW-AgNCs (gray) on MDA-MB-231 breast cancer cells measured by Alamar blue assay after 5 days of incubation.

Confocal Cell imaging

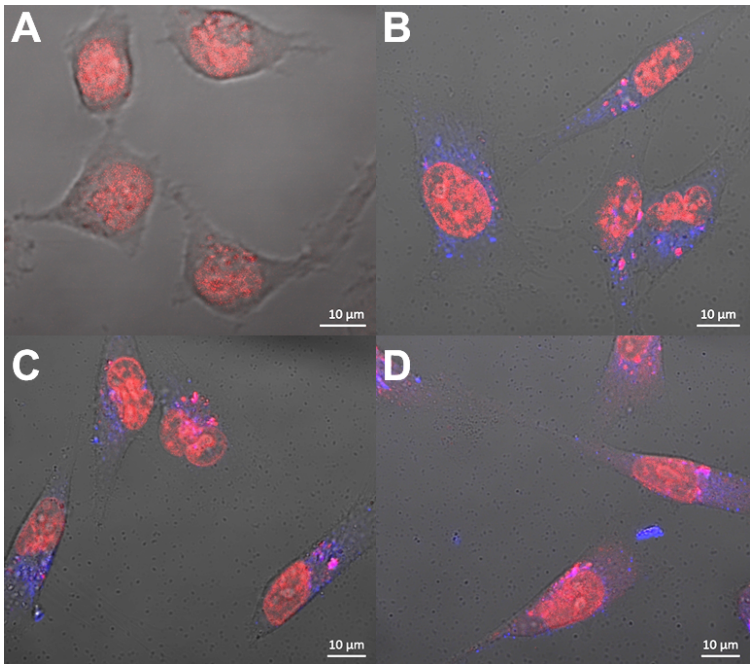


Figure S18. Live confocal fluorescence microscopy images of MDA-MB-231 breast cancer cells incubated metal NCs. Control cells (A) and cells incubated with WHW-CuNCs (B), WHW-AuNCs (C), and WHW-AgNCs (D) for 4h. The Prot-NCs are shown in blue emission under excitation a blue laser (405 nm). In red is shown the nuclear staining with NucRed™ Live 647 upon excitation with a NIR laser (633 nm). Scale bars correspond to 10 µM.

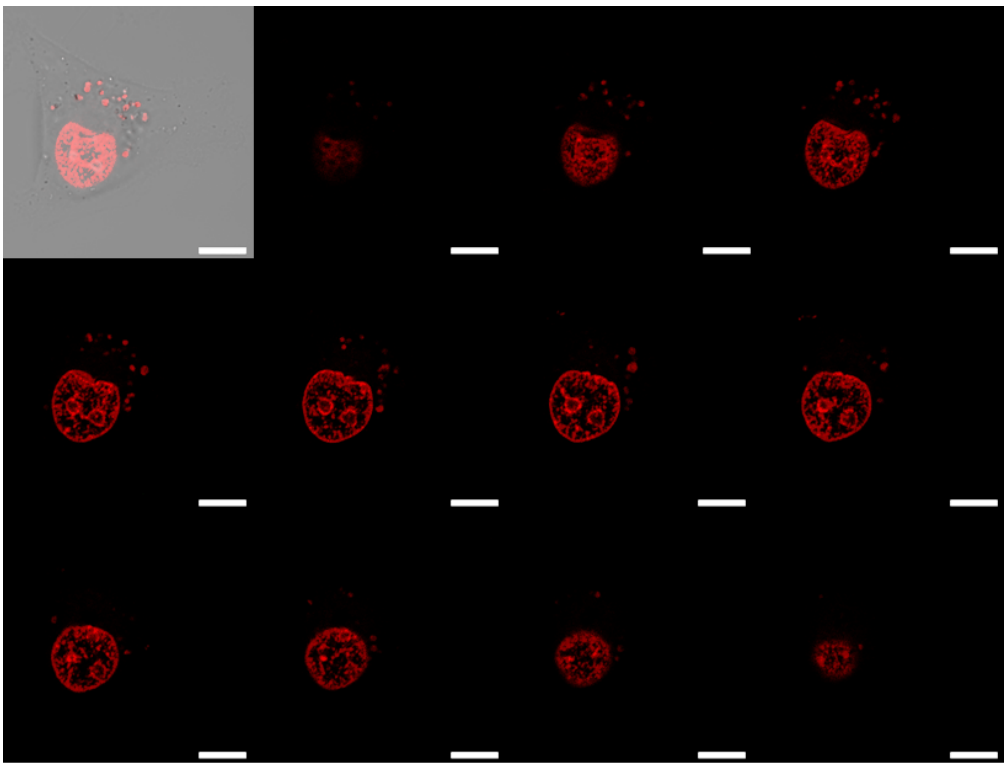


Figure S19. Live confocal fluorescence Z-stack imaging of MDA-MB-231 breast cancer cells incubated fluorescein-labeled WHW protein. Merged and different confocal planes under excitation with blue laser (405 nm), green laser (488nm) and NIR laser (633). In red is shown the nuclear staining with NucRed™ Live 647 upon excitation with a NIR laser (633 nm). Scale bars correspond to 10 µM.

