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Electronical Supporting Information

Protein Capped Nanosilver Free Radical Oxidation: Role of the Biomolecule Capping on Nanoparticle Colloidal Stability and Protein Oxidation

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EXPERIMENTAL METHODS

Chemicals and reagents

Silver nitrate ($AgNO_3$), trisodium citrate, 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]- 2-methyl-1-propanone (I-2959), human serum albumin (HSA), 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), sodium chloride (NaCl) were purchased and used as received from Sigma-Aldrich. All solutions were prepared using Milli-Q water.

Synthesis of citrate capped AgNPs, HSA and radical incorporation

Citrate capped AgNPs were prepared using a similar method as described in the literature.[1-3] Briefly, a deoxygenated (30 min N_2) aqueous solution containing 0.2 mM $AgNO_3$, 0.2 mM I-2959, and 1.0 mM sodium citrate was irradiated with UVA light (8 lamps, in a Luzchem LZC-4 photoreactor at 25.0 ± 0.5°C) for 30 min. Yellow translucent solutions were obtained in all cases and the solutions were kept at room temperature protected from light. For the AgNPs-HSA solutions, a 1:1 v/v mix solution with final concentrations of ≈ 40 nM AgNPs and 1 to 50 μM HSA were prepared. Once mixed, the solutions were pre-incubated overnight at 4°C. Peroxyl radicals were generated in solution by using a 10 mM final concentration of AAPH (0.6 M stock solution) at 37°C.

UV-Vis spectra and kinetics measurements

The plasmon absorption band was followed throughout the absorbance spectra at 37°C in a Libra S50 UV-Vis spectrophotometer (Biochrom, Cambridge, UK). Kinetics were performed following the maximum absorption wavelength of each sample (please refer to Fig. 1A and Fig. 2B).

Tryptophan fluorescence and quenching by AgNP or AAPH addition

The evaluation of Tryptophan 214 (Trp-214; the only Trp residue in HSA) fluorescence was performed in a Shimadzu RF-5301 PC spectrofluorometer (Kyoto, Japan). Fluorescence spectra were obtained using an excitation wavelength of 295 nm, while quenching of Trp fluorescence upon AgNPs (≈ 1 to 16 nM) incorporation was followed at

an emission wavelength of 340 nm at room temperature. The same parameters were used to measured Trp fluorescence quenching by AAPH addition (10 mM) in a Perkin Elmer LS 55 spectrofluorometer (Massachusetts, USA), the fluorescence intensities were followed by up to 2 h at 37°C. In both cases, experiments were carry out in triplicate. Corrections for inner filter effect absorption from the nanoparticle surface plasmon band were carried out by correcting the emission using the equation:

$$F_{corr}=F_{obs}x10^{[(A_{295}+A_{340})/2]}$$

where Fcorr and Fobs correspond to the corrected and uncorrected fluorescence, respectively, and A295 and A340 are the compound absorbances at the corresponding wavelengths.

Physical separation by ultracentrifugation

Ultracentrifugation separations were performed in a Beckman L8M Ultracentrifuge (Beckman Coulter, Inc. USA). Samples solutions (≈12 mL) were centrifuged by 3 h at 40,000 rpm at room temperature. Presence of protein, AgNP, and AgNP-HSA complex in the supernatant were determined by UV-vis and Trp fluorescence (excitation 295 nm, emission 340 nm).

Stopped-flow measurements

An Applied Photophysics SX20 stopped-flow system was used to measure the change in absorbance (l = 1.0 cm) of the nanoparticle when mixing with the AAPH. The kinetics was measured at the wavelength maximum of the AgNp-HSA solution. The experimental temperature was maintained at 37°C with a Lauda RC6 water bath. The solutions were allowed to equilibrate to the temperature of the water bath for 10 min after loading the syringes and before the solutions were mixed. The AgNp-HSA and AAPH solutions were mixed in a 1:1 ratio (dead time = 1 ms). This mixing resulted in a dilution by half of the initial concentrations, leading to a final concentration on AgNPs of ≈ 20 nM, 1 to 50 μM of HSA and 10 mM of AAPH or NaCl (depending on the experiments).

Hydrodynamic sizes and zeta potential measurements

Changes in hydrodynamic sizes and zeta potential for citrate@AgNPs and AgNPs-HSA

solutions before and after (2 h) addition of AAPH were carried out in a Malvern Zetasizer Nano ZS at 20°C in 1.0 cm pathlength disposable plastic cuvettes. Reported values correspond to the average of three independent batches, each measured in triplicate.

