

Biomolecules from Snail mucus (*Helix aspersa*) conjugate Gold nanoparticles, exhibiting potential wound healing and anti-inflammatory activity

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Abstract

In this work, for the first time, snail slime from garden snails “*Helix Aspersa Müller*”, has been used to induce the formation of eco-friendly gold nanoparticles (AuNPs-SS), suitable for biomedical applications. An AuNPs-SS comprehensive investigation was performed observing AuNPs with an average particle size of 14 ± 6 nm, stabilized by a slime snail-based organic layer. Indeed, as recognised in high-resolution MALDI-MS analyses, and corroborated by FESEM, UV-Vis, ATR-FTIR and XPS results, it was possible to assess the main presence of peptides and amino acids as main components of the slime, that, combined with the AuNPs confers them interesting properties. More specifically, we tested, *in vitro*, the AuNPs-SS safety in human keratinocytes and their potential effect on wound healing as well as their anti-inflammatory properties in Murine Macrophages. Moreover, the AuNPs-SS treatment resulted in a significant increase of the urokinase-type plasminogen activator receptor (uPAR), essential for keratinocyte adhesion, spreading and migration, together with the reduction of LPS-induced IL1- β and IL-6 cytokine levels, and completely abrogated the synthesis of the inducible nitric oxide synthase (iNOS).

1. INTRODUCTION

The human body has different ways for protecting itself against potential injuries and infections, by means of the skin characteristics acting as an effective barrier. The physiological process of tissue repair at an injured site is the wound healing, which represents a complex integrated system of biological and molecular processes, such as cell migration, proliferation, extracellular matrix deposition and remodelling.[1] Under normal conditions, the healing process cascade is initiated by an inflammatory phase yielding to granulation tissue accumulation and scar remodelling after epithelial wound closure has been accomplished.[2] A normal wound healing process is constituted by various phases: coagulation (hemostasis), inflammation, cellular migration and proliferation, protein synthesis and wound contraction (fibroplasia), and scar formation (remodelling). Among these phases, three are the most important for a successful wound healing: fibroplasia, angiogenesis and re-epithelialisation. A delay in the development of these mechanisms leads to failure or prolong wound healing process with a crucial part attributable to the wound neovascularisation.[3,4] However, any change

or pathological problem (for example ischemia, diabetes mellitus and so on) may result in the formation of chronic wounds difficult to heal, due to the failure of the normal wound healing process.[3,5] Another very important objective of the wound treatment is the inhibition of all the pathogenic organisms able to cause serious infection, by accelerating the healing process with reducing cicatrices and pain to the patients of all ages.[5] In fact, any delay during the tissue repair process could be defined as chronic injury and can have a highly detrimental impact on human health being more subjected to pathogens. Rapid closure of dermatological wounds is of vital importance in preventing infection and reducing post-treatment side-effects.[6] So, shortening the chronic wound healing process is a prime challenge in modern dermatology.[7]

Several different topical and systemic agents, together with the antimicrobial ones have been developed for improving the cure of surgical and traumatic wounds. However, these functional topical biomaterials might delay re-epithelialisation and might result a risk for oversensitivity and resistance.[4] Hence, some alternative wound treatment methods have been searched for and, in this regard, the use of nanotechnology could be useful as a prominent scientific discipline in the technological revolution of this millennium.[8] Nanobiotechnology-based therapies in the field of wound healing are considered to be a promising area with new benefits based on the utilization of bionanomaterials in promoting wound healing, including antibacterial, antiinflammatory, regulating extracellular matrix production, promoting stem cell proliferation and differentiation, and enhancing growth factors.[9] Researches using gold nanoparticles (AuNPs) of different sizes and morphologies have been proposed in biomedical applications for skin injury therapy with great potential.[6] AuNPs have received great attention due to their low toxicity to animal and microorganism cells compared to other metal nanoparticles,[10] promoting their use as drug delivery systems, markers, and photothermal agents, as well as radiotherapy enhancers as well.[6] Recent studies have established new prospects for developing properly functionalized AuNPs for wound healing, acting as triggers to activate proliferation and migration of wound cells.[7,11,12] The AuNPs use should exhibit benefits including enhanced mechanical stability and resistance against enzymatic degradation when incorporated into tissue scaffolds, in presence of antibodies, growth factors, and peptides, rendering them promising candidates for skin tissue engineering applications.[8]

