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Plasma-assisted synthesis of noble nanoparticles coated with hyaluronan: chemico-physical characterization and safety assessment

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Abstract

Nanosystems are rapidly gaining attention in the biomedical, chemical, material, computer and catalysis fields. Recent research has been focused on synthetic methods to decrease toxicity and side effects as compared to classic formulations. However, harmonization of the interpretative criteria of biological activity and strategies to improve the scalability of synthetic technologies are limited. The present work describes the use of atmospheric plasma as a promising strategy to produce size-controlled and safe noble nanoparticles, which did not show surface toxicity due to the absorption of chemicals during the synthesis reaction. Physisorption with Hyaluronic Acid was used to modulate nanoparticle aggregation kinetics and improve biological properties. Physico-chemical characterization was conducted using NMR spectroscopy, UV-visible and dynamic light scattering. Cytotoxicity on bacterial and Human Umbilical Vein Endothelial Cells was tested. The results demonstrated the efficiency of the plasma synthetic method to control nanoparticle size and toxicity selectively improving antibacterial activity against Gram-negative strain.

1. Introduction

Nanoparticles (NPs) obtained from noble metals (AuNPs and AgNPs) have a free energy surface that depends on temperature, Boltzmann's constant (k_B), saturation of salt solutions (S), molar volume (v), pH and liquid or gas catalysis, which together define their chemico-physical and biological properties ($\Delta G_v = \frac{-k_B T \ln(S)}{v}$) [1-3].

Physisorption with hyaluronic acid (HA) with low (200 kDa) molecular weight (Mw) modulates their aggregation kinetic, prevents secondary collateral phenomena such as coagulation and coalescence and plays a crucial role in morphology, size and biological activity [4-8]. AgNPs and AuNPs represent the most industrially produced and commercialized nanomaterials; their properties are unique and applications are found in different fields including material science, biosensing, organic chemistry, biotechnology, high molecular sensitivity detection, optics, biosensors, computers, medicine, microbiology, and catalysis [3, 9]. Different approaches have been outlined to synthesize NPs, including chemicals, photo- and electro-chemical, extractive, and fermentative methods. The classical methods require high temperature, pressure and energy and involve the manipulation of hazardous chemicals with the need for complex purification steps, and the resulting products usually contain chemical contaminants. Moreover, extractive, and biological sources have a relatively slower rate [10-14].

Here we propose the use of atmospheric plasma as an environmentally sustainable, low energy consumption, affordable and highly efficient method to produce size-controlled and safe noble nanoparticles, which did not absorb toxic chemicals on their surface during the synthesis reaction [15-19]. Cold plasma obtained at atmospheric pressure and room temperature results as being rich with energetic electrons that are capable of dissociating, exciting and ionizing gas molecules [20-22]. Typical reactions include the formation of a Reactive Oxygen and Nitrogen Species (RONS) discharge that can diffuse/dissolve into water during exposure and react with the water molecules, resulting in a cocktail of chemical species whose generation depends on the release of hydrogen ions; moreover, neutral gas molecules subjected to cold plasma treatment are transformed into their oxides and their products can react with hydroxyl radicals, to produce hydrogen peroxide in plasma activated water (PAW) at the gas/liquid interface [23]. PAW was used as the reducing agent for Ag^+ or Au^{3+} ions from Ag/Au salts to obtain AgNPs and AuNPs.

Physisorption with Hyaluronic Acid (HA) at a low molecular weight (200 kDa) and conjugation with the GHHPHGK peptide through an amide bond [24, 25] were performed to improve pro-angiogenic activity [26-28]. The GHHPHGK derives from GHHPH, a 67 kDa peptide repeat present in histidine-proline rich glycoprotein (HPRG, Accession number: P04196, gene ID 3273) [29, 30]. The amide linkage protected the polymer from enzymatic degradation [31, 32]. Successively cold plasma technology as used to synthesize coated noble nanoparticles. Physico-chemical characterization was conducted using NMR spectroscopy, UV-visible and dynamic light scattering [33]. The safety assessment of polymeric nanosystems was evaluated on bacterial strains and HUVEC cell lines; moreover, their capacity to have no effect collagenase activity was tested for their application in wound healing. The resulting NPs can represent a promising technology because of their biological activity, an effective drug delivery system and suitable pharmacokinetic biomodulators [34, 35].

1. Materials and Methods

1.1. Chemicals

HA 200 kDa (HA) was provided by Fidia Farmaceutici S.p.A. (Italy); Sodium hydroxide (NaOH) was purchased from Honeywell Fluka (USA); Silver nitrate (AgNO_3) purity > 99.0%, sodium borohydride (NaBH_4) purity $\geq 99.0\%$, D-(+)-glucose purity $\geq 99.5\%$, Tetrachloroauric (III) acid trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) purity $\geq 99.9\%$, tetrahydrofuran (THF) anhydrous, purity $\geq 99.9\%$, 1-Hydroxybenzotriazole (HOBt), N,N-Diisopropylethylamine (DIEA) Reagent Plus, $\geq 99.0\%$, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl) purity $\geq 99.0\%$, acetone purity $\geq 99.9\%$, Phosphate Buffer Saline (PBS), deuterium oxide (D_2O) 99.9 % atom % D. Water was deionized (resistivity > 18.2 m Ω cm at 25 °C) and purified using a milli-Q unit (Milli-Q plus, Millipore, France). Glassware was first cleaned with aqua regia (HCl: HNO_3 , 1:3 volume ratio) and then rinsed with water before starting.