Transmission electron microscopy (TEM)

Samples for transmission electron microscopy were prepared by delivering ~5.0 μL of a fresh 1/10th diluted samples on Formvar coated copper-carbon grids (400 mesh) and dried in a vacuum system for 72h. Electron microscopy images were obtained using a FEI Tecnai G2 F20 TEM operating with an acceleration voltage of 75 kV.

HSA oxidation profile evaluation

All the assays for the evaluation of the protein oxidation profile were carried out after an overnight incubation of the reactants, where in the case of the oxidation reaction the measurements were performed after 2 h of incubation time at 37°C with AAPH. Electrophoresis was performed employing Mini-PROTEAN TGX precast gels from Bio-rad. 30 μL of a HSA/AgNPs-HSA solution treated, and controls, with AAPH, were mixed with 10 μL of sodium dodecyl sulphate (SDS, Thermo Scientific). Then, 10 μL of the respective solution was loaded per well. Precision Plus Protein Dual Color was used as standard (10 – 250 kD). The samples were run at 80 V for one hour and stained with EZBlue gel staining reagent from Sigma. For concentrations of reagents please refer to Fig. 4.

The concentration of reactants employed for oxygen uptake, carbonyl and peroxide determinations, were 10 μM HSA, ≈ 40 nM AgNPs and 10 mM AAPH. The rate of oxygen uptake was followed by registering the oxygen concentration as a function of time by 10 min at 37°C on an Oxygraph System (Hansatech Instruments, UK). Protein carbonyl concentrations were determined making use of a Protein Carbonyl Colorimetric Assay Kit (Cayman Chemical, USA). Briefly, protein samples were derivatized using 2,4-dinitrophenylhydrazine. Formation of a Schiff base produces the corresponding hydrazone that was analyzed spectrophotometrically at 360 nm.

Peroxide concentration determination on proteins was performed using a Pierce™

quantitative peroxide assay kit (Thermo Scientific, USA). This consists of the oxidation of ferrous to ferric ion in the presence of xylenol orange. Absorbance of the resulting purple solutions were measured in a Synergy H4 Microplate reader (BioTek, USA) at 560 nm.

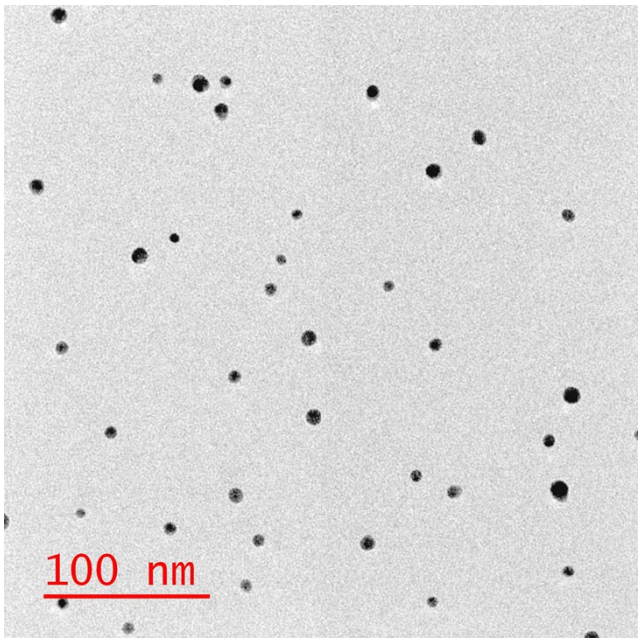


Figure S1. Representative TEM image for 1.0 mM citrate protected nanoparticles. Image obtained using 75 kV acceleration voltage, see experimental.

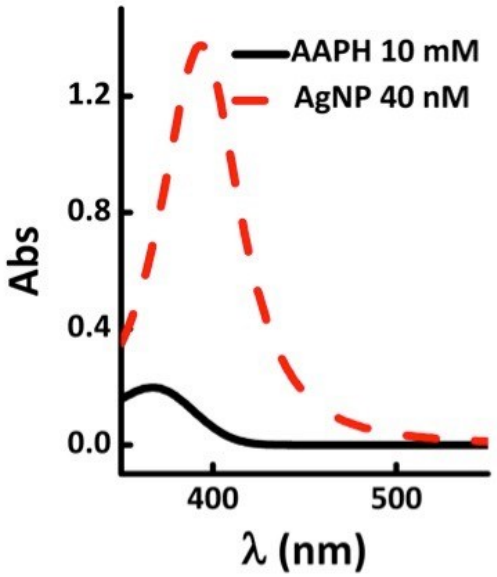


Figure S2. Absorption spectra for a 10 mM AAPH solution (black line) and AgNP ≈ 40 nM red dashed line for solutions at 37°C.