Chemical methods, in which reduction or co-precipitation processes take place for the generation of nanoparticles, have been investigated since ancient times. However, the main drawbacks of these synthetic routes are the use of harsh chemical conditions of work, organic solvents and the production of toxic by-products during the synthesis and/or functionalization of the nanoparticles. For this purpose, many studies are focused on finding alternative therapeutic approaches by adopting biocompatible, safer and greener methods employing plant extracts for the fast and economical synthesis of biogenic nanogold.[13-15] Green synthesized AuNPs can also exhibit anticancer and antimicrobial activity,[16] antioxidant moisture retention, skin lightening properties,[17] and sunscreen ability, enlarging their applicability.[18,19] As reported by Gonnelli *et al.* ,[20] synthetic routes relying on green chemistry show other advantages for large scale production adopting mild conditions of work.[20] For green synthesized AuNPs, additional assembling steps are not necessary since the AuNPs properties are ascribed to main components present on their surface and used to induce their formation. Thus, considering these factors, the snail secretion (SS) from *Helix Aspersa* , currently revolutionizing the world of cosmetics and human skin care, has been used in this work for synthesizing AuNPs (AuNPs-SS), conferring them interesting properties. Indeed, the SS protein content plays a key role in cell regeneration and growth, preventing the effect of the inflammatory disease.[21,22] The SS main compounds are used as reducing agents enabling the AuNPs synthesis via reduction of Au(III), from a HAuCl_4 solution, to Au(0), while forming an organic layer around the AuNPs. AuNPs-SS are thus synthesized by using a single step reaction, avoiding the use of additional and toxic agents. The nanoconfined main components of the SS on the AuNPs surface could be an interesting way for improving the whole process, combining the properties of gold and SS. For example, Conte *et al.* [23] reported that the pharmacokinetics of snail components do not permit their optimal absorption, so the combined use of AuNPs, well-known antibacterial agents, and SS could be a functional ingredient in the treatment of acne and in biotechnology, or used as a preservative in cosmetic products formulation. Considering the overall literature published,[21,22,24-27] although interesting information can be found on the SS use, the presence of fragmentary and not detailed investigations suggests that

this research field has not been extensively explored yet. In a time when cosmetic dermatology and beauty care are increasingly influential,[28,29] we intend to discuss the biogenic formation of AuNPs-SS by characterizing them and by applying as accelerating wound healing and anti-inflammatory agents in biomedicine. More specifically, we tested the effect of AuNPs-SS on improving wound healing in human keratinocytes. Furthermore, considering that gold has been used historically in the treatment of painful inflammatory conditions, such as rheumatoid arthritis, and snail slime has been proved to clear up skin inflammation, we evaluated the potential anti-inflammatory properties of AuNPs-SS in Murine Macrophages.

2. EXPERIMENTAL SECTION

2.1 Chemicals.

HAuCl₄, and KCl used for Zeta potential measurements were purchased from Sigma-Aldrich (Milan, Italy). The Snail Slime was received from the Società Agricola Dap, di Francesco Paolo Perrotta & C. S.a.s - via Diego Rapolla, - 85029 Venosa (Potenza, Basilicata, Italy). NCTC2544 human keratynocytes and RAW 264.7 macrophages were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained at 37°C in DMEM media (Euroclone) in a humidified 5% CO₂ incubator maintained at 37°C (Thermo Scientific, Waltham, MA). For RAW 264.7 cells, the complete media contained L-glutamine, 100 U penicillin or streptomycin/ml, and 10% inactivated fetal calf serum (FBS, Euroclone). In the LPS stimulated macrophage experiments, RAW 264.7 cells were pre-treated overnight with 1µg LPS/mL. The day after, the LPS was removed and the cells replaced with fresh media in presence of AuNPs-SS.

2.2 Mechanical extraction of snail slime.

The snail *Helix Aspersa Muller* is the terrestrial gastropod mollusk that in the adult phase has a shell of a 3.5 cm diameter for a weight of about 15 g. Among mechanical methods that do not damage the animal, the use of a natural gas as ozone was selected to induce the production of snail slime. The mechanical extraction involved the use of an equipment that within a few hours allow to obtain the slime. More specifically, the snails were inserted into a machine in which ozone was nebulized for about an hour; the O₃ generates a sort of excitement that induces the slime production avoiding the stress of the snail.

2.3 AuNPs-SS synthesis.

HAuCl₄ was solubilized in deionized water to obtain a 1.0×10^{-3} stock solution. 1.5 mL of this solution were mixed with 2 mL of water and 500 µL of the received SS. The mixture was moderately stirred at room temperature. The UV-Vis spectra of this solution were carried out soon after the preparation and at 4, 6, 24, 48 and 72 h after, to monitor the AuNPs-SS formation. The induced AuNPs-SS were purified at each acquisition time, and the excess SS and unreacted HAuCl₄ were removed by washing in fresh water and centrifuging (7000 g, for 10 minutes). Before the spectra acquisition, the obtained solutions were properly diluted if necessary.

2.4 UV-Visible measurements.

The Visible absorption spectra of solutions were collected using a Varian CARY 5 UV-Vis-NIR spectrophotometer (Varian Inc., now Agilent Technologies Inc., Santa Clara, CA, USA) in the range 200-800 nm. Measurements were performed using a cuvette with a 1 cm path length.

2.5 ATR-FTIR spectroscopic measurements.

ATR-FTIR spectra were recorded in a 600–4000 cm⁻¹ range, at a resolution of 4 cm⁻¹, using a Fourier Transform Infrared spectrometer 670-IR equipped with an ATR device (Varian Inc., now Agilent Technologies Inc., Santa Clara, CA, USA). 32 scans were summed for each acquisition.

2.6 Field Emission Scanning Electron Microscopy (FESEM).

A Zeiss SUPRA 40 field emission scanning electron microscope was used for the morphological investigation of the AuNPs-SS. FESEM images were acquired using a high resolution in-lens secondary electron detector at the electron acceleration voltage (extra-high tension, EHT) of 20 kV, working distance of 4 mm and

magnification in the range 100–1000k \times . The size distribution of AuNPs as well as the thickness of the organic layer covering the AuNPs was estimated by FESEM images. For FESEM and XPS investigation, AuNPs-SS were dispersed in water, deposited by drop-casting onto small pieces of a Si(100) wafer, and analyzed after solvent evaporation at room temperature.