2.2. Synthesis of Hyaluronic Acid-GHHPHGK conjugate

The conjugation between hyaluronic acid (HA 200 kDa) and the peptide GHHPHGK (Glycine-Histidine-Histidine-Prolyne-Histidine linked to Glycine-Lysine-NH₂) was obtained through an amide bond [25]. For the reaction, 0.372 g of HA were suspended in 15 mL of THF (Tetrahydrofuran, Sigma Aldrich, Italy), under stirring at 4 °C. Then, 0.150 mg of HOObt (3-Hydroxy-1,2,3-benzotriazin-4(3H)-one, Sigma Aldrich, Italy), previously dissolved in 15 mL of THF:H₂O (1:1); 0.0566 g of DIEA (N,N-Diisopropylethylamine, Sigma Aldrich, Italy) previously dissolved in 7.5 mL of THF:H₂O (1:1), and 0.070 g of GHHPHGK-NH₂ previously dissolved in 700 µL of H₂O, were added to this suspension. The reaction mixture was maintained under stirring at 4°C for 30 min. Then, 0.0165 g of EDC·HCl were dissolved in 1.4 mL of water and added to the reaction mixture. After 24 h, the product was precipitated adding 300 mL of acetone. The precipitate was dissolved in a minimum volume of water (65 mL) and dialyzed against water by a dialysis tube (12-14 kDa cut-off); the dialyzed product was then lyophilized (Labconco, Freeze dry system, USA) [24].

2.3. NMR analysis of the HA derivate

1D and 2D ¹H-NMR spectra were recorded at 500 MHz on a Varian Unity Inova spectrometer (Varian, USA). The experiments were performed in D₂O at 27 °C and the chemical shifts reported as δ (ppm), referenced to the resonance of residual HOD. Unequivocal assignments of ¹H resonances were supported by mono- and bi-dimensional experiments gCOSY and gHSQC.

The amount of GHHPHGK peptide (*p*) conjugated with HA was determined from the ratio between the integration value of the signal of the acetyl groups of HA and the -CH₂ Lysine signal or that of the H-2 or H-5 signals of the Histidine imidazole ring.

2.4. Synthesis of AgPNs and AuNPs

2.4.1 Wet chemical synthesis of AgNP and AuNP and HA conjugation

The bare silver nanoparticles (AgNPs) were synthesized following the method described by *Mavani et al.* (2016) [36] Briefly, a solution of 1 mM AgNO₃ was added with 2 mM NaBH₄, see equation (1). The reaction was maintained in an ice bath and under stirring (200 rpm) for 45 minutes. Naked-eye detection was used to confirm the end of the reaction with the chromatic transition of the solution from colourless to yellow.

The bare gold nanoparticles (AuNPs) were synthesized starting from a 0.5 mM HAuCl₄ aqueous solution by adding a 7 mM NaOH and 5 mM Glucose solution, see equation (2). The reaction at 25 °C occurred in 1 minute and its end was detected by the naked eye thanks to the chromatic transition from yellow to purple red.

In order to obtain the coated products, the AgNPs and AuNPs were synthesized solubilizing the AgNO₃ and HAuCl₄ salts in a 0.2 % w/v HA/pHA water solution.

2.4.2 Cold-plasma torch synthesis of AgNP and AuNP and HA conjugation

The plasma method to synthesize AgNPs and AuNPs used atmospheric pressure microplasma which can form the radical species $\text{OH}^\cdot$ that acts as the reducing agent activator for the corresponding metal reduction [17]. Argon (Ar) at a flow rate of 25 sccm was injected from a pressurized stainless-steel capillary tube (0.6 mm inner diameter) whose tip was positioned approximately 2 mm above the plasma-liquid interface (Fig. 1).

a) Plasma technology

b) Nanoparticle aggregation mechanism

Figure 1. a) Schematic representation of nanoparticle synthesis using atmospheric pressure microplasma as the reducing agent activator. The formation of AuNPs consists of four steps: i) *nucleation*, about 1/5 of metal particles present as gold salt start interacting with each other in the first 20s; ii) *growth*, AuNPs size increases to 5.2 nm; iii) *slow further growth*, AuNPs size increases with a controlled growth mechanism where the limiting factor is the metal concentration within the solution; iv) *fast final growth*, a rapid increase in AuNPs size from 5-5.2 nm to 7.7 nm determined by an autocatalytic reduction on their surface [37]. In the absence of a stabilizing agent such as hyaluronic acid, which physioabsorbs on the surface of aggregating particles, the growth of nanoparticles continues even 24 h after the start of the reaction. The use of a stabilizing agent as a nucleation modulator is mandatory to regulate the size and kinetics of gold nanoparticle formation in aqueous media. The nucleation mechanisms have a substantial impact on gold nanoparticle bond length, causing it to decrease from 2.87 Å (calculated on the bulk) to the range of 2.55-2.70 Å. As the reaction progresses, the bond becomes shorter due to the increase in particle size and the decrement of the surface area per gold atom. A secondary mechanism also led to the formation of dimers (Au_2Cl_6) and trimers, with the occasional formation of gold clusters like Au_{13} , the principal cause of coalescence and often considered as an undesired species in reaction mixtures [1]. b) Schematic representation of nanoparticle formation mechanism.

An electric charge of 3 mA (with a resistance value of 140 k Ω) was applied to a 1 mM solution of AgNO₃ for 15 minutes at a temperature of < 5 °C, without stirring. Product formation was observed by the naked eye thanks to a chromatic transition from transparent to yellow brown.

An electric charge of 5 mA (with a resistance value of 140 k Ω) was applied to a 5 mM solution of HAuCl₄ for 15 minutes at a temperature of < 5 °C, without stirring. Product formation was observed by the naked eye thanks to a chromatic transition from yellow to purple red.

For both reactions, a platinum (Pt) foil was immersed in the solution as a counter electrode to promote cathodic reduction reactions at the plasma-liquid interface.

