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Bactericidal activity of silver nanoparticles: An analytical approach based on single cell and single particle inductively coupled plasma mass spectrometry analysis to determine silver species involved

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ABSTRACT

The bactericidal activity of silver nanoparticles is supported by a large number of studies, but their action mechanisms are still a controversial issue due to the role of the silver (I) released from the nanoparticles. In this study, direct analytical methods for detection and identification of dissolved and nanoparticulate silver based on single cell and single particle inductively coupled plasma mass spectrometry (ICP-MS) and hydrodynamic chromatography ICP-MS, in combination with alkaline digestions, have been used. Detection of silver species in *Escherichia coli* bacteria exposed to silver allowed to confirm the different bactericidal activity associated with silver nanoparticles of different sizes. In the case of 10 nm silver nanoparticles, a combined ion-particle action would be responsible for bactericidal effect, since ionic silver was not detected in the culture medium and both dissolved and particulate silver were detected in the exposed bacteria. On the other hand, bacteria did not internalize 60 nm silver nanoparticles and their bactericidal activity was related to the ionic silver released in the culture medium.

1. Introduction

In the first half of the 20th century, until the introduction of antibiotics, metallic silver was widely used in the form of a colloidal suspension to prevent infections, although in some cases, prolonged exposure produced a disease called argyria, gastrointestinal disorders, spasms and even death [1]. Nowadays, silver is used in water treatment systems and medical applications such as dentistry, catheters and burn treatments [2–4]. Likewise, silver ions have been proposed as an effective antimicrobial alternative to antibiotics due to their low cytotoxicity [5,6]. On the other hand, silver nanoparticles are presented as potent antimicrobial agents to fight against antibiotic resistance due to the change in their physicochemical properties and their high surface area-to-volume ratio, which would result in enhanced reactivity. It has been demonstrated that silver nanoparticles are effective against a broad spectrum of resistant bacteria [7] and act as fungicide and antiviral agents [8]. Although the bactericidal activity of silver (I) and silver nanoparticles is influenced by the structure and composition of the bacterial cell wall [9], in the case of silver nanoparticles, their physicochemical properties (size, shape, chemical surface, concentration, coating) also play a relevant role [8–10]. Despite there are many studies related to silver nanoparticle bactericidal activity, most of them evaluate the silver toxicity by using diffusion assays or dilution methods [11], as well as by the observation of membrane alterations, pore formation or accumulation of nanoparticles by transmission (TEM) or scanning electron microscopy (SEM) [12–17]. However, only a few studies have drawn conclusions about the contributions that silver ions released from nanoparticles and the particles themselves play in the mechanism of

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antimicrobial activity [18–24]. The three mechanisms associated to the bactericidal activity of silver nanoparticle are: 1) ion-only action; 2) particle-only action; 3) combined ion-particle action [21]. In the first case, nanoparticles are a passive reservoir of silver ions and the released ions are responsible for the bactericidal activity. In the second one, the activity is solely due to the particles. The third one is a combination of the previous two and it could involve synergistic effects between the released ions and the particles. Stabryla *et al.* reported that 39 % of the studies concluded that bactericidal effect was based on the ion-only action, 16 % on the particle-only action, and 45 % on a combined ion-particle action [21].

Studies reporting conclusions on the different bactericidal activity of silver nanoparticles are based on the use of different analytical techniques such as TEM, SEM, atomic absorption spectroscopy (AAS), inductively coupled plasma optical emission spectroscopy (ICP-OES), inductively coupled plasma mass spectrometry (ICP-MS), UV–visible spectroscopy, dynamic light scattering (DLS) or bioluminescence, and have been summarized in Table 1. Of these techniques, only electron microscopy, bioluminescence, voltammetry and potentiometry with ion-selective electrode allow to obtain direct information about the presence of nanoparticles or released silver (I). On the other hand, electron microscopy provides information about nanoparticle biodistribution, but it is limited to obtain qualitative information in relation to cell interaction with nanoparticles. Sondi *et al.* incubated *E. coli* bacteria with 12 nm silver nanoparticles in Lysogeny broth (LB) medium, observing adsorbed and embedded nanoparticles in the bacterial wall and justifying the bactericidal activity to a particle-only action, since the potential dissolution of the nanoparticles and the occurrence of dissolved silver was not checked [13]. Regarding studies involving voltammetry, Morones *et al.* confirmed the release of silver ions from 16 nm silver nanoparticles, although using a medium different from the one used for bacteria culture [37]. Maurer-Jones *et al.* studied the dissolution of 11 nm silver nanoparticles in culture medium and in the presence of bacteria by monitoring silver ions using an ion-selective electrode. They concluded that ion release was lower in presence of bacteria that in their absence and proposed the ion-only action to explain the bactericidal activity of the nanoparticles due to the different toxicities observed between nanoparticles and silver ions [41]. In a similar way, bioluminescence studies confirmed the presence of intracellular silver ions in bacteria [28,33,36]. In addition, these authors used indirect methods based on ultracentrifugation with atomic absorption spectrometry [28,33,36] and UV–vis spectrophotometry [28] to quantify the release of ions by oxidation of nanoparticles in microorganism culture medium, which allowed to propose a combined ion-particle action [28,33,36].

