

Sputtering-Enabled Intracellular X-ray Photoelectron Spectroscopy: A Versatile Method To Analyze the Biological Fate of Metal Nanoparticles

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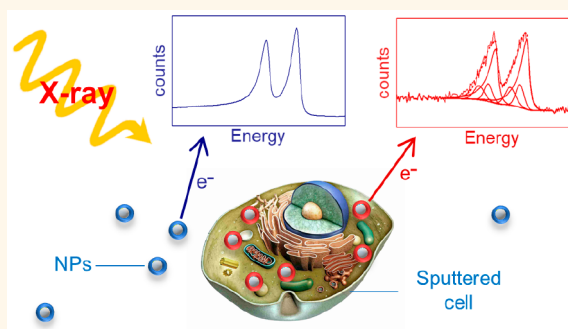
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Supporting Information

ABSTRACT: The investigation of the toxicological profile and biomedical potential of nanoparticles (NPs) requires a deep understanding of their intracellular fate. Various techniques are usually employed to characterize NPs upon cellular internalization, including high-resolution optical and electron microscopies. Here, we show a versatile method, named sputtering-enabled intracellular X-ray photoelectron spectroscopy, proving that it is able to provide valuable information about the behavior of metallic NPs in culture media as well as within cells, directly measuring their internalization, stability/degradation, and oxidation state, without any preparative steps. The technique can also provide nanoscale vertical resolution along with semiquantitative information about the cellular internalization of the metallic species. The proposed approach is easy-to-use and can become a standard technique in nanotoxicology/nanomedicine and in the rational design of metallic NPs. Two model cases were investigated: silver nanoparticles (AgNPs) and platinum nanoparticles (PtNPs) with the same size and coating. We observed that, after 48 h incubation, intracellular AgNPs were almost completely dissolved, forming nanoclusters as well as AgO, AgS, and AgCl complexes. On the other hand, PtNPs were resistant to the harsh endolysosomal environment, and only some surface oxidation was detected after 48 h.

KEYWORDS: nanoparticles, X-ray photoelectron spectroscopy, cellular fate, oxidation state, degradation, nanotoxicology, nanomedicine



The promises of nanomedicine to deliver safe and effective drugs require detailed comprehension of the intracellular fate of nanoparticles (NPs).^{1,2} After their internalization within endolysosomes, NPs are subjected to the action of several small molecules and proteins, a harsh acidic environment, and aggressive proteolytic enzymes.³ However, up to now, there have been limited experimental possibilities to precisely assess the intracellular fate of NPs rather than in simulated biological fluids, leaving many scientific questions unsolved. The main technique to quantify NP internalization is inductively coupled plasma mass spectrometry/atomic emission spectroscopy (ICP-MS/AES)⁴ that has long been used to

determine quantitatively inorganic NP content after digestion of a cell suspension.^{5–7} However, this destructive technique does not provide any information about the NP cellular localization, degradation/biopersistence, and oxidative state. Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS)^{6,7} offers the additional possibility to determine the subcellular localization of metallic NPs,⁸ but also in this latter case, the degradation and oxidation state of the NP

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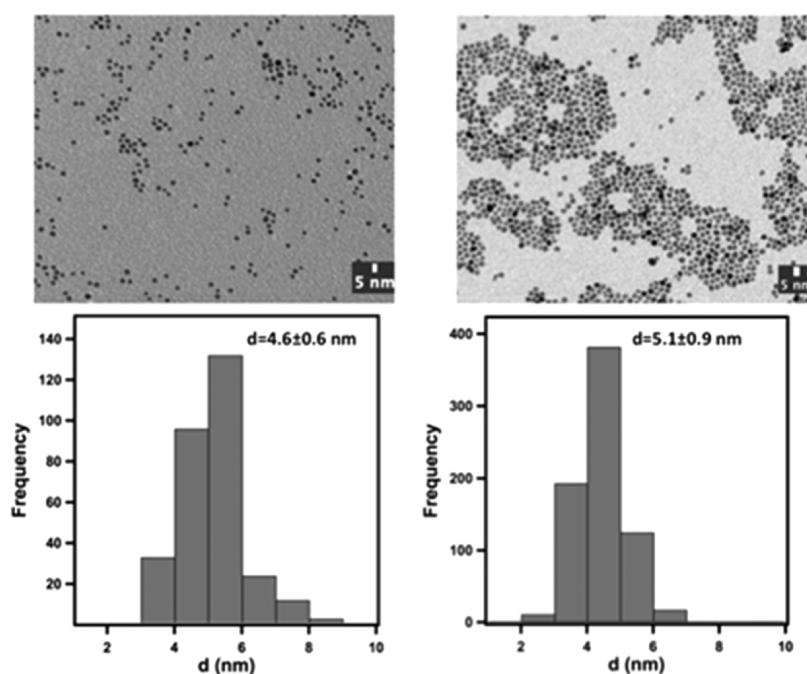


Figure 1. Representative TEM images of citrate-coated AgNPs (top left) and PtNPs (top right) and their size distributions (bottom left and right, respectively).

components cannot be assessed, losing crucial data on their potential toxicity.

