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# Silver and gold nanoparticles produced by pulsed laser ablation in liquid to investigate their interaction with Ubiquitin

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ABSTRACT

The interaction of nanoparticles (NPs) with proteins is widely investigated since it can be a key issue in addressing the problem of nanotoxicity, particularly in the case of biological and medical applications. In this work, silver and gold nanoparticles (AgNPs and AuNPs) were produced in water by Pulsed Laser Ablation in Liquid (PLAL) and allowed to react with Ubiquitin (Ub) (a small human protein essential for degradative processes in cells). NPs produced by PLAL are completely free of undesired contaminants and do not require the use of stabilizers. We found that the NPs + Ub system behaves differently if the NPs are or are not treated with a stabilizer before performing the interaction with Ub, since the presence of capping agents modifies the surface reactivity of the metal-NPs. The surface plasmon resonance (SPR) absorption spectroscopy was employed to monitor the fast changes occurring in the NP colloidal solutions upon interaction with Ub. The results obtained by SPR were confirmed by TEM analysis. Therefore, when Ub interacts with bare NPs a rapid aggregation occurs and, at the same time, Ub undergoes an amyloid transition. Notably, the aggregation of AuNPs occurs at a much greater rate than that of analogous AgNPs and the Ub fibrils that are formed can be imaged by thioflavin T fluorescence.



## 1. Introduction

In the last decade the synthesis of nanoparticles (NPs) by Pulsed Laser Ablation in Liquid (PLAL) has gained wide interest thanks to the possibility to produce NPs completely free of undesired contaminants, to avoid the use of harmful reactants, and to accomplish long-lasting stability without using any stabilizer [1,2]. The stabilizer can affect deeply the chemical reactivity of the NPs surface and its role has to be taken into account when planning biological and medical applications. The peculiarity of the NPs produced by PLAL lies in the particular environment in which they are produced [3,4]: the plasma induced by the laser on a target submerged in water, as well as the subsequent cavitation bubble formation and dynamics, play crucial roles in the NPs formation mechanisms and stability.

The interaction of NPs with proteins has extensively been studied for medical and biological applications [5], since it is known that, once the NPs enter into the biological fluids, a rapid interaction of the NPs with plasma proteins takes place. Adsorbed proteins form the so-called protein corona around the NPs [6]. Such an interaction can alter the normal functioning of a protein, thus leading to unexpected biological reactions [7]. The NPs surface plays a crucial role in the process of corona formation and, if a stabilizer is present in the solution, the surface reactivity can change dramatically. Usually, protein-NPs investigations are carried out with NPs coated with a stabilizer, however, more recently, some studies [8,9] have started to investigate the interaction of different proteins with bare NPs. For instance, AuNPs [10] and AgNPs [11] produced by PLAL have been mixed with bovine serum albumin (BSA) and found to form the classical protein corona. However, in a previous work from our laboratories [12], we demonstrated how bare AgNPs interact with Ubiquitin (Ub) differently from stabilized AgNPs. Thus, while colloidal solutions of citrate-stabilized AgNPs react with Ub forming a protein corona which is stable over time [12,13], in contrast the protein corona formed by interaction of bare AgNPs with Ub leads rapidly to aggregation of the NPs with simultaneous amyloid transition of the protein which appears to be responsible for clustering of the NPs.

Ub is a human protein with important biological functions (e.g., it flags proteins for degradation by the proteasome). Moreover, its relatively small size and well-characterized three-dimensional

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structure render this protein a good model for investigating the interaction between proteins and NPs.

In this work, we performed interaction experiments in which AgNPs or AuNPs produced by PLAL were reacted with Ub, the aim was to unravel if bare NPs of different metals can both aggregate upon interaction with Ub and cause amyloid transition of the protein.

Furthermore, since plaques and inclusion bodies composed of fibrillar deposits of Amyloid β (Aβ) and other proteins are hallmark of several neurodegenerative disorders, the study of an amyloid-like aggregation can have some relevance to the realm of biology and medicine.

AgNPs and AuNPs were produced in pure water and, after selecting the best experimental conditions for PLAL, the produced NPs were characterized and allowed to react with Ub while monitoring the intervening changes as a function of time. The reaction between NPs and Ub was performed both in the presence and absence of stabilizer, so to test the different reactivity of the NPs surfaces. The amyloid transition the protein undergoes by interaction with bare AuNPs was checked by thioflavin T (ThT) assay (indicative of the amyloid form of protein) and by preincubation with transthyretin (an inhibitor of amyloid-type aggregation). Moreover, we tested the interaction with an Ub mutant (E16V), which is unable to form aggregates.

## 2. Experimental set-up

The experimental setup for the production of NPs in water has been extensively described in previous works [12,14]. It consists of nanoseconds Nd-YAG lasers (Quanta System PILS-GIANT and Quanta System Thunder) operating both with fundamental and second harmonic generation (1064 nm and 532 nm, respectively), having a laser frequency of 10 Hz and a nominal pulse duration of 8 ns. Depending upon the experiment, different laser wavelengths (1064 and 532 nm) were used and different lens were employed to focus the laser on a metal target submerged in water. All colloidal solutions were produced in ultrapure Milli-Q water.