On the other hand, silver (I) from nanoparticle oxidation has been indirectly determined by ICP-MS and ICP-OES after separation of nanoparticles from the dissolved silver by ultracentrifugation [29,30], centrifugation [41], ultrafiltration [31,32,34,38,39,42,44] or dialysis [26,35]. Alternatively, other authors have used AAS and a previous separation step by using ultracentrifugation [25,28,33,40,45]. It should be noted that the release of dissolved silver from nanoparticle oxidation was studied mainly in water [35] or in different saline media ($NaHCO_3$ [31,39], NaCl [34], PBS [26], others [38,42,44]), whereas only a limited number of studies were performed in culture media [28–30,32]. Other indirect methods used for studying the oxidation of nanoparticles have been UV–visible spectroscopy [28] or conductivity measurements [27,43]. In any case, studies in which nanoparticle oxidation was determined in media different from the culture one should not be considered representative of the processes undergone by nanoparticles along the microbiological essay. Studies performed in culture media, concluded that a combined ion-particle action was associated with silver nanoparticle toxicity because nanoparticle properties affected the ion released [29,30].

Apart from those described above, there are other analytical techniques that allow the determination of silver species directly, without the requirement of previous sample preparation methods involving separation steps (e.g. (ultra)centrifugation, ultrafiltration or dialysis). Single particle inductively coupled plasma mass spectrometry (SP-ICP-MS) and hydrodynamic chromatography (HDC) coupled with ICP-MS allow the simultaneous discrimination between dissolved species and nanoparticles and therefore, the quantification of both fractions separately and the determination of nanoparticle sizes [46,47]. Likewise, single cell inductively coupled plasma mass spectrometry (SC-ICP-MS) is a technique that allows to detect and quantify the content of different elements in cells and to obtain information on mass distribution of elements per cell [48,49]. To the best of authors knowledge, these techniques have not been used to date to directly determine the silver species involved in the bactericidal activity of silver. The aim of this work is the development and application of an analytical platform that allows the direct detection and quantification of silver species in cultures of *E. coli* bacteria previously exposed to silver (I) and silver nanoparticles, with the objective of providing the chemical information required to elucidate the bactericidal action of different silver nanoparticles. The analytical platform is based on the combination of SC-ICP-MS, SP-ICP-MS and HDC-ICP-MS and it is going to allow: i) the determination of total silver (adsorbed or internalized) in bacteria and its distribution per bacteria, ii) the determination of silver species present in bacteria, iii) the evaluation of the relationship between silver species detected in culture medium and bacteria and their potential role in the bactericidal activity.

2. Materials and methods

2.1. Bacterial cultures with silver

Two different *Escherichia coli* strains were used, *E. coli* ATCC 25922 and *E. coli* K12 strain J62 (F^-, pro, his, trp, lac, Nal^r) [50,51]. The strains were cultured in Müller Hinton Agar (MHA) and grown overnight at 37ºC. The obtained bacteria were diluted in Müller Hinton Broth (MHB) + 2 % Tween 80 [52]. Bacterial suspensions of *E. coli* ATCC and J62 were exposed to 0.5 mg L^{-1} of silver (I) or 60 nm silver nanoparticles during 24 h at 37ºC and 100 rpm. In the case of 10 nm silver nanoparticles, exposure concentrations were 4 and 2 mg L^{-1} for *E. coli* ATCC and J62, respectively. Ag(I) and AgNPs concentrations were selected based on the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC), so that these concentrations were lower than MIC and MBC to avoid bacteria death. Details of standards and reagents are included in Supplementary Information. Bacterial cells non-incubated with silver (control) and a culture medium control (without bacteria or silver) were also treated in the same way. After exposure time, bacterial cultures were centrifuged at 4600 g during 15 min. The supernatants were removed and the bacterial cell pellets were washed three times with phosphate buffer solution (PBS) under the same conditions. Then, the bacterial pellets were resuspended in PBS and the samples were stored at 4 °C until use. To estimate the concentration of bacteria in samples, optical density of bacterial cultures was measured with an UV–vis spectrophotometer (UV-2004 Lan Optics) at 600 nm. Bacteria concentrations ranged 2–26 x 10^9 mL^{-1} for ATCC strain, and 1–11 x 10^9 mL^{-1} for J62 ones. To determine MIC and MBC, bacteria were exposed to half serial concentrations of silver (I) (1–256 mg L^{-1}) and 60 nm or 10 nm AgNPs (0.06–16 mg L^{-1}).

2.2. Acid digestion of bacterial samples

Aliquots of 100 μL of bacterial suspensions were subjected to centrifugation (Thermo Heraeus Multifuge X1R, equipped with a swinging bucket rotor, Walthman, USA) for 20 min at 10,000 g to remove the PBS medium. 500 μL of HNO_3 (69–70 % *w/v*) and 100 μL of H_2O_2 (30 % *v/v*) were added to the resulting pellet and samples were digested for 24 h at room temperature and shaken at 124 rpm. After digestion, the volume was made up to 10 mL with 1 % HNO_3 (*v/v*) and the content of total silver was quantified by ICP-MS (instrument and

Table 1
Studies of bactericidal activity of silver nanoparticles. Direct (D) and indirect (I) evidence of action process. (*) The medium studied was different from culture medium.