2.7 X-ray Photoelectron Spectroscopy (XPS).

XPS analyses were performed using a PHI VersaProbe II spectrometer equipped with a monochromatic Al K α X-ray source (1486.6 eV) operated at a spot size of 100 μ m corresponding to a power of 24.5 W. Survey (0–1400 eV) and high resolution (C 1s, O 1s, N 1s, Au 4f) spectra were recorded in FAT (fixed analyzer transmission) mode at pass energy of 117.4 and 46.95 eV, respectively. All spectra were acquired at a take-off angle of 45 $^\circ$ with respect to the sample normal. Dual-beam charge neutralization was constantly applied during analysis. XPS analysis was repeated on three different spots of the sample. Special attention was devoted to verify that no change in the sample was induced by exposure to the X-ray beam and to the dual-beam used for charge neutralization. Detailed spectra processing was performed by commercial MultiPak software (Version 9.5.0.8, 30-10-2013, Ulvac-PHI, Inc.). Charge correction of the spectra was performed by taking the hydrocarbon (C-C/C-H) component of the C 1s spectrum as internal reference (binding energy, BE = 285.0 eV). The high-resolution spectra were fitted with mixed Gaussian-Lorentzian peaks after a Shirley background subtraction. A maximum relative standard deviation of 10% was estimated on the area percentages of the curve-fitting components, while the determined standard deviation of their position was ± 0.2 eV.

2.8 Zeta potential and size measurements.

The average of the hydrodynamic diameters and the polydispersity index (PDI) of the AuNPs-SS were measured by dynamic light scattering and using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The surface charge of AuNPs-SS was measured as zeta potential by laser doppler velocimetry using the same instrument after dilution with 1 mM KCl.

2.9 Mass spectrometry analysis.

High-resolution mass spectra were acquired on a Bruker (Bruker Daltonik GmbH, Bremen, Germany) solariX XR Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) equipped with a 7T superconducting magnet and a MALDI source. Spectra were acquired with a Time Domain size of 16 megaword, an accumulation time of 0.1 s and a mass range of 100–2000 m/z. Moreover, the average number of scans was set to 50. For the analysis, a laser power of 32% and a number of laser shots of 28 were assumed. 2,5-dihydroxybenzoic acid was used as a matrix, while a solution of this compound and pure snail slime (or AuNPs slime sample), in a 1:1 ratio, was prepared and analyzed. The mass measurement accuracy reached values of less than 0.1 ppm.

2.10 Cell viability determination .

The viability of NCTC2544 human keratinocyte and RAW 264.7 macrophages was determined by trypan blue staining. NCTC2544 cells (2.0×10^5) were seeded in 6-well plates and allowed to attach overnight. On the next day AuNP colloidal solutions were added at the indicated concentrations. 48 h later 20 μ l of cells were aseptically transferred to a 1.5 mL clear Eppendorf tube and incubated for 3 min at room temperature with an equal volume of 0.4 % (w/v) trypan blue solution prepared in 0.81 % NaCl and 0.06 % (w/v) dibasic potassium phosphate. Viable and nonviable cells (trypan blue positive) were counted separately using a dual-chamber hemocytometer and a light microscope. RAW 264.7 cells (3.0×10^5) were seeded in 6-well plates and allowed to attach overnight. On the next day cells were pre-treated overnight with 1 μ g LPS/mL. The day after the LPS was removed and the cells replaced with fresh media in presence of AuNPs-SS. 24h later viable and non-viable cells were counted as described for NCTC2544. The means of three independent cell counts were pooled for analysis.

2.11 Scratch assay.

The migratory potential of the keratinocyte cell line NCTC2544 was assessed with a wound healing scratch assay.[30] NCTC cells were grown to confluence in 60 mm culture plates at a concentration of 2×10^5 cells in complete medium. A straight scratch was made in the confluent monolayer with a p200 pipet tip. Detached cells were removed, and the edge of the scratch was flattened by washing the cells twice with PBS. Cells were replaced with complete media and treated with increasing concentration of AuNPs-SS to monitor cell motility. Scratched areas for each sample were marked and photographed immediately and after 24 and 48 hours with an EVO digital camera (Coolpix 5400, Nikon Corporation, Japan). Using the ImageJ image processing program, the size of the denuded area was determined at each time point from the digital images. The migrated area was calculated by subtracting the wound area at time point $t = 24$ hours and $t = 48$ hours from $t = 0$ h.

2.12 RNA extraction, semiquantitative and quantitative PCR .

Total RNA was prepared using Tri Reagent (Sigma-Aldrich, Saint Louis, Missouri, USA), agarose gel checked for integrity, and reverse transcribed with cDNA synthesis kit (BioRad, Milano, Italy) according to manufacturer's instructions. Selected genes were evaluated by qualitative PCR using Blue Platinum PCR Super Mix (Life Technologies, Monza, Italy). Primer sequences (IDT, TemaRicerca, Bologna, Italy) were as follows:

IL1- β Forward 5'-CCTGCAGCTGGAGAGTGTGGA-3'; Reverse 5'-AGGAGGAACGGA-GACTACCC-3'

iNOS Forward 5' -CCC TTC CGA AGT TTC TGG CAG CAG C-3'; Reverse 5'-GGC TGT CAG AGC CTC GTG GCT TTG-3'