Imaging techniques, such as focused ion beam scanning electron microscopy (FIB/SEM),⁹ dark-field microscopy,¹⁰ and surface-enhanced Raman scattering (SERS) imaging,^{11,12} provide information on the cellular localization of NPs, although with no assessment of the oxidation state of the material and strong limitations in case of small NPs (<10–20 nm). Moreover, transmission electron microscopy (TEM) and related techniques disclose high-resolution data on the cellular localization of NPs as well as on intracellular environment and cellular organelles.^{13–15} However, such approaches require complex preparative steps and are limited to the study of few thin sections of one single cell, not providing direct information about the eventual release of ions from the NPs. On the chemical side, the intracellular presence and distribution of released ions may be assessed through specific chemical probes *via* fluorescence microscopy, but this approach is not quantitative, is not able to detect/discriminate different chemical species/complexes, and is limited to a few metals, such as silver.¹⁶

An interesting innovation is the combined use of synchrotron-based X-ray fluorescence (nano-XRF) and X-ray absorption spectroscopy (XAS), which enables mapping the distribution of metal ions together with the characterization and quantification of ion complexes within cells after NP exposure.¹⁷ A recent report also showed direct measurement of the oxidation state of ceria NPs inside cells and organelles, using correlated scanning transmission X-ray microscopy and structured illumination microscopy.¹⁸ Nevertheless, synchrotron availability is a significant limit to its routine use in the nanomedicine field. The lack of lab-based techniques, relatively accessible and easy to perform, leaves many open questions and restricts the knowledge of NP fate within the cells to a few reports.^{19–22}

X-ray photoelectron spectroscopy (XPS) is one of the most widely used surface analysis techniques in material science and is able to characterize both the elemental and chemical oxidation state on the surface (analyzed depth <10 nm) of virtually any material, including metallic NPs. In the biological field, few papers have suggested the use of XPS in the analysis of yeast, fungi, diatoms, and bacteria.^{23–26} These studies explored changes in the chemical composition of the cell wall, without giving information about the inner parts.^{27,28} To investigate the chemical distribution at higher depths than the cell membrane with XPS, destructive depth profile experiments involving ion etching are required. Usually, in materials science, monatomic ions such as Ar⁺ or Cs⁺ are used to etch a few nanometers of the sample surface that is then analyzed at regular time intervals by XPS. Although this approach is useful for inorganic systems, its application for organic and biological materials has not been explored, as the structural composition of organic compounds is susceptible to damage from the bombardment with ions. The difficulty in performing minimally destructive etching combined with other issues in the cell culture or tissue preparation has increased the prejudice in the use of XPS combined with ion sputtering on complex biological samples. Moreover, an efficient charge compensation for rough and insulating materials is required to guarantee the feasibility and reproducibility of the measurements.^{29,30} To the best of our knowledge, only one preliminary report in the late 1970s combined Ar⁺ sputtering with XPS to identify thallium in the erythrocyte membranes casted on a gold substrate.³¹

In this paper, we propose a versatile method, named sputtering-enabled intracellular XPS (SEI-XPS), based on the combination of XPS, argon sputtering, and efficient charge compensation system,^{30,32–34} which allows to gain detailed chemical characterization of inorganic NPs internalized by cells, with nanoscale vertical resolution. We analyzed two model NPs made of different materials, that is, silver and

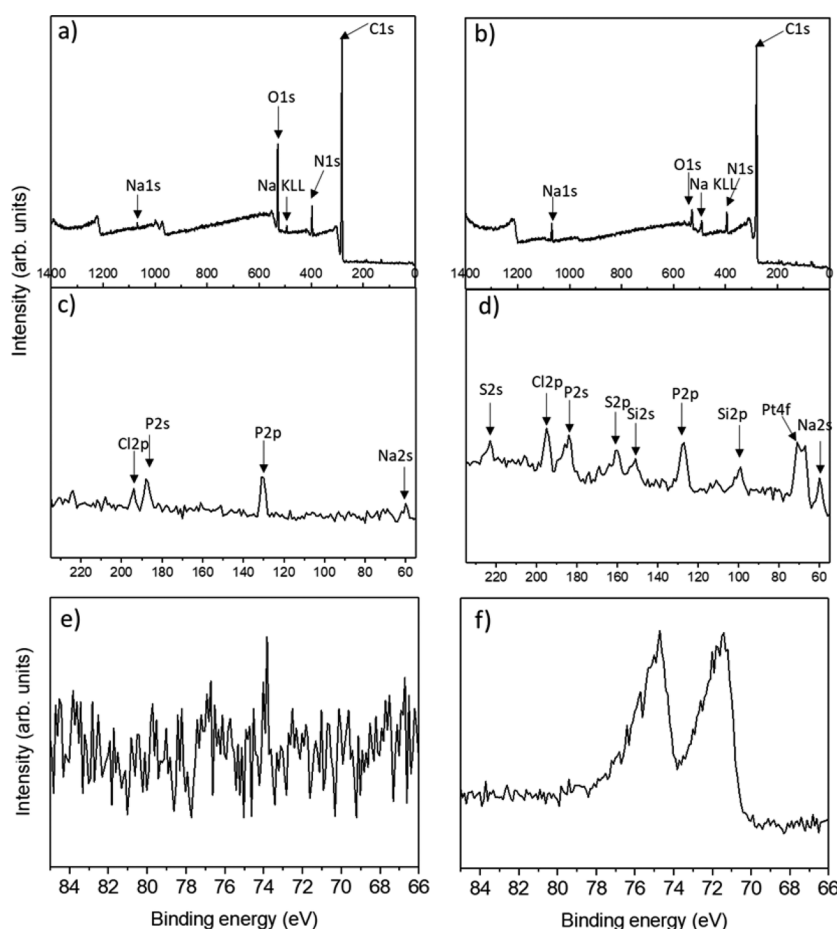


Figure 2. XPS survey spectrum of HeLa cells after exposure to PtNPs for 48 h before (a) and after (b) a single cycle of argon sputtering. The enlarged regions between 225 and 55 eV are presented in (c) and (d), respectively. High-resolution XPS spectra of the Pt 4f region are shown in panels (e) and (f), respectively.

platinum, known to undergo degradation and biopersistence, respectively, once internalized in cells, addressing their intracellular fate over time. In particular, for AgNPs, an extensive amount of literature has demonstrated that they are cytotoxic due to their release of toxic ions within the cells.^{3,16,17,35–37} On the other hand, PtNPs have shown great cytocompatibility and stability within the cellular environment.^{14,38} Notably, we demonstrate that SEI-XPS enables researchers to routinely study, with a widely diffused, lab-based, and easy-to-perform technique, the fate, degradation, and oxidation state of metallic nanomaterials within cells.

