Structure and properties of electrochemically synthesized silver nanoparticles in aqueous solution by high resolution techniques

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Abstract
In this work high resolution techniques as transmission electron microscopy (TEM), scanning electron microscopy (SEM), Zeta Potential measurements, and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) have been employed to deeply investigate about silver nanoparticles (AgNPs) electrochemically synthesized and successfully applied in biological and chemical fields. Strong brightness, as well as the tendency to generate odd number nanoclusters, and the absence of free silver ion in solution have been observed. The chemical and physical properties of the AgNPs seem to be related to their peculiar oxidative state as suggested by X-ray photoelectron spectroscopy (XPS) and X-ray powder diffraction analysis (XRPD). Finally, cytotoxicity of the investigated AgNPs has been tested by MTT assay.

\textbf{Keywords:} silver nanoparticles, X-ray analysis, electron microscopy, oxidative state, cytotoxicity.
1. Introduction

In last decade the range of applications of silver nanoparticles (AgNPs) has been continuously improved due to their unusual properties and features in different fields as catalysts, electronic, magnetic and optical nanomaterials, antibacterial agents, thermally conductive nanofluids up to their inclusion as components into textile and cosmetics products [1-9].

Generally, the experimental conditions by which the synthesis occurs strongly affect size, shape and morphology of metal nanoparticles, as well as their physical and chemical properties [10,11]. Different methods have been proposed to obtain silver nanoparticles, including laser ablation and photochemical approach [12-15] and despite its potential versatility both in organic solvents and aqueous solution [16,17] the electrochemical synthesis is still limited to few applications [18,19]. This method is mainly based on anode oxidation–cathode reduction reactions, hydrogen and oxygen formation from water electrolysis and possible side reactions [20]. A small quantity of hydrogen peroxide is usually needed to reduce silver oxide in solution and stabilizers or coating agents as tetrabutyl ammonium salts or poly-(N-vinylpyrrolidone) are added [21,22] in order to control size and release of silver into the surrounding media [23,24].

It is well known that a single surface plasmon resonance band in the UV-Vis region around 400 nm is related to spherical nanoparticles, even if some factors can affect the extinction coefficients [25]. However, aggregation of silver nanoparticles may easily occur and different techniques can be employed to observe the aggregation phenomena in colloidal systems over time [26-29].

Previously, uncoated and negatively charged AgNPs in aqueous solution have been electrochemically synthesized without employing any stabilizers by using an innovative and fast method based on a home built electron device. The obtained AgNPs are spherical and monodisperse in solution showing high level of stability according to UV–vis spectroscopy analysis, transmission electron microscopy and field emission scanning electron microscopy images, dynamic laser light scattering and zeta potential measurements [30]. Recently, antibiotic activity of AgNPs against planktonic and biofilm cells of Pseudomonas aeruginosa and other cystic fibrosis-associated bacterial pathogens has been observed in vitro [31]. Moreover, the role of these nanoparticles as interactive but not reactive media for the azobenzenes isomerization has been demonstrated by kinetic, spectroscopic and Zeta Potential measurements [32].

The aim of this work has been to explore the surface and core of the investigated nanoparticles by using high resolution technique as scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray photoelectron spectroscopy (XPS), X-ray powder diffraction analysis (XRPD), Zeta Potential measurements and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF).
2. Materials and Methods

2.1 AgNPs preparation
The electrochemical synthesis of AgNPs in aqueous solution is based on 99.9% pure silver electrodes, pure water and a low electrical current as previously described [36]. The power supply and a home-made electronic board were employed to generate 200 V electrical current and 0.5 Hz square wave frequency. Voltage, current, reaction time and total electrical power were optimized and monitored during all the electrochemical process (Patent Application EP 18181873).

2.2 Zeta Potential and ORP measurements
The Zeta Potential analysis were carried out at 25° C using 90PLUS BI-MAS (Brookhaven) equipped with digital correlator at a scattering angle of 15°, with a 35 mW He–Ne laser at the wavelength of 660 nm. For ORP analysis an electrode ORP (mod 98201, Hanna instruments) was used and a dithiothreitol solution (DTT, 20 ug/mL) was employed as standard.