IL6 Forward 5'- CGG AGA GGA GAC TTC ACA CAG GA-3' Reverse 5'- GGA GAG CAT TGG AAA TTG GGG-3'

β -actin Forward 5' GGC ACC ACA CCT TCT ACA ATG-3' Reverse 5'- GGG GTG TTG AAG GTC TCA AAC - 3'

2.13 Western blot analysis.

Harvested cells were resuspended in 20 mM RIPA buffer (pH 7.4) (Merk Millipore, Vimodrone, MI, Italy) containing a cocktail of proteinase inhibitors (Calbiochem, Merck, Darmstadt, Germany) and treated by sonication 19 (Microson XL-2000, Minisonix, Farmingdale, NY, USA). Aliquots of supernatants containing equal amounts of protein (30 μ g) in Laemmli buffer were separated on Bolt® Bis-Tris Plus gels 4-12% precast polyacrylamide gels (Life Technologies, Monza, Italy) and then transferred from the gel to a PVDF nitrocellulose membrane using iBlot 2 system (Life Technologies, Monza, Italy). Blots were stained with Ponceau red to ensure equal loading and complete transfer of proteins. Then, they were blocked for 1 hour, at room temperature, with 5% milk in PBS 0.1% tween solution. Subsequently, the membranes were probed at 4°C overnight with the following primary antibodies: rabbit anti-uPAR (1:500 FL 290, Santa Cruz Biotechnology, Cat# sc-10815); rabbit anti-iNOS (1:1000, Cell signaling, Cat#2982) rabbit GAPDH antibody (1:1000, Cell signaling Technology, Cat# 2118) was used to assess equal amount of protein loaded in each lane. Anti-Rabbit IgG (whole molecule)-Peroxidase antibody (Sigma, Cat#A0545) was used as the secondary antibody; the ECL procedure was employed for development.

3. RESULTS AND DISCUSSION

After mixing, under continuous stirring, appropriate amounts of SS and HAuCl_4 , the formation of AuNPs-SS was observed. More specifically, by diluting the received SS with a dilution factor of 1:8, fixing the HAuCl_4 concentration at 4.0×10^{-4} M, the colour of the reaction mixture changed from light yellow (**Figure 1A** , picture 1) to light purple (**Figure 1A** , picture 2). In particular, extending the contact time to 48h, the colour of these solutions strikingly changed to dark purple indicating the AuNPs-SS formation. As suggested by Yin *et al.* ,[31] the optical properties of nanostructured gold depend strongly on the size, shape, interactions between the nanoparticles and the species adsorbed on their surface. The colour of the colloidal gold is due to the surface plasmon resonance (SPR) absorption, a characteristic signal in the visible absorption spectrum of AuNPs, in general showed in the range from 500 to 600 nm in the UV-Vis absorption spectrum. In this

case, the mixture of HAuCl_4 and SS presents the AuNPs characteristic SPR band, that appeared slightly broadened and centered at 550 nm (**Figure 1B**). In particular, in **Figure 1B**, the AuNPs SPR band time evolution is reported. While in **Figures 1C** and **1D**, the wavelength and absorption values at the maximum of the SPR are showed as function of time compared with the Full Width at Half Maximum (FWHM). A shift towards higher wavelength number values, along with an increase of the A_{max} were observed by extending the contact time. At the same time, the corresponding FWHM became larger than values observed at the beginning of the reaction (**Figures 1C, D**). Always according Yin *et al.*, [31] the variances in the SPR bands can be attributed to a different distribution of the nanoparticles in the solution and to their aggregation. Moreover, the SPR absorption intensity of AuNPs depends on their size and shape as well as their surrounding medium.[31]

Therefore, in agreement with the SPR band position and shape, the spectral observations indicated polydispersed AuNPs that tend to slightly increase their size with elapsing the contact time. Probably, the observed increment in the A_{max} , passing from 4 to 48h, could be attributed to an increase in the AuNPs-SS concentration due to the quite complete reaction of HAuCl_4 with SS. Further, it is worth mentioning that, incrementing the contact time until 72h, the particles tended to aggregate to each other and the SPR signal decreased, becoming barely evident in the UV-Vis spectrum (*data not shown*).

As a whole, the results indicated the reduction of Au(III) to Au(0), forming nanoparticles thanks to the synergistic action, as reducing agents, of main components of snail mucus. Indeed, the chemical reduction of HAuCl_4 aqueous solution is one of the most widely used methods for the synthesis of colloidal gold.[32] Not surprisingly, by looking at the AuNPs-SS spectrum shape line, in particular below 400 nm, the contribution of chemical species, like proteins/peptides and amino acids, mainly present in SS,[33] absorbing in this region, was observed. The finding was confirmed by comparing the UV-Vis spectrum of SS solutions with the AuNPs spectrum (**Figure S1**). The UV-Vis spectra of SS collected at different dilutions (to better evidence the contribution of the absorbance values in the reported wavelength region) clearly displayed the contribution of the main SS components, indicated with arrows, on the AuNPs surface.

A Zeta potential value of approximately -35 ± 2 mV was measured for AuNPs-SS, indicating their good stability in water. Further, **Figure S2** shows the comparison between the UV-Vis spectra of AuNPs-SS collected soon after the synthesis (selecting 24h as contact time) and after 2 months. It is clearly evident as AuNPs-SS are characterized by a long-time stability, as indicated by the presence of the AuNPs SRP signal.