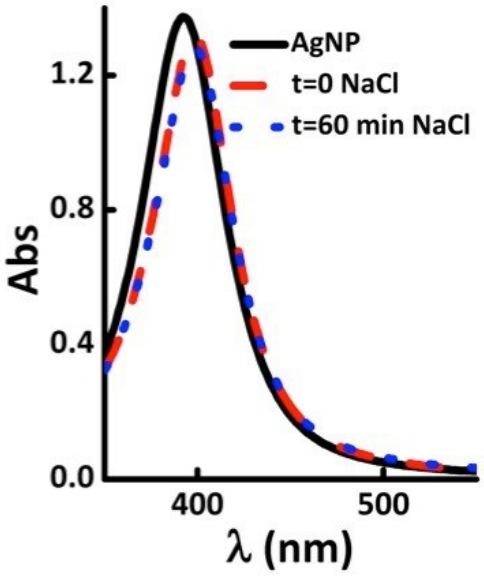


Figure S3. Surface plasmon band variation in the presence of NaCl (10 mM). [AgNP] ≈ 40 nM. Measured at 37°C.

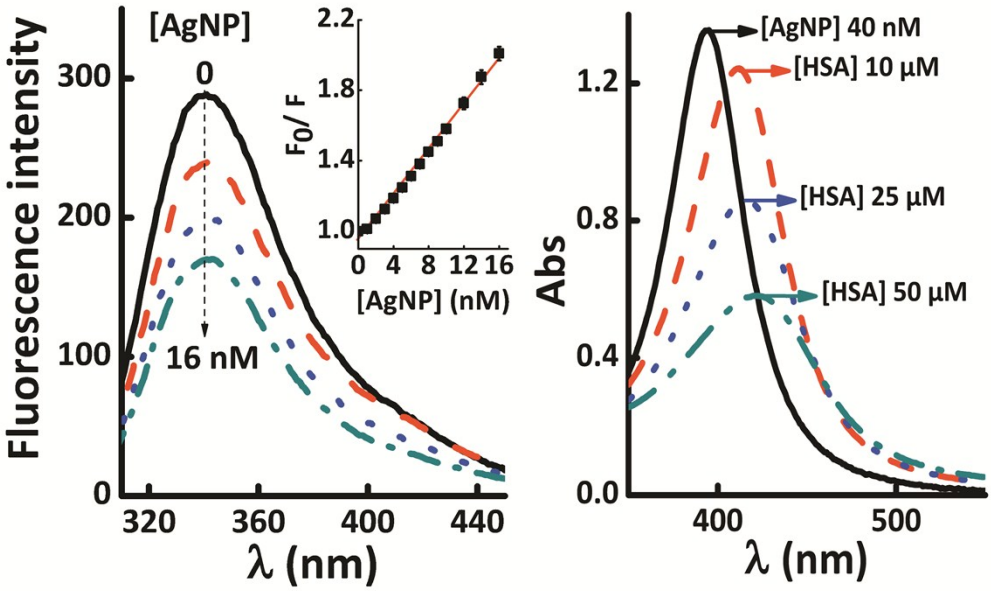


Figure S4. Changes in the intensities of the Trp-214 fluorescence and surface plasmon band of AgNP by HSA-AgNPs composite formation. **Left:** Representative change in the Trp-fluorescence spectra at increasing AgNP concentrations ([HSA] = 10 µM). Inset: Changes in the tryptophan fluorescence in the presence of AgNP (λ_{ex} = 295 nm and λ_{em} = 340 nm) plotted as relative fluorescence intensity vs. AgNPs concentration (data correspond to the average of 3 independent experiments). Fluorescence emission was corrected for the inner filter effect, see SI. **Right:** Nanosilver SPB spectra for AgNPs-protein system at different HSA concentrations ([AgNP] = 40 nM). All measurements were carried out at 37°C in triplicate.

Table ANOVA 1 Comparison of t_{50} values for changes in AgNPs SPB obtained at different concentrations of human serum albumin incubated with 10 mM AAPH, between tree different groups.