Action	Bacteria	NP size (nm)	NP coating	Exposure medium	Analytical technique	Evidence		Ref.
Particle-only	*E. coli*	12	–	LB	TEM/EDS	NPs adsorbed to bacterial wall and inside bacteria	D	[13]
	E. coli	10 56 72	BPEI Citrate PVP	Water	Centrifugation + GF-AAS	Ag(I) released by NP oxidation	I	[25]
	E. coli *S. aureus*	7	Glutathione	PBS	Dialysis + ICP-OES	Ag(I) released by NP oxidation	I	[26]
	Bacillus sp.	10 12	BPEI PVP	NP original medium*	Conductivity	Low NP dissolution	I	[27]
		19	Citrate	BOD	TEM	NP interaction with bacterial wall	D	
		18	Hydrogen		DLS	No NP aggregation	I	
Combined ion-particle	*E. coli*	9–40	Citrate	LB	DLS	NP size increase	I	[28]
		18	PVP		UV–visible	NP dissolution	I	
		23	BPEI		Ultracentrifugation + GF-AAS	Ag(I) released by NP oxidation	I	
					Bioluminescence	Determination of intracellular Ag(I)	D	
					AFM/TEM	NP adsorption on bacterial wall No internalization observed	D	
	E. coli	140	–	LB	Ultracentrifugation + ICP-MS	Ag(I) released by NP oxidation	I	[29]
					TEM	Bacterial wall-associated NPs and NPs in cytosol	D	
	E. coli	24	PEGSH	LB MHB	Ultracentrifugation + ICP-MS	Ag(I) released by NP oxidation	I	[30]
					TEM DLS	NPs preserve their original size	D	
	E. coli	10	Citrate MPA MHA MPS	$NaHCO_3$	Ultrafiltration + ICP- MS	Ag(I) released by NP oxidation	I	[31]
					X-Rays	Ag(I) released by NP oxidation	I	
					SEM	Structural changes in bacteria	D	
					Lysis + ICP-MS	Silver adsorbed on bacterial wall and internalized	D	
	E. coli *Bacillus* sp.	45	Citrate	LB	Filtration + ICP- MS	Ag(I) released by NP oxidation	I	[32]
					FESEM	NP adsorption on bacterial wall	D	
	E. coli *Pseudomonas fluorescens*	10–80	Citrate	Water	Ultracentrifugation + GF-AAS	Ag(I) released by NP oxidation	I	[33]
					Bioluminescence	Determination of intracellular Ag(I)	D	
	Vibrio fischeri	20 200	–	NaCl	Ultrafiltration + ICP- MS	Ag(I) released by NP oxidation	I	[34]
					DLS	NP aggregation	D	
	Synechococcus	20 40 100	Maltose	Freshwater	Dialysis + ICP-MS	Ag(I) released by NP oxidation	I	[35]
					CPS	NP aggregation	I	
	E. coli	<100	–	NaCl	Bioluminescence	Ag(I) released by NP oxidation	D	[36]
	E. coli *V. cholera* *P. aeruginosa* *S. typhus*	16	–	LB $NaNO_3$*	HAADF/STEM TEM Voltammetry	NPs adsorbed and internalized Ag(I) released by NP oxidation	D	[37]
	E. coli	35	Amorphous carbon	NP original medium*	Ultrafiltration + ICP- MS	Ag(I) released by NP oxidation	I	[38]
Ion-only	*E. coli*	3–11 18–70	PEG PVP	$NaHCO_3$	Ultrafiltration + ICP- MS	Ag(I) released by NP oxidation	I	[39]
	E. coli	80 100	Citrate	Water	Ultracentrifugation + GF-AAS	Ag(I) released by NP oxidation	I	[40]
	Shewanella oneidensis	11	Citrate	Ferric citrate	Ion-selective electrode	Ag(I) released by NP oxidation. Lower released in presence of bacteria	D	[41]
					Centrifugation + ICP-MS	Ag(I) released by NP oxidation	I	
					DLS	NP aggregation	I	
					TEM	No morphological changes in bacteria. No internalization of NP	D	
	Nitrosomonas europaea	20 80	Phosphate	HEPES + $(NH_4)_2SO_4$	Ultrafiltration + ICP-OES	Ag(I) released by NP oxidation	I	[42]
					DLS	NP aggregation	I	
	E. coli *S. aureus*	6	Mercapto ligands	Water	Conductivity	Ag(I) released by NP oxidation	I	[43]
					TEM	Ag(I) interaction with bacteria Deposits of Ag(I) inside bacteria	D	
	Nitrosomonas europaea	20–25	Citrate PVP GA	AOB	Ultrafiltration + ICP- MS	Ag(I) released by NP oxidation	I	[44]
					DLS	NP aggregation	I	
					TEM	Membrane damage	D	