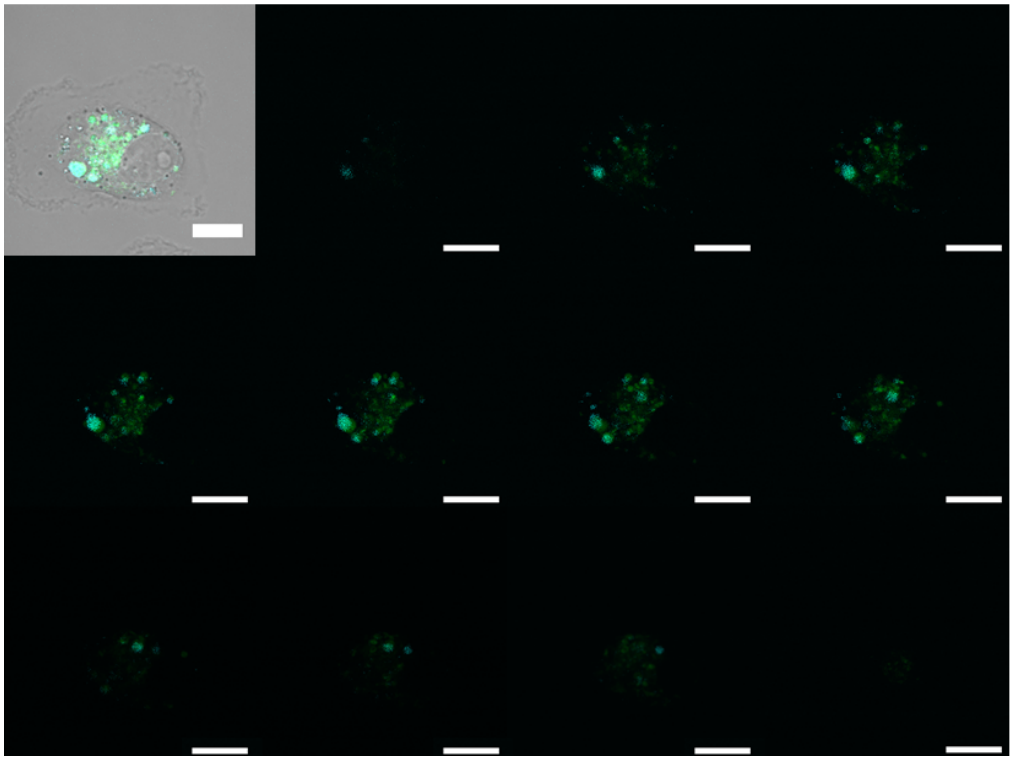


Figure S20. Live confocal fluorescence Z-stack imaging of MDA-MB-231 breast cancer cells incubated fluorescein-labeled WHW-CuNCs. Merged and different confocal planes under excitation with blue laser (405 nm) and green laser (488nm). Scale bars correspond to 10 μM.

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[1] a) E. R. Main, Y. Xiong, M. J. Cocco, L. D'Andrea, L. Regan, *Structure* **2003**, *11*, 497-508; b) T. Kajander, A. L. Cortajarena, E. R. Main, S. G. Mochrie, L. Regan, *Journal of the American Chemical Society* **2005**, *127*, 10188-10190.
[2] A. L. Cortajarena, S. G. Mochrie, L. Regan, *Protein Sci.* **2011**, *20*, 1042-1047.
[3] P. Couleaud, S. Adan-Bermudez, A. Aires, S. H. Mejías, B. Sot, A. Somoza, A. L. Cortajarena, *Biomacromolecules* **2015**, *16*, 3836-3844.

Author Contributions

A.A. and A.L.C. conceived and planned the research, performed experimental research and wrote the manuscript; I.LL. performed confocal fluorescence microscopy experiments; M.M. performed STEM experiments; J.C-S performed time-resolved photoluminescence measurements; J.C-G. contributed to the spectroscopic characterization and to the writing of the manuscript.