Source	Degree Freedom	Sum of Squares	Mean Square	F	P
Model	2	0.44835	0.22418	2.40973	0.10135
Error	45	4.186	0.09303		
Total	47	4.6346			

Estimation of proteins bound to nanoparticles

The concentration of AgNPs was estimated as previously described by Alarcon *et al*.[4] Using this method, at 200 μM of silver ion the concentration of AgNPs is ≈ 80 nM (in the present work, [AgNP] was 40 nM). Thus, the ratio between the protein (assuming all protein is bounded) and nanoparticle concentration give a final estimation of the number of protein units per nanoparticle. As example, at 1 μM HSA:

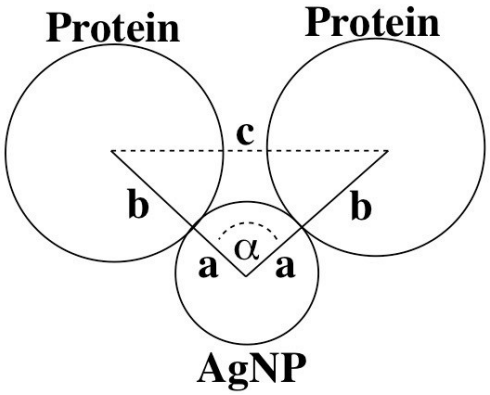
$$[HSA]/[AgNP] = 1 \times 10^{-6} / 0.04 \times 10^{-6} = 23 \text{ protein/nanoparticle}$$

while for 50 μM HSA:

$$[HSA]/[AgNP] = 50 \times 10^{-6} / 0.04 \times 10^{-6} = 1{,}250 \text{ protein/nanoparticle}$$

Geometrical considerations

The estimation above only considers the total number of proteins and nanoparticles, without considering the available surface and limitations due to steric interactions. Thus, to provide an estimate of the number of proteins that can interact with the nanoparticle available surface, we developed a simplified geometrical model, which does not consider the interaction between proteins (see Scheme S1). In this model, we assumed that the volume of a single nanoparticle is 1/3 of the volume of a single protein. As the individual sizes of the nanosilver and protein are known trough dynamic light scattering measurements (Table 1, main text), the values of the radius of the nanoparticle (a) and the radius of the protein (b) can be established. Further, we assume the following limitations to the system: the minimum distance between two proteins (c), corresponds to 2 times the radius of the protein (b, i.e. no overlapping of proteins occurs). The second limitation is that the numbers of proteins are fixed for each system.



Scheme S1. Geometrical model to establish the numbers of proteins interacting with the available surface of a nanoparticle.

Thus, as an example for a system with one NP and 3 proteins we have the following:

We know from DLS measurements that the size of AgNP is ≈ 4.0 nm (Table 1), while the diameter for the HSA is ≈ 6.5 nm. Also, as the system considers the interaction with 3 proteins, the value of the angle (α) is 120° (a whole sphere has 360°). Knowing that (a+b) = 5.25 nm, and that c = 2(a+b) cos (180-α/2), we calculate that the value of the hypotenuse c is ≈ 9.0 nm, which is a value over the conditions of 2 times the value of b (in this case 6.5 nm). Therefore, it is possible to accommodate three proteins around the particle. If we assume the interaction with 4 proteins instead of 3, similar procedure can be follow, but a Pythagoras theorem must be applied to determine the value of c. In this case, c is ≈ 7.4 nm, and it is still feasible to have 4 proteins around the particle. However, when the condition is changed to 5 proteins, the value of c is ≈ 6.2 nm, which is under the limit conditions of 2b.