2.3 TEM and SEM analysis
TEM images were taken after evaporation of a drop of AgNPs diluted solution on 300 mesh formvar coated nickel grids at 75 kV by using ZEISS 109 microscope equipped with Gatan-Orius SC200W-Model 830.10W TEM CCD Camera. SEM images were obtained on the powder from the complete evaporation under nitrogen flow of the AgNPs solution at 40 °C by using Sigma 300 Zeiss microscope equipped with elemental microanalysis apparatus Quantax-200 Bruker.

2.5 XPS and XRPD analysis
XPS analysis was performed using an AXIS Nova spectrometer (Kratos Analytical Inc., Manchester, UK) with a monochromated Al Kα source at a power of 180 W (15 kV×12 mA) and a hemispherical analyzer operating in the fixed analyzer transmission mode. XRPD pattern collected from 4 to 90° of 2θ, with a steps scan of 0.02° and 8 s per step with a Rigaku Ultima IV diffractometer.

2.6 MALDI-TOF measurements
The samples were prepared by adding 0.5 uL of 0.1 % trifluoroacetic acid to 1 uL of 20 ppm AgNPs aqueous solution deposited on the MALDI target. The analysis was performed after drying on the Autoflex Speed TOF/TOF mass spectrometer (Bruker-Daltonics GmbH, Bremen, Germany) equipped with a Smart Beam II 1kHz laser, in Reflectron Positive (RP) mode, in the range 20-1.300
Da. The instrument was controlled through Flexcontrol 3.4.135.0 software. The voltages were set as follows: 19.00 kV and 16.70 kV for the ion source 1 and 2, respectively, 8.55 kV for the lens and 21.00 kV and 9.60 kV for reflector 1 and 2, respectively. Pulsed ion extraction time was set to 130 ns. Laser fluency was kept very low to avoid unwanted fragmentation of sample clusters and adducts. The power attenuator was adjusted before the acquisition to maximize resolution, and a laser frequency of 100 Hz was used. For each sample spot from 5.000 to 15.000 total shots were summed.

2.7 Cytotoxicity assay
Cells (2.500 cells/well) were seeded in 96 well plates and treated with Ag nanoparticles or vehicle (PBS) in dose-response experiments after 24h. Cell viability was assessed after 24-48 h of treatment using the MTT assay, which evaluates the mitochondrial function as index of cell viability by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, Italy) as previously reported [33]. The IC<sub>50</sub> values were calculated using nonlinear regression curve fit analysis with Graph Pad Prism 6.00 (GraphPad Software, San Diego California USA). Data are expressed as the mean ± standard deviation. Cell culture: HEK-293 (human embryonic kidney) cell line was maintained in Dulbecco’s Modified Eagle Medium (DMEM, EuroClone, Italy), containing fetal bovine serum (FBS, Italy) to a final concentration of 10%, 2mM L-Glutamine (EuroClone), and 1% penicillin–streptomycin (EuroClone).

3. Results and Discussion
Generally, high absolute zeta potential value suggests that nanoparticles tend to repulse each other avoiding any aggregation process [34,35]. The investigated AgNPs are shows a good stability in aqueous solution according to the Zeta Potential values in the range -40/-70 mV. Statistical analysis for particle size and distribution performed by TEM on different samples have highlighted high frequency of about 3 nm nanoparticles as shown in Figure 1.
Figure 1. An example of TEM micrograph of the investigated AgNPs (scale bar 100 nm) and particle size distribution histograms.

It is well known that SEM images provide information about structure, surface and composition for nanomaterials and nanoparticles. Examples of SEM images obtained for the AgNPs are reported in Figure 2.

Figure 2. Examples of SEM image of the investigated AgNPs.