3.1. FESEM Analysis

FESEM investigation revealed the formation of irregularly shaped AuNPs (**Figure 2A, B**), with minimum and maximum size of about 4 and 50 nm, respectively, and average size of 14 ± 6 nm (particles size distribution in **Figure 2C**). FESEM images confirmed the hybrid organic-inorganic nature of the AuNPs-SS and, in particular, evidenced the presence of a thin organic layer covering the Au nanoparticles (thickness ranging between 4 and 8 nm, **Figure 2B**).

So, starting from these considerations and considering an AuNPs-SS average size of 14 ± 6 nm, a molar absorption coefficient of $3.7 \times 10^8 \text{ M}^{-1} \text{ cm}^{-1}$ was calculated. As a result, each synthesis produced, adopting 24h as contact time, AuNPs-SS stock solutions with a mean concentration of 5×10^{-9} M. It means 1.64×10^{12} AuNPs/mL.

3.2 XPS analysis

XPS results showed that the surface atomic concentrations of carbon, oxygen, nitrogen and gold are about 64, 18, 11 and 7%, respectively. **Figure 3A** reports the high-resolution Au 4f XPS spectrum consisting of a doublet (i.e., Au 4f_{7/2} and Au 4f_{5/2} at 83.2 and 86.9 eV, respectively) due to spin-orbit coupling, the position being in agreement with literature on AuNPs.[18,34-37] The curve fitting of the Au 4f signal indicates three Au 4f_{7/2} components, i.e., the main component at 83.2 eV (68%) attributed to metallic gold (Au(0)), and the minor peaks at about 84.2 eV (23%) and 85.4 eV (9%) ascribed to Au(I) and Au(III), respectively (the total area of the Au 4f_{7/2} is taken as 100%). [37-40] The high-resolution C1s XPS spectrum in **Figure 3B** is curve-fitted with four peaks and, specifically, the hydrocarbon component at 285 eV (51%), the component

at 286.4 eV (31%) ascribed to both C-N and C-O groups, the peak at 288.0 eV (14.5%) assigned to both C=O and O-C-O moieties and the weak peak at 289.2 eV (3.5%) due to carboxylic functionalities. The O1s spectrum in **Figure 3C** displays two components, i.e., the peak at 531.7 eV (57%) that can be ascribed to O=C moieties and the component at 533.1 eV (43%) associated with O-C groups. The N1s signal (**Figure 3D**) shows one main peak at about 400.1 eV, consistent with the presence of amine and amide groups.[41-43] To unveil the nature of these compounds on the AuNPs surface, ATR-FTIR and MALDI-MS analyses were performed.

3.3 ATR-FTIR spectroscopic measurements

FTIR measurements were carried out to recognize the possible functional groups responsible for efficient stabilization of gold nanoparticles prepared by using SS. For this purpose, a comparison between the ATR-FTIR spectra of SS before and after the formation of AuNPs, and the FTIR spectrum of AuNPs-SS was performed (**Figure 4**).

Starting from as received SS, bands around 1700 cm^{-1} and 1580 cm^{-1} were detected, and in accordance with literature,[33,44,45] these peaks indicated the main presence of amide bonds of peptides and proteins as glycoaminoglycans and proteoglycans. Further, the bands at about 1388 cm^{-1} and 1700 cm^{-1} could suggest the presence of carboxylate moieties associated with acid derivatives of sugars and amino acids chains. Indeed, the peaks at 1192 cm^{-1} and at lower wavenumber values (below 900 cm^{-1}) indicated the O-glycosidic linkages.[33] In this region, the contribute of sulphated, acylated and ester based compounds should be also taken into account. The shoulder at 1360 cm^{-1} with a partial contribute at 1380 cm^{-1} indicated the presence of carbon chains of proteins and carbohydrates.[33,44,45] Accordingly, there was evidence at 2850 cm^{-1} and 2930 cm^{-1} of CH_2 and CH_3 arisen from protein skeleton and lipidic component of the slime.[30] The observed bands between 2900 and 3500 cm^{-1} can be ascribed to the presence of a large amount of OH and NH moieties. The contributes of polyphenols, mainly in the range 1500-3500 cm^{-1} , cannot be excluded.[18] Overtones of the benzene rings of phenylalanine and tyrosine were detected above 1800 cm^{-1} and 2600 cm^{-1} . [33] Interestingly, after the contact with HAuCl_4 solution, and thus after the AuNPs formation, the SS spectrum appeared almost unchanged as a whole in comparison with the one observed at the beginning of the reaction. In particular, the signal at 1580 cm^{-1} disappeared, but others main vibrations at 1700 cm^{-1} and 1192 cm^{-1} were retained. Then, if, on one hand, the disappearance of the of amide bond signals from peptides and proteins features was indicative of their role in the formation of AuNPs-SS, on the other hand, the counterpart of the spectrum ascribed to sugar derivatives did not show important changes. The wavenumber region 2800-3500 cm^{-1} appeared also affected, suggesting the contribute of biomolecules having OH and NH functionalities. At the same time, the bands intensity at 2850 cm^{-1} and 2930 cm^{-1} was reduced further suggesting the role of proteins in nanoparticles formation. These results were confirmed when the FTIR-ATR of the AuNPs-SS was collected. Indeed, the main signals at 1645 and 1545 cm^{-1} , indicated the presence of a protein component based on the Amide I (C=O stretching) (1645 cm^{-1}) and II (1545 cm^{-1}) (N-H bending and C-N stretching) bands.[44] Interestingly, if these bands were compared with signals of SS and ascribed to the same vibration modes, the observed shift towards lower wavenumber values could be attributed to their coordination on the AuNPs surface. The characteristic absorption in the region 1200–1300 cm^{-1} , which represented the amide III, due to the C-N stretching and N-H deformation bands, was also detected. Accordingly, signals between 3300 and 3750 cm^{-1} arise also from the AuNPs-SS surface due to N-H amine group stretching vibrations superimposed on the side of O-H hydroxyl group stretching bands.[46] Once again, comparing the position of these bands with those observed in the as received SS, a clear shift towards higher wavenumbers was observed confirming that the molecules in the SS could coordinate to AuNPs through formation of Au-N or Au-O covalent bonds. In the spectral region from 750 to 1400 cm^{-1} only very weak signals can be observed. The lacking of clear peaks at 1192, 1388 cm^{-1} and 1700 cm^{-1} could suggest the absence O-glycosidic linkages and carboxylate moieties associated with acid derivatives of sugars and amino acids chains. The signal at 2850 cm^{-1} and 2930 cm^{-1} observed in the ATR-FTIR of AuNPs-SS could confirm the presence of protein skeleton. So, from the ATR-FTIR analyses it has been possible to assess the main presence of peptides and aminoacids on the surface of AuNPs-SS, acting as reductant agents during the formation of AuNPs.