AFM: atomic force microscopy; AOB: ammonia oxidizing bacteria; BOD: biological oxygen demand; BPEI: branched polyethylenimine; CPS: centrifugal particle sedimentation; DLS: dynamic light scattering; EDS: energy-dispersive x-ray spectroscopy; FESEM: field emission scanning electron microscopy; GA: gum arabic; GF-AAS: graphite furnace atomic absorption spectroscopy; HAADF/STEM: high-angle annular dark-field scanning transmission electron microscopy; LB: Lysogeny broth; MHA: mercaptohexanoic acid; MHB: Müller-Hinton Broth; MPA: mercaptopropionic acid; MPS: mercaptopropanesulfonic acid; PBS: phosphate buffer saline; PEG: polyethylene glycol; PEGSH: polyethylene glycol dithiol; PVP: polyvinyl pyrrolidone.

data acquisition details are included in Supplementary Information). Five replicates of each sample were analyzed.

2.3. Alkaline digestion of bacterial samples

In the same way as acid digestion, aliquots of 100 μL of bacterial suspensions were subjected to centrifugation to remove the PBS medium. 400 μL of $CaCl_2$ 25 mM and 1.6 mL of TMAH (25 % *w/w*) were added to the resulting pellet and samples were digested for 24 h at room temperature and darkness. After digestion, the volume was made up to 10 mL with ultrapure water so that TMAH final concentration was 4 %. The total silver content was quantified by ICP-MS and the presence of the different chemical species of silver in bacteria was determined by SP-ICP-MS and HDC-ICP-MS (instrument and data acquisition details included in Supplementary Information – Table S1 and S2). Five replicates of each sample were analyzed. Bacterial control samples spiked with 5 μg L^{-1} of silver (I) or 60 nm silver nanoparticles were also subjected to the alkaline digestion procedure.

2.4. SP-ICP-MS measurements and data processing

The determination of silver species after the incubation of silver nanoparticles in the culture medium was analyzed by SP-ICP-MS (Table S1). Silver nanoparticles of 10 and 60 nm (2 and 0.5 mg L^{-1}, respectively) were incubated in MHB+2% Tween 80 medium for 24 h at 37ºC and shaken at 130 rpm in an orbital incubator (OPAQ I10-OE, Ovan). Before analysis by SP-ICP-MS, both suspensions of nanoparticles incubated in culture medium and samples digested with TMAH were diluted in ultrapure water to get a particle number concentration of approximately 10^8 particles L^{-1}. Suspensions were measured in single particle mode using the Syngistix Nano-Application module version 2.5 (PerkinElmer Inc.) and the recorded signals were processed by applying a 5-sigma threshold calculated as five times the square root of the mean baseline intensity of the time scan [53,54]. Nebulization efficiency was calculated according to the methods developed by Pace *et al.* [55] by using the ultra-uniform gold nanoparticle standard described in Supplementary Information. In the study of nanoparticles incubated in culture medium, the nebulization efficiency (size method) was 5.90 ± 0.03 % (n = 3), whereas in the analysis of digested samples with TMAH, the nebulization efficiency was 5.72 ± 0.02 % (n = 6). Sample flow rate was measured gravimetrically.

2.5. Analysis by HDC-ICP-MS

The determination of silver species after the incubation of silver (I) and silver nanoparticles in the culture medium was also analyzed by HDC-ICP-MS (Table S2). Silver (I) (0.5 mg L^{-1}) and silver nanoparticles of 10 and 60 nm (2 and 0.5 mg L^{-1}, respectively) were incubated in MHB+2% Tween 80 medium for 24 h at 37ºC and shaken at 130 rpm in an orbital incubator. Before analysis by HDC-ICP-MS, both suspensions of nanoparticles incubated in culture medium and the samples digested with TMAH were diluted in ultrapure water to get a concentration of approximately 100 μg L^{-1}. Volumes of 50 μL of each sample were directly injected in the HDC column. The mobile phase consisted of 0.45 mM sodium dodecyl sulphate and 1 mM penicillamine [47]. The mobile phase was previously filtered through 0.22 μm filter and degassed through an online vacuum degasser. OriginPro 2019 (OriginLab, Northampton, MA, USA) was used for processing the chromatograms.

2.6. SC-ICP-MS measurements and data processing

Bacterial cell suspensions obtained from the exposure experiments with silver were analyzed by SC-ICP-MS (instrument and data acquisition details included in Table S1) to detect and quantify silver in bacteria [49]. Aliquots of 100 μL of bacterial suspensions were subjected to centrifugation for 20 min at 10,000 g to remove the PBS medium. The obtained pellet was resuspended in ultrapure water to get a cell number concentration of approximately 10^8 cells L^{-1}. Suspensions were measured in single cell mode using the Syngistix Single Cell-Application module version 2.5 (PerkinElmer Inc.). The recorded signals were processed by applying a 5-sigma threshold calculated as five times the square root of the mean baseline intensity of the time scan [53,54]. Nebulization efficiency was calculated according to the methods developed by Pace *et al.* [55] by using the ultra-uniform gold nanoparticle standard described in Supplementary Information. Similar results were obtained for the frequency and the size methods (60.4 ± 0.3 % and 57.6 ± 0.6 %, respectively, n = 6). Sample flow rate was measured gravimetrically.