RESULTS

PtNPs and AgNPs Characterization and Cytotoxicity.

Citrate-coated AgNPs and PtNPs were characterized by transmission electron microscopy (TEM), and the size distributions were calculated by measuring the diameters of at least 300 particles (mean size of 4.8 ± 0.6 nm and 5.1 ± 0.9 nm, respectively) (Figure 1). The identical coating and similar dimensions of the two different NPs suggested the same cellular internalization efficiency and pathway.

Confluent HeLa cells, adherent on a glass substrate, were exposed to PtNPs and AgNPs for 3 and 48 h. Cellular internalization of the NPs was assessed by high-angle annular dark-field scanning transmission electron microscopy (HAADF STEM) analysis after 48 h incubation, showing the presence of NPs inside vesicular compartments (Figure S1). The

concentrations used were 100 $\mu\text{g}/\text{mL}$ for PtNPs and 50 $\mu\text{g}/\text{mL}$ for AgNPs, doses able to avoid strong effects on cell confluency and viability (Figure S2). After abundant washing to remove NPs physically adsorbed on cell membranes, the samples were fixed and directly analyzed by XPS without the need of complex preparative procedures.

XPS Characterization of Cells Exposed to PtNPs. One of the hardest challenges to obtain reproducible XPS spectra in organic materials is related to charging problems during the analysis, that is, the creation of a positive charge on the sample, due to the photoelectrons lost during the photoemission process. As a consequence, the binding energy (BE) of the emitted photoelectrons can increase during the measurement, resulting in a gradual shift of the observed peaks and overall perturbation of the results. To avoid such issues, we employed an efficient charge compensation system³⁴ to counterbalance the charging effects occurring on the cellular surface during the analysis. More in detail, the system employed (described in Materials and Method section) is fitted with an electron only charge compensation system to minimize the effect of induced charge during the analysis of electrically insulating materials and avoid the potential damage to the sample unlike low-energy ions systems. This advanced electron only charge neutralizing system is suitable for use on all electrically insulating materials, regardless of the physical state of the sample. In particular, it is formed by an electrostatic mirror/electron source coupled with magnetic immersion lens that are

able to force overfocused and under-focused photoelectrons back to the sample surface, making them available to neutralize surface charge. Therefore, the neutralizer is self-regulating, and many sample types may be analyzed without optimization of neutralization system settings.^{39–43} Furthermore, as the electrons are not produced by a focused source, they may reach the sample from many directions simultaneously. Hence, insulating samples with roughness up to hundreds of micrometers can be analyzed without the problems associated with shadowing of neutralizer electron/ions.⁴⁴ This permits reproducible analysis on different nanomaterials^{39–43} as well as on different cell types.

Figure 2a–c shows a representative survey spectrum of HeLa cells after exposure to PtNPs for 48 h, in which all the characteristic signals of elements present in a cell membrane, such as C, O, N, P, Na, and Cl, can be observed.³¹ Si signals were not detected, meaning that the glass substrate was continuously and homogeneously covered by cells on the analyzed area (*ca.* $700 \times 300 \mu\text{m}^2$), corresponding to about 100 cells. Moreover, Pt 4f signals were absent (Figure 2e), suggesting that PtNPs were not present on the uppermost cellular layer for a depth lower than ~ 10 nm, compatible with the thickness of the cellular membrane or its close proximity. This indicated that the washing procedure before the measurement was efficient in removing the aspecifically adsorbed NPs on the cell surface.

After the preliminary characterization of the cell surface, argon sputtering was employed to etch the superficial layer and expose the intracellular environment. Sputtering parameters, such as time and ion gun potential, were selected to elicit a small and progressive removal of the uppermost material. Atomic force microscopy (AFM) was exploited to characterize quantitatively the corrosion rate of a sputtering cycle (300 s), measuring the thickness of the cell film before and after sputtering. An average removal of 360 ± 23 nm after each cycle was optimized (Figure S3). Figure 2b–d shows a representative spectrum of the cell surface appearance after a single sputtering cycle. Interestingly, the intensities of photoelectron lines relative to S appeared, and the signals due to Cl, P, and Na became more prominent, indicative of the exposure of the internal part of the cells. Moreover, weak Si 2p signals (Figure 2b–d) were present, due to the perturbation of the homogeneity and continuity of the cellular layer, previously observed. However, comparing the intensity of the Si 2p electrons from the glass substrate after argon sputtering with that from a clean substrate and assuming that the intensity of Si 2p signal is inversely proportional to the degree of surface coverage, we estimated that the cultured cells still covered about 96% of the glass surface, confirming that the selected conditions were able to progressively expose the inner part of the cell.