In order to obtain the coated products, the AgNPs and AuNPs were synthesized solubilizing the AgNO₃ and HAuCl₄ salts in a 0.2 % w/v HA/pHA water solution.

As required by some experimental methods, after washing with ultrapure water, the concentrated NPs were obtained by centrifugation for 15-20 minutes at 15 °C and 8000-13000 rpm (Eppendorf Centrifuge 5417R, FA453011 Rotor, Italy). The NPs coated with HA were centrifuged for 4 minutes at 15 °C and 10000 rpm using a filter with a 30 kDa molecular weight cut-off (Merk Millipore, USA).

2.5 Physicochemical characterization of bare and HA/pHA coated AgNPs and AuNPs

2.5.1 UV-Visible nanoparticle characterization

UV-Visible characterization was conducted at 25 °C using a Lambda2S spectrophotometer (Perkin Elmer, USA). The measurements were performed using quartz cuvettes (1.0 or 0.1 cm path length). The absorbance and maximum wavelength were recorded. The assay was performed in triplicate twice and the reproducibility of the synthesis was evaluated as a percentage of relative standard deviation (% RSD), as given in equation (3):

$$\% \text{ RSD} = \frac{SD}{\bar{x}} \times 100 \quad (3)$$

where $\bar{x}$ is the average value of the measured variable, and SD is the standard deviation.

The theoretical size of NP diameters was calculated from equation (4) as described by Haiss et al. (2007) [38], considering each force involved in the system, including plasma frequency and collision frequency.

$$\sigma_{ext} = \frac{2\pi}{|k|^2} \sum (2L + 1) \text{Re}(a_L + b_L) \quad (4)$$

where σ_{ext} is the cross section of a spherical particle with R as the radius embedded in a medium with dielectric function ϵ_m at a defined wavelength λ ; $k = \sqrt{\epsilon_m}/\lambda$ is the wave vector; $a_L(R, \lambda)$ and $b_L(R, \lambda)$ are the scattering coefficients in terms of Ricatti-Bessel functions.

2.5.2 Dynamic light scattering nanoparticle characterization

The NPs hydrodynamic diameter size was investigated by Dynamic Light Scattering (DLS), measuring the particle size distribution at 25 °C with a 650 nm light source (laser diode, 5 mW and photo multiplier tube detector) by using DLS HORIBA LB-550, USA [33]. The measurements were performed using quartz cuvettes (1.0 cm path length). The assay was performed in triplicate twice.

2.5.3 ATR-FTIR nanoparticle characterization

The chemical structure of the functional groups was determined by Fourier-transform infrared spectroscopy (ATR-FTIR) following the method described by Sanfilippo et al. (2020) [39]. Briefly, the silicon wafers are cut to give a surface of 0.8 cm x 1 cm and washed with methanol and iso-octane. 100 μ L of the sample (HA, NP or HA-NP) were put on the cleaned silicon surface and left to dry at room temperature. The ATR-FTIR spectra was recorded using a Spectrum Two™ FTIR Spectrometer (Perkin Elmer, USA).

2.5.4 Atomic Force Microscopy (AFM) nanoparticle characterization

The surface morphology and nanomechanical properties were studied using Atomic Force Microscopy (AFM), following the method described by Golek et al. (2014) [40]. Briefly, 10 μ L of NPs were put on freshly cleaved muscovite mica (Ted Pella, Inc.) and incubated at room temperature for 5 minutes. The mica surface was immediately imaged, after a drying treatment under a gentle nitrogen stream, subsequent to washing with ultrapure water. Scans were recorded using a Cypher atomic force microscope (AFM - Asylum Research, Oxford Instruments, Santa Barbara, CA), operating in a tapping-mode and furnished with a scanner at an XY scan range of 30/40 μ m (closed/open loop). AFM images without filtering were obtained and produced with silicon tetrahedral tips, fitted on 30 μ m long rectangular cantilevers (Olympus AT240TS, Oxford Instruments),

with contact AFM operating in a constant force mode, with a nominal spring constant of 2N/m and driving frequencies of 70 KHz. NP size was analyzed with MFP-3DTM software.

2.5.5 Diameter size determination by transmission electron microscope (TEM)

The morphology, composition and size of the NPs were verified by means of a transmission-electron microscope (TEM) using a Philips CM20 microscope 200 kV operating with a tungsten electron gun, using the method described by Haiss et al. (2014) [38]. The Feret diameter of the particles was determined automatically using “Image Tool” software.

2.6 Biological properties of bare and HA/pHA coated AgNPs and AuNPs

2.6.1 Bacterial strains and culture conditions

The strains used were *Escherichia coli* ATCC 9637 and *Staphylococcus aureus* ATCC 29213 from the Bacteriological Laboratory collection of Fidia Farmaceutici S.p.A. (Noto, Italy). For the assays, frozen stocks of each strain were retrieved from a – 80 °C freezer and plated on Mueller Hinton (MH) agar plates. After 24 h of incubation at 37 °C in aerobic conditions (Memmert incubator, GmbH + Co. KG, Äußere Rittersbacher Straße), a single colony for each strain was picked to inoculate 10.0 mL of MH broth and incubated overnight (16-18 h) at 37 °C. The overnight broth cultures were then used for the assay.