3. Results and discussion

3.1. Determination of silver species during the incubation process: Stability of nanoparticles

The antimicrobial activity of silver nanoparticles is influenced by their size and other specific properties such as surface charge, coating, chemical composition, shape, pH and ionic strength of the medium among others [8,56]. Nanoparticles in contact with culture medium could undergo transformations such as aggregation or dissolution/oxidation. Therefore, it is necessary to determine these changes and the silver species that could be present during the incubation process, which would be responsible for the bactericidal action of silver nanoparticles. Ionic silver and silver nanoparticles were subjected to incubation processes for 24 h in the culture medium (in absence of bacteria), as previously described in the Methods section (2.1), and the silver species were determined by SP-ICP-MS and HDC-ICP-MS. In addition, a control sample of the same nanoparticles diluted in ultrapure water was also analyzed.

The results obtained by SP-ICP-MS for 60 nm silver nanoparticles diluted in ultrapure water and after incubation in the culture medium showed distributions with similar mean sizes in both cases (Figure S1). The nominal size of incubated nanoparticles was 58.3 ± 0.1 nm, whereas the obtained size for control nanoparticles was 59.7 ± 0.4 nm. These values are in agreement with the nominal size certified by the supplier (60 ± 7 nm). This fact indicated that 60 nm silver nanoparticles kept their original size after incubation and did not undergo aggregation. Number concentration recoveries of control and incubated nanoparticles were 91 ± 3 % in both cases (Table S3), confirming previous information. Additionally, it was determined that the original 60 nm silver nanoparticle suspension contained a small fraction of silver (I) (3.9 ± 0.3 %), whereas after their incubation in the culture medium, this ionic silver fraction increased up to 9.2 ± 0.1 %, showing that further dissolution of the nanoparticles resulted during the incubation process.

On the other hand, 10 nm silver nanoparticles incubated in the culture medium could not be quantitatively determined by SP-ICP-MS, since the achieved detection limits were 10.7 nm. In order to obtain complementary information about 10 nm silver nanoparticles and the presence of a dissolved fraction in the original suspensions and after incubation, samples containing these nanoparticles were analyzed by

HDC-ICP-MS. Fig. 1a shows the chromatograms corresponding to a sample containing Ag(I) and 10 nm silver nanoparticles incubated in the culture medium for 24 h. It was observed that, in both chromatograms, a single peak corresponding to dissolved silver (red distribution) and 10 nm nanoparticles (blue distribution), respectively, was obtained. This fact indicates that the original state of the silver species incubated in the culture medium was preserved in both cases, and in the case of the nanoparticles, they were not dissolved to Ag(I). Fig. 1b shows the chromatogram corresponding to 60 nm silver nanoparticles incubated in the culture medium, where two peaks were observed. The first peak at 8.15 min corresponds to nanoparticles of 60 nm, while the second one at 8.50 min corresponds to ionic silver. Thus, in the case of these nanoparticles, it was confirmed their partial oxidation to Ag(I) during the incubation process. A comparison between samples and control samples diluted in the mobile phase is included in Supplementary Information.

Therefore, results obtained by both techniques showed that after incubation of 60 nm silver nanoparticles in the culture medium, the presence of the nanoparticles themselves and a fraction of dissolved silver was detected, the latter being a consequence of the nanoparticle oxidation in the culture medium. In contrast, in the case of incubation with 10 nm silver nanoparticles no further presence of dissolved silver was observed after incubation in the culture medium, indicating that 10 nm nanoparticles were not significantly oxidized during this process.

3.2. Accumulation of silver in E. coli bacteria: Determination of total silver

The uptake of silver (I) and silver nanoparticles by *E. coli* bacteria was assessed after their exposure to different concentrations of them during 24 h following the conditions described previously. Exposure levels were chosen based on the results obtained for the determination of MIC and MBC. MIC and MBC are defined as the lowest concentration of an antimicrobial that inhibits the visible growth of a microorganism and the lowest concentration of antimicrobial that kills 99.9 % of the inoculum, respectively [57,58]. Thus, the concentrations to which bacteria were exposed were lower than the MIC and MCB in order to avoid their death. The experimental MIC and MBC values obtained for Ag(I) and 60 nm AgNPs were 8 and 16 mg L^{-1}, respectively, against both strains of *E. coli* (ATCC 25,922 and J62). In the case of 10 nm AgNPs, MIC values of 16 mg L^{-1} (ATCC 25922) and 8 mg L^{-1} (J62) were obtained. MBC values for these particles were around 16 mg L^{-1} for both strains.