Figure 2f shows the high-resolution spectrum of Pt 4f region present in the internal part of the cells after 48 h incubation. The two peaks at BE of 71.4 and 74.7 were ascribable to Pt 4f photoemission, which is split in two peaks with well-defined ratio, due to the spin–orbit coupling effects in the initial state. The presence of these signals was due to the Pt atoms present within the cell, confirming the internalization of the nanomaterial after incubation. Analogous results were obtained also after 3 h of incubation with PtNPs. To better characterize the stability and oxidation state of internalized PtNPs, fitted high-resolution XPS data of the Pt 4f region after 3 and 48 h of incubation were reported in Figure 3b,c, respectively. As a

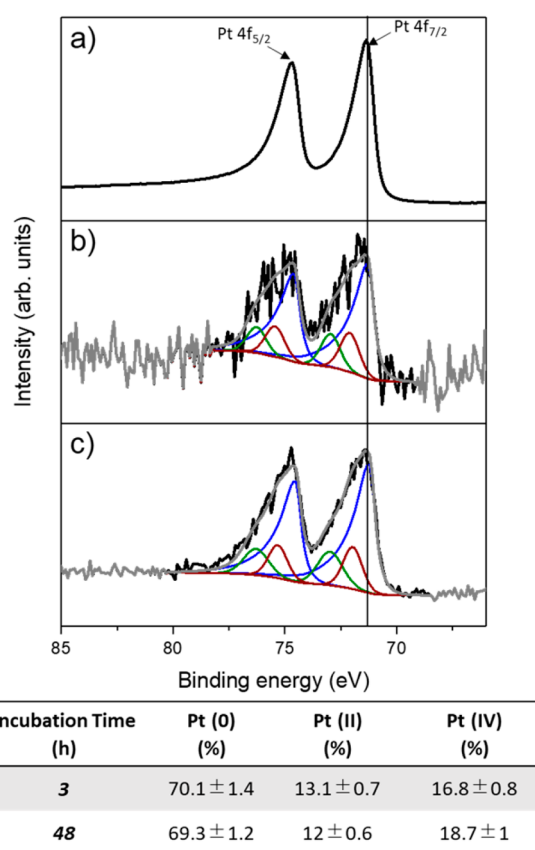


Figure 3. High-resolution XPS spectra of the Pt 4f region for freshly prepared PtNPs deposited on glass substrate (a), HeLa cells exposed to PtNPs for 3 (b) and for 48 h (c), after a single cycle of argon sputtering. Acquired (black lines) and fitted peaks (blue for Pt (0), red for Pt (II) and green for Pt (IV)) are shown as thin solid lines; fitted peaks sums (gray) are shown as solid lines. The bottom panel reports relative percentage of signal intensities after a single sputtering cycle of Pt (0), Pt (II), and Pt (IV) recorded on HeLa cells after exposure to PtNPs for different incubation times.

reference, a high-resolution spectrum of as-synthesized PtNPs was also included (Figure 3a), characterized by a Pt $4f_{7/2}$ signal at 71.2 eV, related to zerovalent platinum in PtNPs. On the other hand, the Pt 4f spectra of PtNPs inside cells could be fitted into three pairs of doublets (Figure 3b,c) with binding energies for Pt $4f_{7/2}$ of 71.2, 72, and 73 eV.⁴⁵ The relative intensities of the three components were quantified to be $69.3 \pm 1.2\%$, $12 \pm 0.6\%$, and $18.7 \pm 1\%$ after 48 h of incubation (see bottom panel in Figure 3). The signals at 72 and 73 eV were ascribed to Pt (II) and Pt (IV), respectively. The presence of these ion species was related to the surface oxidation of the nanomaterial, due to the intracellular environment. The percentage of the oxidized Pt was equal to 30%, regardless of the incubation times, and suggested the partial oxidation of the outermost layer of 5 nm diameter NPs.

Further argon sputtering cycles permitted to gradually expose other portions of the cells, as evident by the Si 2p signal increase, due to removal of cellular material from the glass substrate (Figure S4). However, the process did not cause significant changes in the proportion between the three components of Pt $4f_{7/2}$, indicating that this procedure did not significantly modify the oxidation state of PtNPs and that