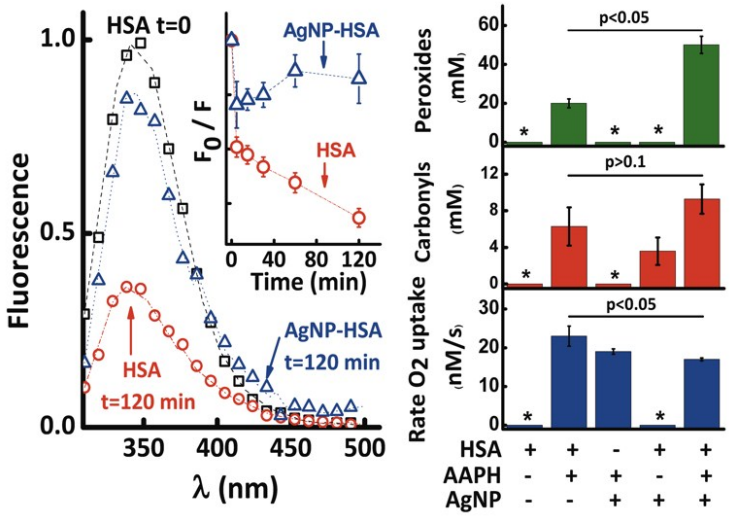


Figure S5. Oxidation profile for HSA in the presence/absence of AgNP. Left: Trp fluorescence intensity change in the presence of peroxyl radicals (10 mM AAPH) with and without AgNP (40 nM). Inset:Trp fluorescence deactivation measured under the same experimental conditions. Experiments were carried out for 2 h, at 37°C; λex=295 nm and λem=340 nm (n=3). Right: Selected protein oxidation markers: Peroxides, carbonyl, and oxygen consumption. [HSA] = 10 μM; [AgNP] ≈ 40 nM and [AAPH] = 10 mM. Oxidation reaction was carried out for 2 h at 37°C. Values reported corresponds to three independent measurements. *Non-detected.

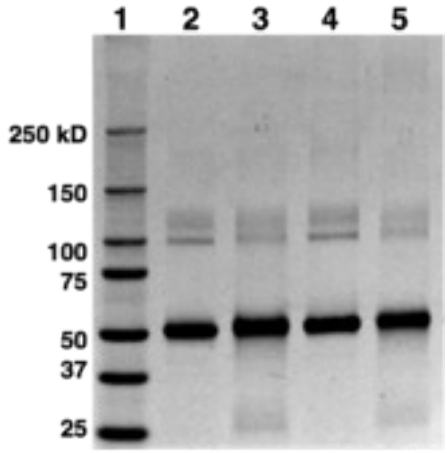


Figure S6. Representative image for a SDS-electrophoresis experiment. Lanes from 2 to 5 correspond to samples containing human serum albumin. **(1)** Molecular weight standard (10 - 250 kD); **(2)** HSA 1 μM; **(3)** HSA 1 μM + AAPH; **(4)** HSA 1 μM + AgNP; and **(5)** HSA 1 μM + AgNP + AAPH. The concentrations in all cases were: 40 nM for [AgNP] and 10 mM for[AAPH]. The gel was run for 1 h at 80 V, posterior staining was performed with EZblue gel staining and pictures captured in a FluorChem IS-8900 (Alpha Innotech, USA).

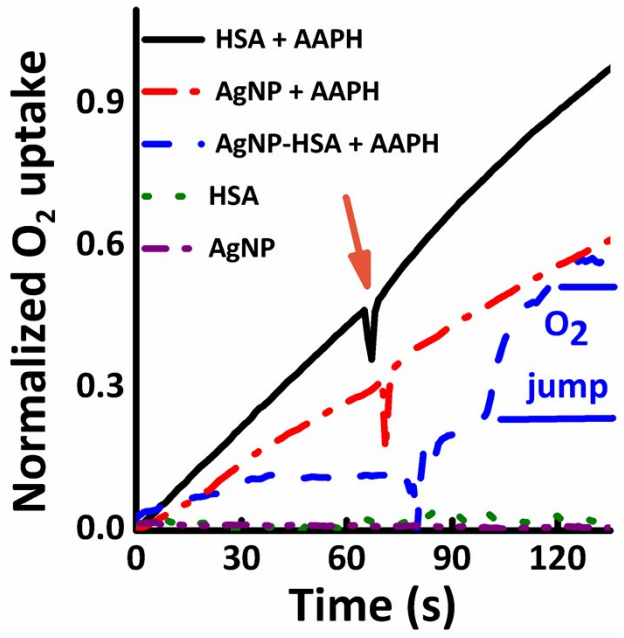


Figure S7. Oxygen consumption plots for the nanosilver-protein composite (black) and its individual components (colored) by the reaction with peroxyl radicals, see Scheme 1 main text, in the presence of catalase. The orange arrow indicates the time of catalase (100 U/mL) addition to the solutions. [AAPH] 10 mM; [HSA] 50 μM; and [AgNP] 40 nM. Note that the oxygen uptake "jump" in presence of catalase was only observed for the composite nanosilver-protein. Measurements were carried out at 37°C in triplicate.

References

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