Soon after production, colloidal solutions were monitored as a function of time by surface plasmon resonance (SPR) absorption spectroscopy using an Ocean Optics (USB2000 + XR) spectrometer with a light source (Mini Deuterium Halogen Light Source DT-Mini-2-GS). The transmission electron microscopy (TEM) analysis was performed using a Philips Morgagni 282D TEM, operating at 60 kV.

Fluorescence imaging of the samples was performed by means of Laser Scanning Confocal Microscopy (LSCM) using the TCS SP8 SMD confocal microscope by LEICA equipped with a blue diode laser (excitation wavelength of 405 nm). Samples were observed through a 20× dry objective and the emission in the range 420–600 nm was collected by a hybrid detector; at the same time the transmitted light was independently collected by a conventional PMT detector.

## 3. Materials and methods

The best experimental conditions for NP production by PLAL were chosen on the basis of reproducibility, size distribution, and stability in time. After the production by PLAL, the NPs were monitored for at least two days with SPR absorption spectroscopy, this time being sufficient for the NPs to reach the equilibrium.

For the production of AgNPs in water, a 532 nm NdYAG (Quanta System PILS-GIANT) laser was focused on a silver target immersed in a cuvette filled with 3 ml of water by using 4 cm focal lens, irradiance of 17 GW/cm$^2$, and laser ablation time of 3 min. The silver target was purchased from Goodfellow Cambridge Limited.

For the production of AuNPs in water, a 1064 nm NdYAG (Quanta System Thunder) laser was focused on a gold target immersed in a vessel filled with 25 ml of water by using of a 5 cm focal lens, irradiance of 71 GW/cm$^2$, and a laser-ablation time of 10 min. The gold target was purchased from Kurt J. Lesker Company.

The molar concentrations (mols of NPs/liter) of the NPs solution were calculated by applying the Lambert-Beer law to the absorbance spectra of the colloidal solutions. The extinction coefficients, which depend upon the NPs size, were measured via appropriate calibration curves for AgNPs and AuNPs. The standard solutions used for calibration were: AgNPs of 10 nm particle size in 2 mM citrate purchased from Nanocomposix (10 nm Citrate NanoXact™ Silver, 0.022 g L$^{-1}$); AuNPs of 10, 20, 40, 60, 80 and 100 nm particle size in 2 mM citrate purchased from Sigma Aldrich (ranging from 0.06 g L$^{-1}$ to 0.04 g L$^{-1}$). The concentrations of NPs solution used in this work were 2.2 and 2.4 nM for AgNPs and AuNPs, respectively.

The NPs size was determined by TEM and SPR absorption spectroscopy and resulted to be 10 ± 3 and 15 ± 4 nm for AgNPs and AuNPs, respectively.

The incubation of NPs with wild-type Ub and the Ub mutant Glu16Val (E16V) was performed by dissolving the lyophilized protein in ultrapure water and adding an aliquot to the NP solution to obtain a final protein concentration of 25 μM. Ub was always added to the NP solution after 2 days from PLAL preparation. Wild-type Ub and E16V used in this work were prepared as described in ref. [12].

The sample treatment for TEM analysis (sample deposition on a grid with or without negative staining) as well as the sample preparation for the thioflavin T assay have also been described in ref. [12].

The colloidal solutions incubated with wild-type Ub, E16V mutant, or preincubated with transthyretin (TTR) have been monitored, as a function of time, by SPR absorption spectroscopy.

## 4. Production and characterization of silver and gold nanoparticles

### 4.1. Pulsed laser ablation in liquid

To produce NPs a laser pulse is focused on a target immersed in a liquid (pure water in our experiments). If the laser irradiance overcomes the breakdown threshold (so to induce a breakdown of the material) a plasma is generated. Once the plasma is formed it expands supersonically driving a shockwave and then extinguishes in several hundreds of nanosecond. Usually, the laser-induced plasma is characterized by high excitation temperature (≈10,000–6000 K) and high electron density (≈$10^{17}$ cm$^{-3}$). The plasma energy is rapidly transferred to the surrounding liquid, thus inducing a vaporization at the front head of the plasma itself, which leads to the formation of a cavitation bubble. In these conditions the plasma is subjected to a strong confinement effect, and consequently a fast plasma cooling occurs with a strong increase of the rate of the recombination phenomena (the total amount of emitting species decreases because atoms aggregate to generate the NPs and are subtracted from the collisional plasma [3]). The ablated material can condense in small NPs that are transferred to the cavitation bubble. The subsequent cavitation bubble dynamics consists of an expansion and a shrinking phase with a lifetime of hundreds of microseconds (one order of magnitude bigger than the plasma lifetime). The extreme conditions of temperature and pressure [15] reached in the plasma are believed to be responsible for the formation of the NPs, while the bubble dynamics are connected to the release of the NPs and their stabilization in solution [3].

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