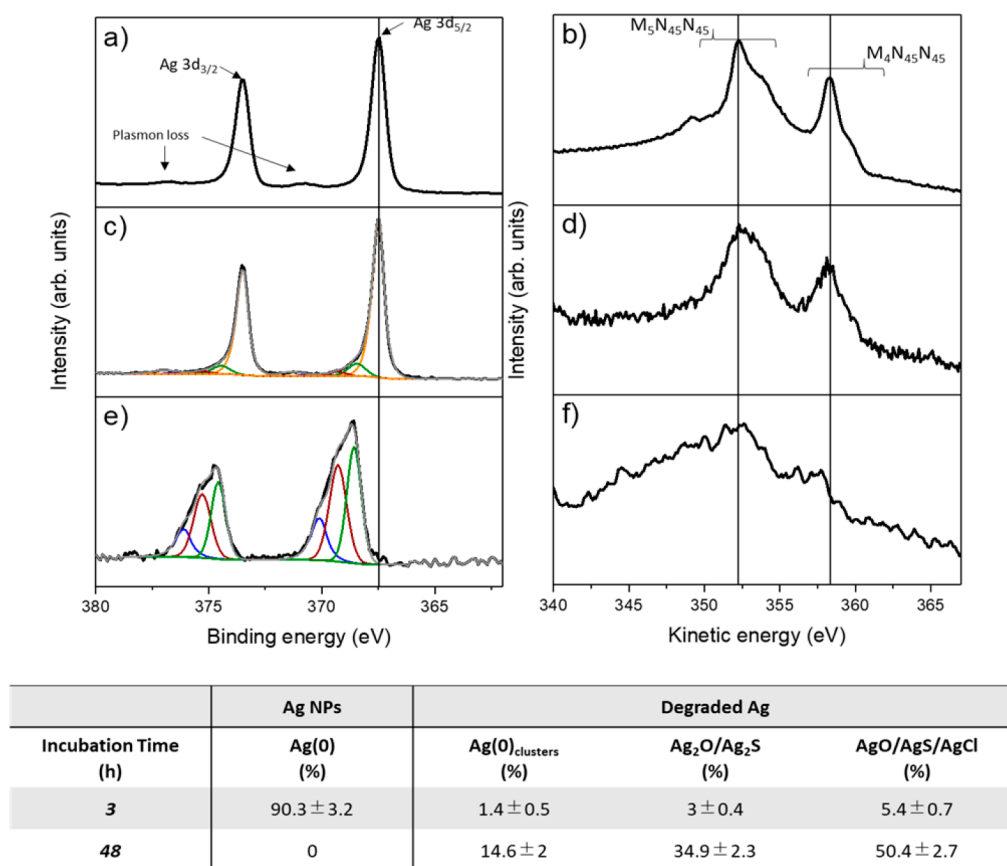


Figure 4. High-resolution XPS spectra of the Ag 3d region and Ag MNN Auger region for freshly deposited AgNPs (a and b, respectively), for HeLa cells exposed to AgNPs for 3 h after a single cycle of argon sputtering (c and d, respectively) and for HeLa cells exposed to AgNPs for 48 h after a single cycle of argon sputtering (e and f, respectively). Acquired (black) and fitted peaks (orange for Ag(0), green for AgO/AgS/AgCl, red for Ag₂O/Ag₂S and blue for clusters Ag(0)) are shown as thin solid lines; fitted peaks sums (gray) are shown as solid lines. The bottom panel reports the relative percentage of signal intensities after a single sputtering cycle of Ag(0), Ag₂O/Ag₂S, and AgO/AgS/AgCl recorded on HeLa cells after exposure to AgNPs for different incubation times.

the component ratio was constant over the entire portion of the cells (Figure S5).

The ratio between Pt components was also constant for the two incubation times, suggesting a quite rapid surface oxidation process of the intracellular PtNPs, followed by their stability/biopersistence within cellular vesicles (Figure 3, bottom panel). Notably, in cell culture medium, the spectrum of PtNPs did not show detectable differences from pristine PtNPs, where the zerovalent platinum signal was predominant (Figure S6). This means that, apart from the formation of classical protein corona surface layers (Figure S6), PtNPs were not affected by the cell culture medium and that the observed changes in the oxidation state of the nanomaterial happened only within the endolysosomal environment.

Furthermore, SEI-XPS is also able to provide semi-quantitative information about the efficiency of NP internalization over time. As a proof of principle, we estimated the amount of the intracellular metallic species, normalized to the signal of the carbonaceous material related to the cellular environment, calculated as (Pt 4f)/(C 1s) area ratio. As shown in Figure S7a, the experimental data demonstrated an increase of internalized PtNPs as a function of incubation time, with an order of magnitude difference between 3 and 48 h of cellular exposure. Interestingly, SEI-XPS internalization data were in qualitative agreement with standard inductively coupled

plasma atomic emission spectroscopy (ICP-AES) measurements (Figure S7b).

XPS Characterization of Cells Exposed to AgNPs. The same procedure described above was used for the analysis of AgNPs treated cells. Similarly to the above observations on PtNPs, survey spectrum without sputtering showed the characteristic signals of cells, such as C, O, N, P, Na, and Cl. Also in this case, the Si signals were not present before sputtering (Figure S8). Moreover, high-resolution spectrum of Ag 3d region evidenced a weak signal, due to the presence of some Ag species associated with cellular membranes (Figure S9). Figure 4a shows a high-resolution spectrum of Ag 3d region, originating from reference as-synthesized AgNPs, and Figure 4c–e reports the spectrum of AgNPs exposed cells (3 and 48 h incubation, respectively) after a single cycle of argon sputtering (survey spectrum in Figure S10).

The BE of Ag 3d_{5/2} core electrons of freshly deposited AgNPs was 367.4 eV, in agreement with previous literature.^{46,47} Moreover, the presence of plasmon loss peaks accounted for the metallic character of AgNPs. As observed in the case of PtNPs, the peaks position and shape of AgNPs dispersed in cell culture medium for 1 h at 37 °C (Figure S11) did not differ from that of as-synthesized AgNPs, confirming that culture medium did not significantly degrade the AgNPs and did not affect their oxidation state on this time scale. In the AgNPs exposed to the intracellular environment after 48 h of