2.6.2 Agar diffusion assay

The putative inhibitory activity of the NPs was investigated determining the zone of inhibition by the agar diffusion assay, modifying the method described by CLSI M7-A7 for bacteria [41]. Briefly, 200 µL of each NP were deposited in 6.0 mm wells, previously punched into MH agar, where 100 µL of tested bacterial suspension were spread at the concentration of about $1.0 \times 10^{5-6}$ CFU/mL, determined spectrophotometrically (OD₆₃₀, using a spectrophotometer Synergy HT, Biotek, USA) [42]. After 24 h of incubation at 37 °C under aerobic conditions, the inhibitory effect was detected by an inhibition zone around the wells. The chloramphenicol (32 µg mL⁻¹) was used as the reference antimicrobial agent. The results were the mean of three individual experiments. The interpretative criteria used for the agar diffusion assay were as follows: very

strong = diameter of inhibition zone ≥ 18.0 mm; strong = diameter of inhibition zone (18.0; 13.0] mm; good = diameter of inhibition zone (13.0; 11.0] mm; weak = diameter of inhibition zone (11.0; 9.0] mm; very weak = diameter of inhibition zone (9.0; 8.0] mm; no activity = diameter of inhibition zone < 8.0 mm. The chemically synthesized NPs were tested at the following concentration ranges: AgNP from 9.25 nM ($= 1.05 \cdot 10^{-2}$ $\mu\text{g/mL}$) to 1.16 nM ($= 1.31 \cdot 10^{-3}$ $\mu\text{g/mL}$); HA-AgNP from 14.0 nM ($= 1.30 \cdot 10^{-2}$ $\mu\text{g/mL}$) to 1.75 nM ($= 1.63 \cdot 10^{-3}$ $\mu\text{g/mL}$); pHA-AgNP from 20.5 nM ($= 1.66 \cdot 10^{-2}$ $\mu\text{g/mL}$) to 2.56 nM ($= 2.08 \cdot 10^{-3}$ $\mu\text{g/mL}$); AuNP from 0.585 nM ($= 1.45 \cdot 10^{-5}$ $\mu\text{g/mL}$) to 0.07 ($= 1.81 \cdot 10^{-6}$ $\mu\text{g/mL}$); HA-AuNP from 0.55 nM ($= 1.37 \cdot 10^{-5}$ $\mu\text{g/mL}$) to 0.07 nM ($= 1.71 \cdot 10^{-6}$ $\mu\text{g/mL}$); pHA-AuNP from 0.24 ($= 5.93 \cdot 10^{-6}$ $\mu\text{g/mL}$) to 0.03 nM ($= 7.41 \cdot 10^{-7}$ $\mu\text{g/mL}$). The plasma synthesized NPs were tested at the following concentration ranges: AgNP from 5.82 nM ($= 3.17 \cdot 10^{-4}$ $\mu\text{g/mL}$) to 0.73 nM ($= 3.96 \cdot 10^{-5}$ $\mu\text{g/mL}$); HA-AgNP from 5.51 nM ($= 2.61 \cdot 10^{-4}$ $\mu\text{g/mL}$) to 0.69 nM ($= 3.27 \cdot 10^{-5}$ $\mu\text{g/mL}$); pHA-AgNP from 3.60 nM ($= 1.02 \cdot 10^{-4}$ $\mu\text{g/mL}$) to 0.45 nM ($= 1.27 \cdot 10^{-5}$ $\mu\text{g/mL}$); AuNP from 29.80 nM ($= 7.36 \cdot 10^{-4}$ $\mu\text{g/mL}$) to 3.72 nM ($= 9.20 \cdot 10^{-5}$ $\mu\text{g/mL}$); HA-AuNP from 19.54 nM ($= 4.83 \cdot 10^{-4}$ $\mu\text{g/mL}$) to 2.44 nM ($= 6.04 \cdot 10^{-5}$ $\mu\text{g/mL}$); pHA-AuNP from 2.04 nM ($= 5.04 \cdot 10^{-5}$ $\mu\text{g/mL}$) to 0.25 nM ($= 6.30 \cdot 10^{-6}$ $\mu\text{g/mL}$); chloramphenicol was tested at the concentration range from 124 to 0.5 $\mu\text{g/mL}$.

2.6.3 Broth microdilution assay

A broth microdilution assay using 96-well plates was performed for determining the MIC (Minimum Inhibitory Concentration) End Points for each NP against standard strains *Escherichia coli* ATCC 9637 and *Staphylococcus aureus* ATCC 29213, according to CLSI M7-A7 [41]

For the assay, individual bacterial colonies, pre-cultured overnight on MH agar (Oxoid, Italy), were checked for purity and suspended in 5.0 mL of sterile NaCl 0.85% w/v solution, until they reached a density corresponding to McFarland 1 (3.0×10^8 CFU/mL) with an equivalent OD_{630} of 0.16 – 0.2 (Biotek Synergy HT, USA). The saline bacterial suspension was then diluted in MH broth to obtain a final concentration of about 5.0×10^5 CFU/mL in each well. The positive control was MH broth inoculated with the tested strain; the negative control was sterile MH broth. The 96-well plates were incubated as described by CLSI M100-S23 [43]. The absence of growth was confirmed by spreading 100 μL from each well on MH agar in which bacterial growth seemed inhibited. The experiments were carried out twice in duplicate.

2.6.4 Cell lines and treatment culture and maintenance

Human Umbilical Vein Endothelial Cells (HUVEC - CRL-1730 - from America Type Collection) were grown in tissue-culture Corning flasks (Sigma-Aldrich, St. Louis, MO) using Human Large Vessel Endothelial Cell Basal Medium (Medium 200) supplemented with Low Serum Growth Supplement (LSGS, composed of 2% v/v fetal bovine serum, 1 µg/mL hydrocortisone; 10 ng/mL human epidermal growth factor; 3 ng/mL basic fibroblast growth factor; 10 µg/mL heparin; Thermo Fisher Scientific). The incubation was performed in a humidified atmosphere (95% Air, 5% CO₂) at 37 °C (Heraeus Hera Cell 150C incubator). The maintenance of the cells was performed according to the protocol described by Koob et al. (2014) [44]. Before the assay, the HUVEC cells at a density of 1×10^4 cells/well were seeded in 96-well cell culture plates (Cellstar, Sigma Aldrich, USA). For the treatment, the NPs pellets were diluted using Medium 200, added with cell lines and incubated for 24 hours at the cell growth conditions.