The amount of Ag accumulated in bacteria was determined by ICP-MS analysis after alkaline digestion of the bacterial samples exposed to silver. The alkaline treatment described in section 2.3. allows the digestion of the biological samples while preserving the nanoparticles

Table 2
Total silver concentration after alkaline and acid digestion of *E. coli* bacterial samples. Mean ± standard deviation ($n = 5$).

Sample	Silver concentration µg/L	
	Alkaline digestion	Acid digestion
E. coli ATCC 25922 control	< 4	< 0.4
E. coli ATCC 25922 + 0.5 mg L^{-1} Ag(I)	330.9 ± 53.0	305.1 ± 49.1
E. coli ATCC 25922 + 4 mg L^{-1} 10 nm AgNPs	4410.9 ± 950.8	4385.2 ± 152.8
E. coli ATCC 25922 + 0.5 mg L^{-1} 60 nm AgNPs	121.7 ± 10.4	130.1 ± 24.5
E. coli J62 control	< 4	0.9 ± 0.2
E. coli J62 + 0.5 mg L^{-1} Ag(I)	495.0 ± 8.2	492.6 ± 58.9
E. coli J62 + 2 mg L^{-1} 10 nm AgNPs	74.1 ± 12.0	73.3 ± 9.4
E. coli J62 + 0.5 mg L^{-1} 60 nm AgNPs	494.3 ± 59.1	451.7 ± 93.6

[59]. A procedure based on Clark *et al.* [60] was followed. Control bacteria (without exposure to silver) were spiked with Ag(I) or 60 nm silver nanoparticles to check if the bacterial matrix affected the silver recovery. Recoveries obtained for Ag(I) and 60 nm AgNPs were higher than 91 %, indicating that the biological matrix did not affect the silver quantification.

Table 2 shows the total silver content accumulated in *E. coli* bacteria exposed to Ag(I) and 10 and 60 nm silver nanoparticles and subjected to alkaline digestion. To validate these results, an acid digestion of the same bacterial samples was performed. As shown in Table 2, there was a good agreement between results obtained by both methods and no significant differences were observed ($p < 0.05$). The results obtained demonstrated that both bacteria were able to accumulate silver after their exposure to silver (I) and silver nanoparticles of different sizes. Differences observed in the results could be explained by the inherent differences between the bacteria strains, probably related to the structure and composition of their wall. The standard deviations obtained were high due to the heterogeneity of the samples, as the amount of silver accumulated per bacteria shows a high variability.

3.3. Study of bactericidal effect of silver (I) and silver nanoparticles

As previously discussed, bactericidal properties of silver nanoparticles are influenced by properties such as the size. In this study, negatively charged citrate-stabilized silver nanoparticles of different sizes, 10 and 60 nm, were used in order to study the influence of nanoparticle size on their interaction with *E. coli* bacteria.

3.3.1. Detection of silver by SC-ICP-MS

Bacterial samples previously diluted with ultrapure water were analyzed by SC-ICP-MS to detect the presence of silver in bacteria. In

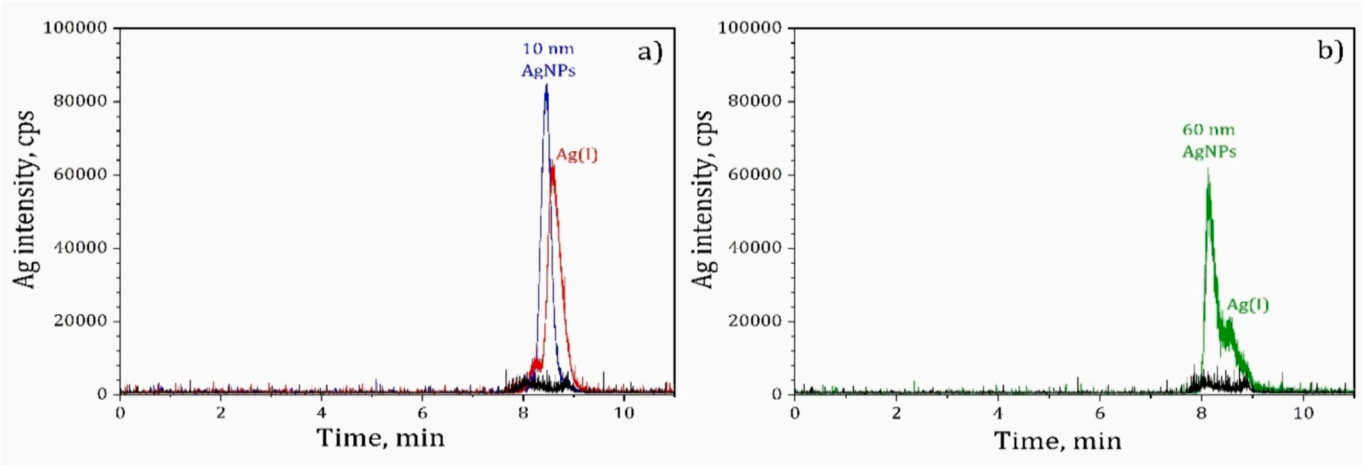

Fig. 1. HDC-ICP-MS chromatograms of samples subjected to incubation process: a) Ag(I) (red) and 10 nm AgNPs (blue) and b) 60 nm AgNPs (green). Culture medium control (black). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