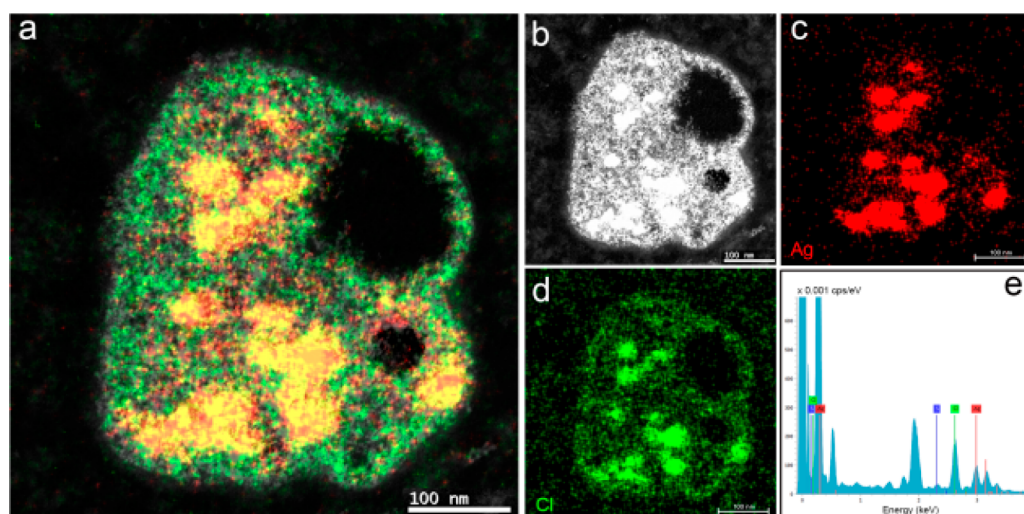


Figure 5. EDXS analysis on HeLa cells incubated with AgNPs after 48 h. (a) EDXS maps of Ag (red) and Cl (green) channels superimposed to a STEM image of an endosome. (b) Single HAADF-STEM image of the endosome shown in (a) (note that the image contrast is inverted). (c,d) The single Ag and Cl compositional maps superimposed in (a). (e) EDXS spectrum of the region mapped in (a).

incubation, the Ag 3d peaks shifted toward higher BE with respect to reference AgNPs, reaching a value for Ag 3d_{5/2} of 368.6 eV. Such an energy shift was reasonably attributed to the decrease of AgNP dimensions.^{48,49} The dependence of the BE on NP size is related to core–hole screening by conduction electrons. When the size of metal clusters is small enough, the quantum confinement effects become significant, and, hence, a net reduction in final-state screening leads to higher BE.⁵⁰ Notably, the observed value of the Ag 3d_{5/2} peak was close to that observed for silver clusters (of *ca.* 5–10 Å size),^{48,51–54} suggesting strong intracellular degradation of AgNPs after 48 h incubation. The BE shift due to AgNP dissolution was poorly detectable after 3 h of incubation, indicating that the majority of the AgNPs maintained their original dimension at short incubation time. A more detailed analysis showed that, after 3 h of incubation, the Ag 3d peaks exhibited a broadening at higher BE and, by a curve fitting procedure, at least three more spin–orbit pairs of components were evidenced (Figure 4c). The barely visible signal of Ag 3d_{5/2} at 370.1 eV was assigned to metallic silver atoms in the clusters.^{48,51–54} The second spin–orbit pair at 369.4 eV was attributed to Ag₂O and Ag₂S.^{55–62} The third spin–orbit pair (368.7 eV) was assigned to AgO/AgS/AgCl.^{59–61,63} The plasmon loss peaks were still present and correlated with the metallic component of Ag 3d_{5/2} at 367.4 eV. Contrary, a substantial change was observed after 48 h incubation (Figures 4e). The Ag 3d peaks showed an increase in the intensity of the three components at lower BE. Moreover, the components and plasmon loss peaks relative to metallic pristine NPs disappeared. The relative intensities for each pair of components were quantified to be 14.6 ± 2% for Ag (0) in the bulk of clusters, 34.9 ± 2.3% for Ag₂O/Ag₂S, and 50.4 ± 2.7% for AgO/AgS/AgCl, as reported in the bottom panel (Figure 4). It is important to point out that also for AgNPs the repeated cycles of argon sputtering did not provide significant changes in the relative ratio between the components and peaks position of Ag 3d (Figure S12).

Figure 4 right panel shows Ag MNN Auger structure of AgNPs, before and after cell incubation. For freshly deposited AgNPs, the Ag M₄N₄₅N₄₅ peak maximum occurred at a kinetic energy (KE) of 358.2 eV (Figure 4b), in good agreement with the KE values previously reported for silver metal. Moreover,

the Auger spectrum had the typical peak shapes of metallic Ag.^{61,62,64,65} Despite the presence of silver oxide species and metallic clusters after 3 h of incubation, Auger spectra (Figure 4d) did not significantly change, probably due to the small amount of oxide species with respect to pristine metallic NPs that were still more than 90% of the Ag 3d signal⁶⁵ (see bottom panel in Figure 4). After this short incubation time, the degradation of the nanomaterial just began and involved a small percentage of the AgNPs. After 48 h, the Auger spectra were characterized by two broad peaks. The small shift and the line-shape of the AgM₄N₄₅N₄₅ spectrum were consistent with those obtained for mixed oxidized Ag species.^{60,61,66,67} All these results clearly prove that, for longer incubation times, AgNPs within the cellular environment are substantially degraded, with the release of ion species responsible for the toxicity of the nanomaterial.^{3,35}

SEI-XPS data on intracellular NPs were validated by high-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) and energy dispersive X-ray spectroscopy (EDXS) imaging. Such analyses confirmed that PtNPs were stable in endolysosomal environment, even after 48 h incubation (Figure S13). On the other hand, AgNPs underwent extensive intracellular dissolution, and, after 48 h, different and heterogeneous silver ion complexes, including AgCl and AgS, were formed in the endolysosomal environment (Figure 5 and Figure S14).