2.6.4.1 Determination of bare and HA/pHA coated AgNPs and AuNPs toxicity

To find a safe and non-toxic amount of bare and HA/pHA coated AgNPs and AuNPs the 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazoliumbromide (MTT) assay was performed. The reaction was carried out by adding 0.5 mg/mL of MTT to each well. The absorbance was measured at 490 nm (Varioskan® Flash Spectral Scanning Multimode Readers, Thermo Scientific, USA) after 90 min when the reaction was stopped using 150 µL of DMSO for each well. The results were expressed as the percentage of viable cells referred to the concentration of each compound.

2.6.5 Effects of NPs on collagenase activity

The effect of tested nanoparticles on the enzymatic activity of collagenase was determined spectrophotometrically by quantifying the fragment produced by the enzymatic reaction against an external standard. The assay was performed by modifying the method described by Wunsch–Heidrich et al. (1963) [45, 46] Briefly, the enzymatic activity of collagenase was detected spectrophotometrically (absorbance of fragment at 320 nm) by measuring the production of the glycil-L-prolil-D-arginine fragment (yellow) produced from the PZ-L-prolil-L-leucyl-glicil-L-prolil-D-arginine (PZ = 4-phenylazobenzyloxycarbonyl) synthetic

substrate, after extraction with a solution of ethyl acetate acidified with citric acid. For the assay, a collagenase solution (ranging from 0.35 to 1.2 nkatal/ml) was prepared using a buffer solution of 25 mM TRIS-HCl and 10 mM CaCl₂ (pH 7.1 adjusted using HCl). The enzymatic reaction between collagenase and 1.23 mM of substrate solution (PZ-L-prolil-L-leucyl-glicil-L-prolil-D-arginine) was carried out for 15 minutes at 37 °C in a water bath and two fragments, PZ-L-prolil-L-leucine and glicil-L-prolil-D-arginine, were obtained. The glicil-L-prolil-D-arginine (yellow) fragment was extracted using ethyl acetate solvent in the presence of citric acid. The organic solution was dehydrated using anhydrous Na₂SO₄ and the absorbance was determined spectrophotometrically (at 320 nm, using a UV-1200 spectrometer). The substrate solution mixed with the buffer solution was used as the blank. The enzymatic activity was quantified as follows:

$$\text{Activity [nkat/mL]} = \frac{\text{Sample Abs}_{320} \times \text{Standard Conc. } [\mu\text{moles/mL}]}{\text{Sample Abs}_{320}} \times \frac{50 \times 1000}{900} \times df \quad (5)$$

where *standard concentration* was 0.02 $\mu\text{moles/mL}$, 50 was the sample *dilution factor*, 1000 was the *conversion factor* from μmoles to nmoles , 900 was the number of seconds in 15 minutes, *df* was the dilution factor of collagenase to obtain a solution with a final activity ranging from 0.35 to 1.2 nkat/mL.

The results are expressed as a relative percentage of collagenase activity (%A) calculated as follows:

$$\%A = \frac{\text{Activity of treated sample}}{\text{Activity of untreated sample}} \times 10 \quad (6)$$

3. Results

3.1 Chemical and spectroscopical characterization of HA conjugate (pHA)

The ¹H-NMR spectrum of Hyaluronic Acid-GHHPHGK-conjugate (pHA) is shown in Fig. 2 and displays the following characteristic signals: 8.68 ppm (s, 3H, H-2) referred to the imidazole ring, 7.41, 7.35 and 7.31 ppm (s, 3 H totally, H-5) referred to the imidazole ring, 5.03 ppm (br s, CH of H), 4.6-4.5 ppm (br m, H-) referred to glucuronic acid and H-1 referred to N-acetylglucosamine, 4.31 ppm (br s, CH of Lysine), 4.01 ppm (br s, CH of P), 3.9-3.2 ppm (br m) H-2, H-3, H-4 and H-5 referred to glucuronic acid; H-2, H-3, H-4, H-5, H-6 referred to N-acetylglucosamine, CH₂ of G, CH₂ 5 a and b of P, CH₂ of H), 3.13 ppm and 3.03 ppm (br m, ϵ

CH₂ of Lysine), 2.36 ppm (br m, CHa-3 of P), 2.11-1.95 ppm (br m, CHa-4 of P and CH₃ referred to N-acetylglucosamine, 1.9-1.7 ppm (br m, CHb-3, CHb-4 of P and β CH₂ of Lysine), 1.49 ppm (br m, δ CH₂ of K), 1.10 ppm (br m, γ CH₂ of Lysine) (Fig. 2). The amide bond formation between the free carboxylic group of HA residue and the amino group of the Lysine side chain was underlined by a resulting upfield shift ($\Delta\delta = -0.10$ ppm) of ϵ -methylene protons ($\delta = 3.13$ ppm) relative to the Lysine-NH₂ moiety, as compared to the corresponding resonance of the non-conjugated peptide ($\delta = 3.03$ ppm).

The conjugate shows all the expected signals from the protons of peptide and hyaluronic acid units; all proton resonance signals were completely assigned. The amount of GHHPHGK peptide conjugated with HA results as being $\sim 15\%$.