3.4 MALDI (+) FT-ICR MS Analysis.

The presence of amino acids and peptides was supported by MALDI (+) FT-ICR MS experiments (**Figure 5**). The MALDI (+) FT-ICR MS spectrum for as received SS sample (**Figure 5A**) shows a huge number of MS signals, thus revealing the complexity of the sample in terms of its metabolic profile. Thanks to higher levels of accuracies reached by HRMS technique,[47] it was possible to unequivocally identify several protonated amino acids, i.e. threonine ($[\text{C}_4\text{H}_{10}\text{NO}_3]^+$, m/z 120.12747), tryptophan ($[\text{C}_{11}\text{H}_{13}\text{N}_2\text{O}_2]^+$, m/z 205.23364), arginine ($[\text{C}_6\text{H}_{15}\text{N}_4\text{O}_2]^+$, m/z 175.20953), hystidine ($[\text{C}_6\text{H}_{10}\text{N}_3\text{O}_2]^+$, m/z 156.16258), asparagine ($[\text{C}_4\text{H}_9\text{N}_2\text{O}_3]^+$, m/z 133.12607), cysteine ($[\text{C}_3\text{H}_8\text{NO}_2\text{S}]^+$, m/z 122.16670), glutamine ($[\text{C}_5\text{H}_{11}\text{N}_2\text{O}_3]^+$, m/z 147.15341), proline ($[\text{C}_5\text{H}_{10}\text{NO}_2]^+$, m/z 116.13903) and serine ($[\text{C}_3\text{H}_7\text{NO}_3]^+$, m/z 106.10083), already found in snail slime sample.[48] 6198 peptides could be identified showing the presence of at least one unit of cysteine. The presence of peptides showing high contents of cysteine, proline, serine and glycine was accessed too, these ones very well-known in the literature for their high antimicrobial activity.[48] Instead, analysis of AuNPs-SS sample (**Figure 5B**) shows a very different situation. In this case, MALDI (+) FT-ICR MS spectrum of the sample shows a lower number of MS signals in comparison to the pure sample (3158 and 209694 signals, respectively). No free amino acids were found and only 3 free peptides could be identified, thus suggesting a drastic shrinking of the range of free peptides present in the sample, indicative of their role in the formation of AuNPs.

3.5 pH dependence of synthesized AuNPs.

The AuNP stability, after their synthesis, was investigated under different pH values ranging from 2 to 12. Once again, by observing the SPR of AuNPs, the wavelength and FWHM were monitored. Results, reported in **Figure 6A**, indicated that both the position and the FWHM were stable in the pH range from 4 to 10 and slight changes were detected at the lowest and the highest investigated pH value, *i.e.* pH 2 and 12. More specifically, at pH 2 the SPR signal shift towards higher wavelength values indicating the contribution of larger AuNPs that under this condition tend to agglomerate.[31] Yin *et al.* [31] suggested that a large number of H^+ in the aqueous solution could be adsorbed onto the surface of the AuNPs, modifying their surface and their charges. Accordingly, at the same time, the FWHM increased. Then, AuNPs tended to agglomerate easily and consequently the final particle size was larger. Conversely, at pH 12 the contribute of smaller AuNPs occurred in agreement with their stabilization, under this condition of work. The FWHM in this case reduced its value, indicating that the particles were more monodisperse. These findings were confirmed by performing Zeta potential measurements as function of pH values, as reported in **Figure 6B**. It is worth mentioning that the Zeta potential gives information about the AuNP suspension stability, since a higher electric surface charge of the AuNPs prevents aggregation. Indeed, the AuNPs were negatively charged in the range of pH from 4 to 12 and, in particular, the charge decreased at the increasing of the pH value. On the other hand, at pH 2 a positive Zeta potential value, of approximately +25mV, was observed, affecting the AuNPs surface as previously described. The presence of functional groups arisen from the main components of SS, mainly the carboxylic moieties of proteins and aminoacids (and/or polyphenols) present on the AuNP surface, produced a negative electrical charge and the observed dependence on the pH. At pH values below 4, the organic moieties were in the protonated form and the AuNPs slightly aggregated.