Silver nanoparticles appear no spherical in shape and exhibit strong brightness. Generally, brightness is related to the lack of conductivity for metal nanoparticles in the so-called charging effect [36] due to the accumulation of static electric charge onto the particle surface. The results indicate that the AgNPs are non conducting nanoparticles and could be considered as insulating materials. Moreover, ORP analysis has revealed a value of + 400 mV associated to silver nanoclusters in comparison to -90 mV obtained from the DTT standard solution, suggesting an oxidative rather than reducing properties of the investigated AgNPs. The absence of free silver ion has been demonstrated by using ISE analysis [37].
The chemical state of silver has been determined by the position of the peaks associated to the binding energy of the emitted photoelectron in the XPS spectrum. In particular, the bulk value of silver corresponds to 368.1 eV and the core level binding energy shifts provide directly the metal electronic properties [38]. One of the XPS spectra obtained for the AgNPs is reported in Figure 3 as an example.

![Figure 3. X-ray photoelectron spectrum of AgNPs. The binding energies are referred to Ag 3d₃/₂ and Ag 3d₅/₂ in metallic and oxidized state.](image)

The peaks at 374.53 and 373.32 eV are associated to metallic and oxidized Ag 3d₃/₂, respectively, while the peaks at about 368.53 and 367.32 eV are associated to metallic and oxidized Ag 3d₅/₂ [39]. The binding energy of metallic silver is higher than that observed for oxidized silver suggesting that the former represents the dominant state for the investigated AgNPs [40]. Moreover, the presence of Ag₃⁺ is suggested by the shifting of the values toward higher binding energies accordingly to previous studies [41,42].

X-ray powder diffraction analysis has been performed in order to evaluate the oxidative state of the AgNPs. The XRPD pattern can be observed in Fig. 4. The patterns peaks at 2θ values of 38.10, 44.28, 64.42, 77.40, and 81.50 can be ascribed to the reflection of (111), (200), (220), (311), (222) planes of the face-centered cubic silver, respectively [43,44].

The diffraction profiles of as-prepared AgNPs are broadened as compared with bulk silver, revealing the formation of silver nanoparticles [45].
Figure 4. The XRPD pattern for the investigated AgNPs. The peaks marked with * and • are due to Ag₃O₄ and AgO respectively.

Other labeled diffraction peaks may be attributed to Ag₃O₄ and AgO crystalline phases on the surface of the AgNPs [43, 46].

Silver nanoclusters can be pointed out by MALDI-TOF mass spectroscopy analysis. Interestingly, these kind of aggregates are mainly composed of odd number atoms (Table 1).

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<tr>
<th>Ag Species Detection</th>
<th>MALDI-TOF MS Analysis</th>
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<td>Ag</td>
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<td>Ag₂</td>
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Table 1. Detectable (+) and Not Detectable (-) Ag species by MALDI-TOF mass spectrometry.
Finally, the cytotoxicity of the investigated AgNPs has been tested by MTT assay on HEK-293 (human embryonic kidney). The dose-dependent curve, shown in Figure 5, has demonstrated lower toxicity of AgNPs in comparison to AgNO₃.

![Figure 5. Dose-response curves of the effects of AgNPs and AgNO₃ on HEK-293 viability, evaluated by MTT assay after 48 h of treatment. Data represent the mean ± SEM of independent experiments, performed in quadruplicate.](image)

4. Conclusions

AgNPs electrochemically synthesized without adding any stabilizers or coating agents are negatively charged and monodisperse in aqueous solution. High population of about 3 nm in size and strong brightness have been observed. Metallic rather than oxidized silver is the dominant state for the investigated nanoparticles as suggested by the X-ray photoelectron spectrum, while the presence of oxides and the tendency to form nanoclusters has been demonstrated by XPRD analysis and MALDI-TOF measurements, respectively. Finally, the cytotoxic effect observed by MTT assay confirms that the investigated AgNPs could improve the use of nanoparticles in aqueous media for a wide range of applications both in chemical and biological fields.

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Notes

The authors declare no competing financial interest.

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