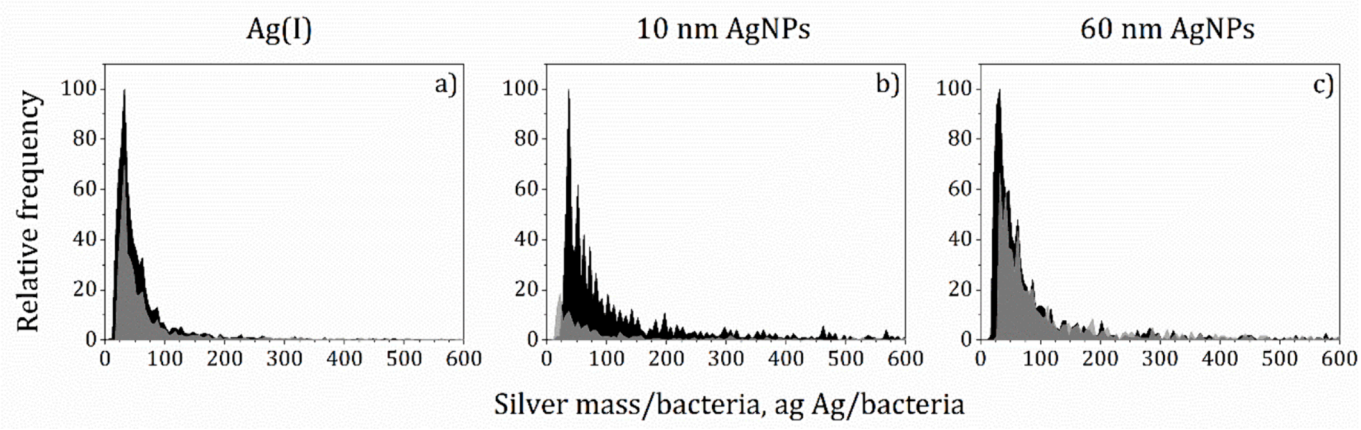

Fig. 2. Silver mass per particle distributions analyzed by SC-ICP-MS for samples of *E. coli* ATCC 25922 (black) and J62 (grey) bacteria exposed to: a) 0.50 mg L^{-1} Ag (I), b) 4 and 2 mg L^{-1} 10 nm AgNPs, respectively, and c) 0.50 mg L^{-1} 60 nm AgNPs.

addition, bacterial and incubation medium control samples were measured. Fig. 2 shows the silver mass per particle distributions from *E. coli* bacteria exposed to silver (I) and 10 and 60 nm silver nanoparticles. Under the corresponding measurement conditions, the element mass per particle limit of detection was 12 ag of silver. For both samples of bacteria exposed to silver (I) (Fig. 2a), the mass per particle distribution range was 12–400 ag of silver, being the distribution of *E. coli* ATCC 25922 bacteria slightly higher. For bacteria exposed to 10 nm silver nanoparticles (Fig. 2b), the silver mass per particle distributions ranged, approximately, between 14–600 ag/bacteria and 14–200 ag/bacteria for *E. coli* ATCC 25922 and *E. coli* J62, respectively, being greater the first distribution, which could be due to the fact that the initial concentration of exposed nanoparticles was higher than in the case of *E. coli* J62, so that a greater amount of silver could be taken up by bacteria. In the case of bacteria exposed to 60 nm silver nanoparticles (Fig. 2c), the distributions were similar for both strains and the distribution range was 12–400 ag of silver per bacteria. Therefore, these results demonstrated that both strains of *E. coli* bacteria had internalized or adsorbed silver as a consequence of their incubation with silver (I) and 10 and 60 nm silver nanoparticles. In a previous study [49], SC-ICP-MS analysis of spheroplasts obtained by enzymatic digestion of the bacterial cell with lysozyme, allowed to quantify the intracellular silver and to study the silver biodistribution in individual bacteria. In the case

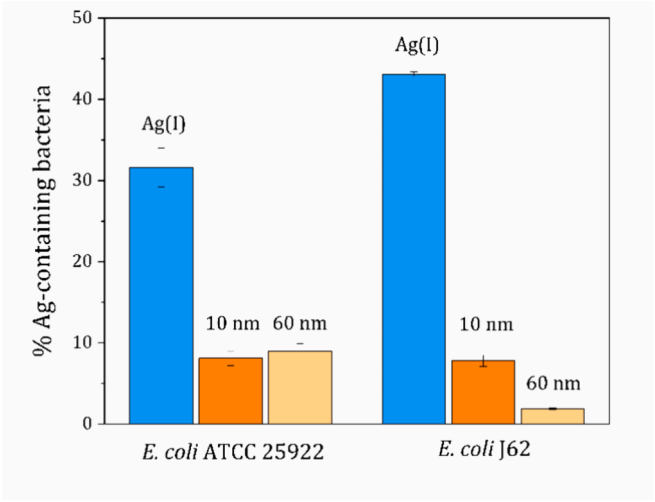

Fig. 3. Percentage of silver-containing bacteria *E. coli* ATCC 25922 and *E. coli* J62 in relation to total bacteria. Exposed silver concentration: 0.50 mg L^{-1} Ag (I), 4 and 2 mg L^{-1} 10 nm AgNPs, respectively, and 0.50 mg L^{-1} 60 nm AgNPs.

of *E. coli* bacteria exposed to ionic silver, all the silver was internalized, whereas in the case of *E. coli* bacteria exposed to 10 nm AgNPs, this percentage was limited to about 46 %.