DISCUSSION

The experimental results presented in the above section prove that SEI-XPS is a powerful tool to directly investigate *in situ* the intracellular fate of metallic NPs, without any complex preparative procedure. The technique can provide detailed data on the degradation of the nanomaterial as well as the oxidation state of the ion species released. SEI-XPS also gives semiquantitative information about the cellular internalization of the metallic species. Additionally, it is possible to discriminate the NPs present on the cell membrane from the internalized ones, thus distinguishing the biointeractions specifically elicited by the intracellular environment with respect to protein corona effects, and avoiding uptake overestimation due to adsorbed nanomaterial on the cell

surface. As shown, such precise discrimination is possible with direct SEI-XPS measurements, without the need of any pretreatments, such as chemical etching.⁶⁸

The common idea about argon sputtering as a destructive technique has discouraged to date its use for the analysis of biological samples and nanomaterial internalization. Here, we present an effective procedure obtained by careful optimization of argon sputtering parameters, through the fine-tuning of time and ion gun potential, together with the analysis of relative signals ratio coming from the substrate, the cell surface, and the internalized nanomaterial. AFM measurements were employed to further characterize the sample partial removal and optimize the sputtering efficiency. Moreover, this protocol combined together with cell homogeneity, confluency, and fixation gives the possibility to perform XPS analysis both on the uppermost layer and in the intracellular environment. This allows to analyze chemical modification and degradation of metallic NPs, with high precision (nanometer vertical resolution) and statistical robustness. XPS measurements on cultured cells are particularly challenging, as these materials have rough surfaces and insulating properties. These issues have been overcome using an efficient and self-regulating electron charge compensation system in the XPS setup, to avoid the overcharging and damage of the surface, thus guaranteeing high reproducibility and reliability of the measurements.

As a proof of principle, AgNPs and PtNPs fate within cells was investigated over time, and particle degradation as well as oxidized Ag and Pt atoms were quantitatively assessed. We observed that, after cellular internalization, PtNPs and AgNPs revealed a completely different behavior, due to their different stability in the intracellular environment, despite the same dimension and coating. In agreement with STEM/EDXS analyses, our XPS data clearly show that PtNPs are stable after 48 h of incubation, even within endosomes/phagolysosomes, with no detectable NP degradation and ion release; only some surface oxidation was rapidly induced by the intracellular environment. The percentage of the oxidized Pt was constant (about 30%), regardless of the incubation times, and compatible with a partial oxidation of atoms present on the surface of 5 nm diameter NPs. Within the acidic environment of the endolysosomes, it is reasonable that the outer layer of the PtNPs is oxidized, while the citrate coating is replaced by thiolated molecules abundantly present within cells^{69,70} that strongly bind to the surface of the NPs.⁷¹

SEI-XPS data also provide semiquantitative information about the amount of PtNPs internalized within cells, offering the possibility to estimate the variation of nanomaterials within the biological samples. The relative amount of PtNPs within cells increased over time by almost 1 order of magnitude, proving the ability of the biological sample to incorporate an increasing amount of nanomaterial without manifesting toxicity effects.

Contrary to PtNPs, SEI-XPS data demonstrated that AgNPs undergo strong degradation in an endolysosomal environment, forming new nanoclusters as well as AgO/AgS/AgCl complexes. Such a biotransformation process massively occurs within 48 h, involving the majority of the NP atoms. More than 85% of the nanomaterial was oxidized within cells, indicating that the degradative process does not involve only the surface but also the core of AgNPs, leading to complete dissolution and ion release. SEI-XPS data showed the presence of a metallic residue (around 10%) that is related to the

formation of Ag clusters.⁷² The rate of the dissolution calculated by our method was in close agreement with STEM/EDXS analyses and also in line with the synchrotron-based technique (XAS-XANES), considering that XAS-XANES was performed using different AgNP sizes, cell lines, and incubation times.¹⁷ Furthermore, XPS data prove that the dissolution of AgNPs mainly occurs within the cellular environment, as the interaction with the cell culture medium poorly affected the NP oxidation state.

CONCLUSIONS

In conclusion, SEI-XPS is a powerful experimental tool able to provide detailed information about NPs–cell interaction process. This approach has high versatility, as it permits the analysis of different NPs and cells, with roughness up to hundreds of micrometers, offering the possibility to assess the NP dissolution/biopersistence and, hence, the mechanism behind toxicity. Considering the increasing interest for biomedical application of metallic NPs and the growing amount of NPs of different shape, size, and material, SEI-XPS can meet the demand of a relatively easy-to-use and easy-to-access technique to investigate the cellular fate of nanomaterials.

MATERIALS AND METHODS

Materials. Silver nanoparticles were bought from NanoComposix. TEM images are shown in Figure 1a. Platinum nanoparticles (Figure 1b) have been synthesized following a modified protocol reported elsewhere.

Pt nanoparticles were synthesized by adding H₂PtCl₆ (160 μ L, 0.5 M) to 79.65 mL of Milli-Q water together with a solution of sodium citrate (192 μ L, 0.5 M). Under vigorous stirring, 5 mL of an aqueous solution of 0.06 M NaBH₄ was slowly added. The vessel was then moved to a silicon oil bath at 75 °C. The reaction was kept under stirring for 30 min. After being cooled to room temperature, the Pt nanoparticles were thoroughly purified using 10 K Amicon filters with 2 mM sodium citrate solution.

Cell Culture. Human cervix epithelioid carcinoma (HeLa) (ECACC) cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma-Aldrich), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich). Cells were maintained in an incubator at 37 °C under a humidified controlled atmosphere and 5% CO₂.

STEM/EDXS Analyses. HeLa cells after incubation with 5 nm Pt nanoparticles were processed as reported elsewhere.¹⁴ Briefly, the cells after incubation were fixed for 1 h in 1.2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), post fixed in 1% osmium tetroxide in the same buffer and en bloc stained with 1% uranyl acetate aqueous solution. The cells were then dehydrated in a graded ethanol series and embedded in epoxy resin (Epon 812, TAAB). Semithin and thin sections of the embedded cell monolayer were cut with an ultramicrotome (UC6, Leica) equipped with a diamond knife (Diatome). Both projection images and tilt series were acquired in STEM mode working in HAADF geometry, using a FEI Tecnai F20 transmission electron microscope operating at 200 kV and equipped with a Schottky field-emission gun.