Figure 2. ¹H-NMR spectrum of the Hyaluronic Acid-GHHPHGK conjugate (pHA)

3.2 Physicochemical characterization of bare and HA/pHA coated AgNPs and AuNPs

3.2.1 UV-Visible and Dynamic light scattering nanoparticle characterization

Fig. 3 shows the absorbance in dependence of λ for chemically (*a* and *a.1*) and plasma (*b* and *b.1*) synthesized AgNP, AuNP, HA-AgNP, pHA-AgNP, HA-AuNP, pHA-AuNP. The surface plasmon resonance is visible as a peak in the range between 395.3 and 397.5 nm for bare AgNP and between 535.0 and 546.0 nm for bare chemically synthesized AuNP (c AgNP, c AuNP) and those coated with HA and pHA (HA- c AgNP, pHA- c AuNP). The AgNP peaks showed a decrease in Abs_{max} compared to the bare ones when coated with HA and pHA.

The surface plasmon resonance is visible as a peak in the range between 407.5 and 411.1 nm for bare AgNP and between 523 and 534 nm for bare AuNP prepared by plasma assisted synthesis (p AgNP, p AuNP) and those coated with HA and pHA (HA- p AgNP, pHA- p AuNP). All plasma prepared NPs were more polydispersed and red-shifted (Fig. 3). The red-shift between chemically and plasma synthesized HA-AgNP ($\Delta\lambda_{\text{max}}$ of 2.2 and 3.6 nm, respectively) and pHA-AgNP ($\Delta\lambda_{\text{max}}$ of 1.8 and 1.2 nm, respectively) was noteworthy. This effect could be due to the extra interactions between the histidine group of the peptide and the Ag⁺ ions during seed formation, resulting in a thicker shell for pHA with respect to HA. The FWHM (Full Width at Half Maximum) of the plasmon peaks ranged between 63 and 72 for c AgNPs and from 87 and 99 for plasma prepared ones; the larger size of the optical diameters was due to the HA and pHA shell around the metallic core. The peak of bare p AgNPs was damped with respect to those obtained by chemical synthesis. The peaks of HA- c AgNP, pHA- c AgNP were almost overlapped. The c AgNP shows a linear variation of the λ_{max} as a function of HA presence following a different trend compared to p AgNP which produced a high redshift when HA is conjugated with antiangiogenic peptide GHHPHGK (pHA).

The Abs_{max} peak of AuNPs was right shifted with respect to those of AgNPs for both chemically and plasma synthesized ones. The Abs_{max} peak of p AuNPs was left shifted with respect to the chemically synthesized ones. For both syntheses it is possible to observe a red shift of the λ_{max} of HA-AuNP compared to AuNP, relative to the increase in NP optical diameter (Fig. 3). This red shift is more intense for pHA-AuNP (1.1 nm for both chemically and plasma synthesized), a linear variation of the λ_{max} was observed for c AuNP with respect to plasma synthesized ones, as a function of the HA presence. The broad plasmon band for pHA- c AuNP is evidence of the presence of several populations of various sizes. On the contrary, p AuNP and HA- p AuNP

exhibited a smaller size than the $^p\text{AgNPs}$. The $p\text{HA-}^p\text{AuNP}$ plasmon band points to the formation of a monodisperse solution of larger NPs. These differences could be due to the different charge of the respective metal precursors salts, monovalent silver and trivalent gold. The hydrodynamic diameter is higher than the optical size except for glucose-capped $^c\text{AuNP}$ and $\text{HA-}^c\text{AuNP}$ which showed quite similar values. The glucose used as reducing agent for synthesis of $^c\text{AuNP}$ could contribute to forming a dense and rigid shell around the nanoparticles which roughly corresponds to the hydration shell around the NPs.

Parameters	Chemical synthesis						Plasma synthesis					
	AgNP	HA-AgNP	pHA-AgNP	AuNP	HA-AuNP	pHA-AuNP	AgNP	HA-AgNP	pHA-AgNP	AuNP	HA-AuNP	pHA-AuNP
$\lambda_{\max}$ (nm)	395.3	397.1	397.5	535.0	543.0	546.0	407.5	408.7	411.1	523.0	524.0	534.0
$\text{Abs}_{\max}$	0.261	0.154	0.163	0.327	0.262	0.162	0.144	0.217	0.171	0.238	0.246	0.208
FWHM	66	63	72	53	70	101	87	89	99	51	58	59
d_0 (nm)	12	14	14	55	76	84	33	35	38	22	24	52
Δd_0 %	-	14	14	-	28	38	-	6	13	-	8	58
$[\text{nM}] \times 10^{-9}$	1.58	1.08	0.93	0.03	0.03	0.01	0.07	0.09	0.06	0.83	0.58	0.09
[NP/mL]	$1.67 \cdot 10^{10}$	$7.58 \cdot 10^9$	$5.87 \cdot 10^8$	$2.42 \cdot 10^3$	$6.53 \cdot 10^2$	$3.42 \cdot 10^2$	$3.69 \cdot 10^7$	$4.00 \cdot 10^7$	$1.85 \cdot 10^7$	$1.26 \cdot 10^9$	$6.16 \cdot 10^8$	$1.22 \cdot 10^7$

3.2.2 ATR-FTIR nanoparticle characterization

The ATR-FTIR spectra of chemically synthesized HA-AgNP (HA-^cAgNP) are 3311 cm^{-1} (-OH stretching; -NH stretching), 2923 cm^{-1} (-CH₂ stretching), 1605 cm^{-1} (-C=O stretching, -NH bending), 1408 cm^{-1} (-CH₂ bending), 1027 cm^{-1} (-C-O-C- stretching). The variations in -OH/-NH and -CH₂ stretching with respect to the corresponding HA indicate the different electronic interaction of this group of HA with the metal ^cAgNPs (Fig. 4).