3.6 AuNPs-SS enhance scratch wound closure of NCTC 2544 keratinocytes.

To determine whether AuNPs-SS could affect cell motility, scratch assays were performed in a confluent monolayer of keratinocytes and evaluated at 0h, 24h and 48h after treatment with increasing doses of AuNPs-SS. By 24h, even though the keratinocytes normally showed an active migratory phenotype, scratches treated with AuNPs-SS displayed a significant acceleration of wound closure as compared with the untreated (CTRL) wound (**Figures 7A, B**). By 48h the scratch treated with the highest AuNPs dose was completely closed, thus showing a significant increase in closure rate from 24h while no statistically significant change in cell viability was observed on AuNPs-SS treatment as shown in **Figure 7C**. Moreover, western blot analysis displayed that AuNPs-SS treatment induced a significant increase of the urokinase-type plasminogen activator receptor (uPAR), which is essential for keratinocyte adhesion, spreading and migration *in vitro* and *in vivo* (**Figure 8C**).[49,50]

3.7 AuNPs-SS reduce the inflammatory response of LPS-activated macrophages.

To further assess possible effects of the AuNPs-SS on biological inflammatory response, RAW 264.7 macrophages were pre-treated with LPS and the day after loaded with two doses of AuNPs-SS. Morphological changes and an inflammatory gene panel were evaluated at the end of the sequential treatments. As shown in **Figure 8A**, the mouse macrophage cells took up AuNP regardless of treatment with LPS. Cells without LPS treatment were more homogeneous in size, and AuNPs appeared to be dispersed throughout the cytoplasm. After LPS treatment, macrophages became activated as seen by variations in size. The higher the AuNP concentration, the more activated cells appeared, presenting extended pseudopods. It was found there was no statistically significant change in macrophage viability (**Figure 8B**). Gene expression of cytokines IL-1 β , IL-6 and inducible nitric oxide synthase (iNOS), showed that, in the absence of LPS treatment, there were moderate changes in cytokines expression after AuNPs-SS treatment. LPS induced a massive increase in cytokines and iNOS expression. Interestingly, the AuNPs-SS synergistically reduced these elevated cytokine levels and completely abrogated the synthesis of iNOS (**Figure 8C**). The effect of AuNPs-SS on iNOS levels before and after LPS stimulation was confirmed also by western blot analysis (**Figure 8D**). Altogether these results suggest that AuNPss-SS may have modulatory effects that could be beneficial in reducing inflammatory or pathogenic responses.

4. CONCLUSIONS

For the first time, during this work, the Snail Slime was used to induce the formation of AuNPs, proposing in literature a novel and green approach for their synthesis. A comprehensive investigation was performed in our laboratory by adopting several complementary techniques such as UV-Vis and ATR-FTIR spectroscopy, DLS, XPS, and high-resolution MALDI-MS analyses. AuNPs 14 \pm 6 nm wide were observed through the FESEM investigation that evidenced the formation of the organic layer Snail Slime-based surrounding AuNPs. The latter was confirmed by ATR-FTIR, XPS and MALDI-MS analyses. Indeed, the main presence of peptides and aminoacids was observed at the surface of the AuNPs giving them a high Zeta potential value in water (-35 \pm 2 mV), indicative of their great stability. Indeed, the temporal and pH stability of the proposed colloidal gold solution was investigated, which resulted in a suspension stable for a long time and appeared only slightly affected under hard acidic pH value (pH 2), far from biological conditions, in which aggregation of AuNPs was observed. With the prospect of using the AuNPs for biomedical devices, we studied the effect of AuNPs on wound healing in human keratinocytes and evaluated their potential anti-inflammatory properties in Murine Macrophages. Our findings demonstrated that the accelerated wound closure promoted by AuNPs-SS was associated with an increased expression of the receptor for urokinase (uPAR) which, besides that canonical conversion of plasminogen to plasmin and extracellular matrix (ECM) degradation, directly controls cell adhesion, differentiation, proliferation and migration through non-proteolytic mechanisms.[49,50]

It has been established that in the classic process following infection or injury, macrophages play a key role as regulators of inflammation. However, if the inflammatory response of macrophages is not properly controlled, it may lead to severe diseases such as atherosclerosis,[51] rheumatoid arthritis (RA), vascular injury and cancer.[52] Accordingly, to moderate inflammation, the macrophages have been targeted for specific ablation, inhibition of infiltration and reduction of the pro-inflammatory cytokines release. In this work, we demonstrated for the first time the AuNPs-SS were able to modulate the inflammatory response induced by LPS in murine macrophages by significantly reducing the IL-1 β , IL-6 cytokines levels. It is known that macrophages isolated from chronic wounds in diabetic patients exhibit a proinflammatory phenotype with the release of IL-1 β , MMP-9, TNF- α , and IL-6. In a recent study Mirza and coworkers[53] showed that IL-1 β sustains the proinflammatory macrophage phenotype observed in poorly healing wounds of humans and mice. The authors of the study demonstrated that targeting the IL-1 β pathway upregulated expression of pro-healing factors in wounds of diabetic mice and improved healing of these wounds. Indeed Sumbayev et al. reported that citrate-stabilized gold nanoparticles were able to down-regulate the inflammatory process by selectively targeting the IL-1 β dependent pathway[54].