The EDXS analysis was performed using the EDX detector Bruker XFlash 6T30 on plastic embedded ultrathin sections of HeLa cells incubated with NPs. The compositional analysis has been performed in dark-field scanning transmission electron microscopy using a high angular annular detector (HAADF-STEM) on a Thermo Fisher Scientific Tecnai G2 F20 scanning transmission electron microscope equipped with a 200 kV field emission gun (FEG).

XPS Sample Preparation. Glass coverslips, carefully cleaned with acetone, ethanol, and finally washed with PBS for three times, were deposited on a 24-well multiwell plate (Corning), and HeLa cells

were seeded at high confluence (6×10^5 cells per well). The samples were incubated overnight, leading cells adhesion and growth. Then, NPs stock solutions (1 mg/mL) were diluted into complete DMEM medium at a final concentration of 100 $\mu\text{g/mL}$ (or 50 $\mu\text{g/mL}$), and 1 mL of this solution was deposited on the HeLa samples maintained in the incubator for 48 h. After incubation, the medium was removed, and the samples were abundantly washed with PBS for three times in order to remove the NPs excess. The cells were then fixed using paraformaldehyde 4% (PFA, Sigma-Aldrich) for 20 min and again washed carefully with PBS to remove PFA residues. Finally, the samples were washed with Milli-Q water to remove salt residues and left to dry under the air stream of the hood at least for 1 h in the dark. The control samples were prepared by depositing a drop of the NPs stock solutions on the glass slides cleaned and dried in the same way. The samples were then immediately mounted on the XPS vacuum chamber for the analysis.

The effects of the cells medium on the NPs oxidation were evaluated by incubating 100 μL of 1 mg/mL NPs stock solution (PtNPs or AgNPs) with equal amount of complete DMEM medium for 1 h at 37 $^\circ\text{C}$. The solution was then centrifuged at 80,000g for 45 min. The pellet was suspended in Milli-Q water and deposited on a glass slide for XPS analysis.

XPS Analysis. XPS measurements were performed with an AXIS ULTRA DLD (Kratos Analytical) electron spectrometer using a mono-Al K α source (1486.6 eV) operating at 225 W (15 kV, 15 mA). Survey scan spectra were recorded using a pass energy of 160 eV and a 1 eV step. Narrow scans of single regions were acquired using a pass energy of 20 eV and a 0.1 eV step. The hybrid lens mode was used for all measurements. In each case, the area of analysis was about $700 \times 300 \mu\text{m}^2$. The base pressure in the analysis chamber was 2×10^{-9} Torr. During the acquisitions, a system of neutralization of the charge, consisting of a filament and electrode plate mounted at the bottom of the electrostatic input lens system, was used. The magnetic field, generated by the magnetic immersion lens, confines low-energy emitted electrons from the filament and transports them to the sample surface. The charge balance electrode directs the neutralization electron from the filament into the magnetic field.³⁴ The BE scale was referenced to the C 1s line of alchilic carbon (285 eV). Processing of the spectra was accomplished with CasaXPS Release 2.3.16 software.

Sputtering was performed by leaking Ar gas into the chamber with an ion gun (Kratos Ar Gas Cluster Ion Source) at 3 kV, at a fixed distance from the sample surface with a rastered area of $1 \text{ mm} \times 1 \text{ mm}$. Sputtering time for each cycle is 300 s.

AFM Analysis. AFM measurements were carried out using a commercially available microscope (Agilent Technologies, 6000 ILM) at room temperature in air, working in dynamic tapping mode. Commercially available silicon cantilevers (Mikromasch NSC35 B, nominal spring constant 16 N/m) were used, working at low oscillation amplitudes with a half free amplitude set point. High-resolution images ($30 \times 30 \mu\text{m}^2$, 512×512 pixels frames) were acquired at 0.5 lines/s scan rate. Sample thicknesses have been calculated as the difference between the cells surface before and after sputtering and a flat scratched area of the surface cleaved using a scotch tape and exposing the bare glass below. The line profile of the samples was measured on a $90 \times 90 \mu\text{m}$ region and mediated on 512 lines. The measurements were performed on three independent samples, and the standard deviation has been calculated. Agilent Pico Image software was used for data processing.

Quantification of the NP Intracellular Uptake. The amount of internalized NPs per cell was quantified by ICP-AES (Agilent 720/730 spectrometer). Five $\times 10^5$ HeLa cells per well were seeded in 6-well plates (EuroClone) and treated with 100 $\mu\text{g/mL}$ PtNPs for different time points (1, 3, 6, and 48 h). After the treatment, 3 wells were pooled together. Untreated cells were used as a control. After incubation, cells were washed three times with phosphate buffered saline (PBS) (Sigma-Aldrich), detached by 100 μL Trypsin 1 $\times$ (Sigma-Aldrich), and suspended in 900 μL PBS. The number of harvested cells was determined by using a TC10 automatic cell counter (Biorad). After centrifugation (300g for 5 min), cell pellets were frozen, dried in oven at 80 $^\circ\text{C}$, and, finally, digested overnight

with 1 mL of aqua regia in a CEM Discover SP Microwave synthesizer. The samples were then diluted to 10 mL with Milli-Q water and analyzed by ICP-AES. The values obtained were then normalized to the cell number. Results were reported as the mean $\pm$ standard deviation (SD) of six different replicates.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.8b01612.

HAAD-STEM images, cell viability assays, AFM characterization, additional XPS spectra, ICP-AES measurements, STEM/EDXS images (PDF)

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Notes

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