When the HA-AgNP is synthesized by plasma (HA-^pAgNP) the IR spectra values are: 3460 cm^{-1} (-OH stretching; -NH stretching), 3289 cm^{-1} (-CH₂ stretching), 1604 cm^{-1} (-C=O stretching, -NH bending), 1405 cm^{-1} (-CH₂ bending), 1095 cm^{-1} (-C-O-C- stretching). (Fig. 4). There is a noteworthy variation in -CH₂ stretching of HA-^pAgNP, in -C=O stretching, -NH bending and in -C-O-C- stretching for HA-^pAuNP with respect to the corresponding signal for HA. These results could be due to the different interactions between NP plasma synthesized with the carboxylate (-COO⁻) and amide (-NH) groups of HA (Fig.4).

The IR values for HA-^cAuNP are: 3277 cm^{-1} (-OH stretching; -NH stretching), 2905 cm^{-1} (-CH₂ stretching), 1605 cm^{-1} (-C=O stretching, -NH bending), 1409 cm^{-1} (-CH₂ bending), 1027 cm^{-1} (-C-O-C- stretching) (Fig. 4). The IR values for HA-^pAuNP are: 3459 cm^{-1} (-OH stretching; -NH stretching), 2943 cm^{-1} (-CH₂ stretching),

1669 cm^{-1} (-C=O stretching, -NH bending), 1467 cm^{-1} (-CH_2 bending), 1156 cm^{-1} (-C-O-C- stretching) (Fig. 4).

Figure 4. IR/ATR spectra of chemically synthesized NPs coated with HA (200 kDa). (a) AgNPs and (b) AuNPs. (c) Schematic illustration of primary interaction between synthesized AgNP plasma and hyaluronan.

Parameters	Chemical synthesis					Plasma synthesis				
	HA	AgNP	HA-AgNP	AuNP	HA-AuNP	HA	AgNP	HA-AgNP	AuNP	HA-AuNP
-OH/-NH stretching	3279	-	3311	-	3277	3414	-	3460	-	3459
-CH ₂ stretching	2890	-	2923	-	2905	2895	-	3289	-	2943
-C=O stretching -NH bending	1603	-	1605	-	1605	1556	-	1604	-	1696
-CH ₂ bending	1399	-	1408	-	1409	1372	-	1405	-	1467
C-O-C stretching	1033	-	1027	-	1027	1011	-	1095	-	1156

Table 2. Peak position (cm^{-1}) of the characteristic transmittance spectra of the hyaluronic acid coating chemically and plasma synthesized NPs

3.2.3 Atomic Force Microscopy nanoparticle characterization

The atomic force microscopy (AFM) images of HA (200 kDa) show a layer of macromolecules < 0.5 nm thick, indicating an aggregation force between chains, as well as rather strong hydrophilic interactions between the mica substrate and HA, typical of the low molecular weight. The stiffness of the HA molecules is apparent and

is due to dehydration (Fig. 5 a-c). HA-AgNP and HA-AuNP show the HA monolayer around the core of aggregated NPs, especially for HA-AuNPs with respect to HA-AgNP. The dimensions of the height images were 25 nm and 60 nm for HA-AgNPs obtained by chemical synthesis and 27 nm and 30 nm for plasma synthesized ones, with different microscopic characteristics typical of globular hybrid-polymer metal nanoparticle systems (Fig. 5).

3.2.4 Diameter size determination by transmission electron microscope (TEM)

The chemically and plasma synthesized AgNPs and AuNPs showed two different aging trends: AgNP aging (with and without HA) shows $\lambda_{\max}$ red-shift of the plasmonic peaks (in all cases the $\Delta\lambda_{\max} < 1$ nm) The synthesized AuNP plasma showed a $\Delta\lambda_{\max}$ decrease and a $\Delta\lambda_{\max} < 1$ nm in all cases, except for HA-AuNP which shows a $\Delta\lambda_{\max} = 1.3$ nm, at an aging time of 15 days. The AuNP suspension contained aggregates of various sizes at the initial growth stage. The TEM images (Fig. 5) show a size decrease for the HA-AgNP plasma synthesized after three days of aging and a tight size range (20-30 nm) for 46% of the NPs; 20% showed a size ranging between 10 and 20 nm, while 0% of NP showed a range between 0 and 10 nm.

Figure 5. Microscopy characterization of NPs. AFM images of chemically and plasma synthesized HA, HA-AgNP, HA-AuNP, AgNP, AuNP. (a) Phase, (b) amplitude; (c) height images. TEM image of plasma synthesized (d) HA-AuNP, (e) and (f) HA-AuNPs aged 3 days.

3.3 Biological properties

3.3.1 Antibacterial activity

The MIC values of all the tested NPs were lower than those of chloramphenicol (CHL); NPs were more active against *Escherichia coli* ATCC 9637 and no significant differences were observed for HA and *p*HA coated NPs. AuNP was less toxic with respect to AgNP.

With the agar diffusion method, the antibacterial activity of NPs was shown to be lower with respect to that observed using the broth microdilution method and AuNPs did not show any inhibitory activity. These results could be due to interactions between NPs and the agar mesh. All chemically synthesized AgNPs (^cAgNPs, HA-^cAgNPs, *p*HA-^cAgNPs) showed a clear inhibition zone for both tested strains (Fig. 6 A). Plasma synthesized AgNPs (^pAgNPs) did not show any inhibitory activity. However, when coated with HA and *p*HA (HA-^pAgNPs, *p*HA-^pAgNPs), *p*HA-^pAgNP showed a very strong (ZOI = 18.0 mm) inhibitory activity against *E. coli* ATCC 9637 and a strong (ZOI = 15.0 mm) inhibitory activity against *S. aureus* ATCC 29213. This activity is six-fold higher than that of *p*HA-^cAgNPs. HA has a notable effect on antibacterial activity for plasma synthesized nanoparticles. The HA-^pAgNPs showed a strong (ZOI = 15.0 mm) inhibitory activity against *E. coli* ATCC 9637 and a good (ZOI = 13.0 mm) inhibitory activity against *S. aureus* ATCC 29213. This activity is two-fold higher than those of *p*HA-^cAgNPs.