We also observed a massive drop of the inducible nitric oxide synthase (iNOS) levels, which is essential for the macrophage activation and thus for cytokines release. Regulation of iNOS gene expression is, through

transcriptional regulation, particularly influenced by NF- κ B activation. Indeed, in mice, iNOS gene promoter contains two NF- κ B binding sites, both of which need to be bound in order to get full induction of iNOS by LPS stimulation

Hence, a novel horizon in the field of biotechnology is opened with this work, proposing an innovative nanoplatform that should have other properties, under investigation in our research groups. The applications in cosmetic formulations of AuNPs as an additional ingredient would give the possibility to avoid, for example, the use of preservatives, due to the potential antimicrobial activity of gold that, at least, should also preserve the snail slime components.

In conclusion, our findings demonstrated that AuNPs-SS promote effective wound healing and may pave the way to accelerate the regeneration of difficult-to-heal wounds. A more in-depth understanding of the effects of NPs on macrophage activation may lead to a more potent and efficient control of inflammatory-related diseases.

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Figure Captions

Figure 1: Camera Pictures of a HAuCl_4 solution at 4.0×10^{-4} M in absence (1) and presence (2) of SS (A); UV-Vis spectra of solutions containing AuNPs (diluted 1:5), observing the typical SPR, collected at different incubation time from the mixture of HAuCl_4 and SS (B); Time evolution of SPR absorbance intensity (A_{\max}) versus the related FWHM (C); Time evolution of SPR wavelength registered in correspondence of the A_{\max} versus the SPR FWHM (D).

Figure 2: FESEM images of AuNPs-SS (**A**, **B**); AuNPs size distribution determined from FESEM images (500 particles) (**C**).

Figure 3: High resolution XPS spectra of the AuNPs-SS and their relative curve-fitting: Au 4f (**A**), C 1s (**B**), O 1s (**C**), N 1s spectra (**D**).

Figure 4: ATR-FTIR spectra of SS, AuNPs-SS and SS after the AuNPs formation.

Figure 5: Positive ion MALDI-FT-ICR MS of the snail slime (**A**) and AuNPs snail slime (**B**) samples with DHB as matrix

Figure 6: Effect of pH on SPR wavelength position and FWHM (**A**) and on ζ -potential values (**B**).

Figure 7: Confluent monolayers of NCTC2544 were wounded by manually scratching as described in Materials and Methods and incubated with increasing concentrations of AuNPs-SS. Representative pictures of the wound gap taken at 0h, 24h and 48 h (**A**); Histograms reported the percentage of gap closure compared with time 0h(**B**); histogram representing viable cells (trypan blue negative) counted with the aid of a Burker chamber, as described in M&M(**C**); Western blot analysis of uPAR levels quantified by densitometric analysis; GAPDH was also examined to ensure equal loading of samples in each lane. For all the panels in the figure, data in the graphs represent the mean \pm SD of at least three independent triplicate experiments. Asterisks ($*p < 0.05$) indicate significant differences of AuNPs-SS treated cells, from CTRL (untreated)(**D**).

Figure 8: Optical images of untreated, LPS treated, or AuNPs-SS treated cells (**A**); histogram representing viable cells(**B**); RT-PCR analysis of mRNA levels of IL1 β , IL6, iNOS in cells treated as in (A) (**C**). Amplification of cDNA was carried out for 30 cycles and β -actin was used as the reference gene; results were from a typical experiment out of three. Densitometric analysis of the expression gene levels is reported at the bottom. Significance was assessed by one-way ANOVA test followed by Newman-Keuls post test. Error bars indicate mean \pm SD; Asterisks ($*p < 0.05$) indicate significant differences of LPS+AuNPs-SS treated cells from LPS, while Number sign ($\#p < 0.05$) indicates significant differences from CTRL (**D**) western blot analysis of iNOS and p65. Histograms on the right represent densitometric analysis of the iNOS and p65 protein levels. Asterisks ($*p < 0.05$) indicate significant differences of LPS+AuNPs-SS treated cells from LPS. Results were from a typical experiment out of three.

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Figure 5.docx available at <https://authorea.com/users/331555/articles/458202-biomolecules-from-snail-mucus-helix-aspersa-conjugate-gold-nanoparticles-exhibiting-potential-wound-healing-and-anti-inflammatory-activity>

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Figure 6.docx available at <https://authorea.com/users/331555/articles/458202-biomolecules-from-snail-mucus-helix-aspersa-conjugate-gold-nanoparticles-exhibiting-potential-wound-healing-and-anti-inflammatory-activity>

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Figure 7.docx available at <https://authorea.com/users/331555/articles/458202-biomolecules-from-snail-mucus-helix-aspersa-conjugate-gold-nanoparticles-exhibiting-potential-wound-healing-and-anti-inflammatory-activity>

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Figure 8.docx available at <https://authorea.com/users/331555/articles/458202-biomolecules-from-snail-mucus-helix-aspersa-conjugate-gold-nanoparticles-exhibiting-potential-wound-healing-and-anti-inflammatory-activity>