Using the broth microdilution method, NPs were shown to be more active against *E. coli* ATCC 9637, confirming the results obtained using the agar diffusion method (Fig. 6 A.1). The highest inhibitory activity was shown towards *E. coli* ATCC 9637 by *p*HA-^cAuNP (MIC = 0.24 nM) followed by HA-^cAuNP (MIC = 0.55 nM), ^cAuNP (MIC = 0.58 nM) and *p*HA-^pAgNP (MIC = 0.90 nM). The ^cAuNP, HA-^cAuNP and *p*HA-^cAuNP showed the same MIC values against *S. aureus* ATCC 29213. AgNPs, HA-^pAgNPs and *p*HA-^pAgNPs did not show any activity in broth solution. These results are dependent on HA molecular weight (MW); in fact, using a high MW, HA was shown to be less active (data not shown). Moreover, the surface characteristics of ^pNPs improve their antibacterial activity, particularly against Gram-negative strain, which have a thin peptidoglycan layer and an outer lipid membrane in their cell walls

3.3.2 Cytotoxic activity on Human Umbilical Vein Endothelial Cells (HUVEC)

The results of the MTT (tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on Human Umbilical Vein Endothelial Cells (HUVEC) showed that cell viability is a function of NPs and of the final yield depending on the method used for the synthesis (Fig.6 B). The NPs obtained by the chemical method showed a higher toxicity with respect to those obtained by plasma technology (cell viability $\geq 69\%$ and $\geq 83\%$, respectively). The HA and HA conjugated with GHHPHGK (*p*HA) at the highest tested concentration increased the cytotoxicity of chemically synthesized AgNP (80 % and 69.11 % vs 84.47 %). The bare and *p*HA coated NPs reduced HUVEC viability by $\sim 20\%$ ($p < 0.01$). The plasma synthesized NPs did not show significant cytotoxicity with respect to the untreated control.

3.3.3 Effect on collagenase activity

After treatment with AgNPs, collagenase activity was reduced by 20% (Fig. 6 C). In the presence of plasma synthesized AgNPs, collagenase activity is higher respect to what determined in presence of chemically synthesized NPs (91% vs 84,57%). Nevertheless, collagenase activity stays at high levels when chemically synthesized AgNPs are coated with HA (HA-AgNP).

Figure 6. Biological properties of chemically and plasma synthesized NPs. (A) Clear inhibition zones of chemically and plasma synthesized NPs. (a) Mueller Hinton (MH) agar plate spread with *Escherichia coli* ATCC 9637. (b) MH agar plate spread with *Staphylococcus aureus* ATCC 29213. (a.1 and b.1) Treatment with chemically synthesized AgNP (9.25 nM = $1.05 \cdot 10^{-2}$ $\mu\text{g/mL}$); (a.2 and b.2) treatment with chemically synthesized HA-AgNP (14.0 nM = $1.30 \cdot 10^{-2}$ $\mu\text{g/mL}$); (a.3 and b.3) treatment with chemically synthesized pHA-AgNP (20.5 nM = $1.66 \cdot 10^{-2}$ $\mu\text{g/mL}$); (a.4 and b.4) treatment with plasma synthesized AgNP (5.82 nM = $3.17 \cdot 10^{-4}$ $\mu\text{g/mL}$); (a.5 and b.5) treatment with plasma synthesized HA-AgNP (5.51 nM = $2.61 \cdot 10^{-4}$ $\mu\text{g/mL}$); (a.6 and b.6) treatment with plasma synthesized pHA-AgNP (3.60 nM = $1.02 \cdot 10^{-4}$ $\mu\text{g/mL}$). (A.1) MIC values of chemically and plasma synthesized NPs against *E. coli* ATCC 9637 and *S. aureus* ATCC 29213, expressed in nM and $\mu\text{g/mL}$, shown in round brackets. (c) Microplate, after 1 h of incubation the wells contain plasma synthesized AuNP, HA-AuNP and pHA-AuNP. A turbidity probably due to bacterial cell destruction is visible. (B) Cell viability percentage of Human Umbilical Vein Endothelial Cells (HUVEC) after 24 h of incubation with NP, HA-NP and pHA-NP at different concentrations. The black and grey full bars are for chemically synthesized NPs; the bars with black and grey diagonal stripes are for plasma synthesized NPs. The AgNP, HA-AgNP, pHA-AgNP, AuNP, HA-AuNP, pHA-AuNP at highest tested concentration are black colored, the lowest tested concentrations for each NPs are grey colored. The untreated cells showed 100% viability. The results are expressed as the mean $\pm$ SD of triplicate experiments and normalized to the values for the untreated control. The statistical significance with respect to the untreated control calculated by one-way analysis of variance was: * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$. (C) Percentage of collagenase activity after treatment with chemically (black solid bars) and plasma (empty bars) synthesized nanoparticles compared with collagenase alone (ctrl). The significant differences in collagenase activity compared to the control are indicated as follows: * = $p < 0.05$; ** = $p < 0.01$.

4. Conclusion

The results collected in this study lead to the consideration of cold plasma technology as an efficient method for the synthesis of gold and silver nanoparticles, which is also applicable in the industrial field. The nanoparticles obtained were stable and the physisorption with Hyaluronic Acid contributed to modulating their aggregation kinetic and stabilizing their biological properties, especially against Gram-negative bacteria, maintaining their safety profile when tested on HUVEC cells. Our research on the synthesis of nanoparticles composed of noble metals provides promising bioactive compounds with reduced side effects, using substances that could constitute a reference for the fight against bacterial resistance, the elusive mechanisms of onco-related pathologies and other ailments where these systems have been shown to be applicable.

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7. Reference

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