In addition, the percentage of bacteria that had accumulated silver in comparison to total bacteria present in the suspension was determined. Fig. 3 shows the fraction of bacteria containing silver above 12 ag (mass per particle limit of detection) in relation to the total bacteria in the suspension. In the case of bacteria exposed to the same concentration (0.5 mg L^{-1}) of silver (I) and 60 nm silver nanoparticles, it was observed that, in the first case, the fraction of silver-containing bacteria was significantly higher (approximately 30–40 %) than in the case of samples exposed to silver nanoparticles (approximately 2–9 %). This fact indicated that silver ions had a higher reactivity than silver nanoparticles for a similar initial concentration of *E. coli* bacteria. It was in agreement with the MIC and MBC results obtained previously. The electrostatic attraction between the negative charge of the bacterial cell membrane [61,62] and the positive charge of silver ions could facilitate the adsorption of the ions to the cell wall and their uptake inside bacteria, resulting in an increased silver accumulation in them. On the other hand, it has been demonstrated that AgNPs are more active against gram-negative bacteria, as the *E.coli* used in this work, than against gram-positive ones, most likely due to the thicker layer of peptidoglycan present at the latter [8,63]. Although AgNPs are commonly synthesized by the reduction of silver ions to neutral silver atoms, leading to the formation of nanoparticles with strongly negative zeta potential [64–66], as the citrate stabilized nanoparticles used here [67], their surface can be modified to carry a positive charge [64]. In this sense, some authors have shown positively charged AgNPs have a higher bactericidal effect [27,68] due to the electrostatic attraction with cell membrane. Samples of bacteria exposed to 10 nm silver nanoparticles could not be compared with bacteria exposed to silver (I) and 60 nm silver nanoparticles because the initial silver concentration was different. However, it is acknowledged that AgNPs smaller than 10 nm can alter cell permeability by entering into bacterial cells and causing cell damage [69].

3.3.2. Detection of silver nanoparticles in bacteria after alkaline digestion by SP-ICP-MS

SC-ICP-MS is limited to determine the total content of specific elements in individual cells, meaning it is not possible to differentiate whether the silver is in its ionic or nanoparticulate form. For this purpose, samples of *E. coli* bacteria exposed to silver, previously digested with TMAH and $CaCl_2$, were analyzed by SP-ICP-MS to determine the different silver species present in bacteria. Silver nanoparticles of 10 and 60 nm diluted in the same final medium (TMAH 0.2 % y $CaCl_2$ 0.05 mM) as samples were used as reference. Fig. 4 shows the size distribution of

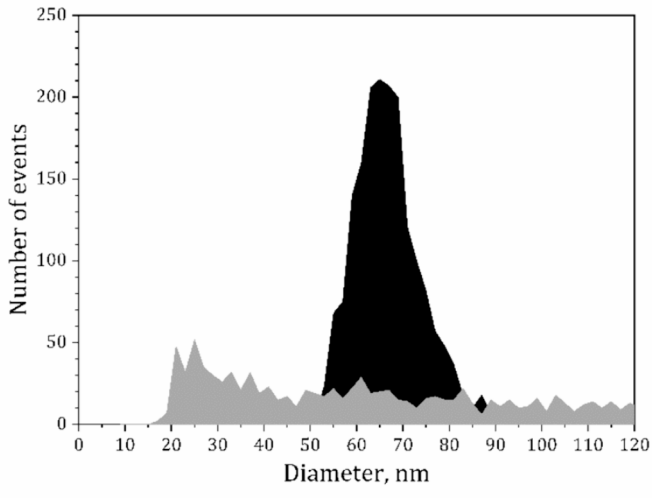

Fig. 4. Size distribution obtained by SP-ICP-MS for *E. coli* J62 bacteria exposed to 60 nm silver nanoparticles (grey) and 60 nm silver nanoparticles reference suspension (black).

E. coli J62 bacteria exposed to 60 nm silver nanoparticles and subjected to alkaline digestion. No distribution corresponding to 60 nm nanoparticles was observed for the sample studied, indicating that nanoparticles were not significantly internalized or adsorbed by bacteria in their original form. In addition, a baseline higher than reference nanoparticles was observed, which indicated that the chemical species accumulated in bacteria was ionic silver, as a result of partial oxidation of silver nanoparticles or the presence of silver (I) in the original suspension.

In the case of bacteria exposed to 10 nm silver nanoparticles and subjected to alkaline digestion, only qualitative results could be obtained because the size detection limit (14.6 nm) was larger than the mean size of 10 nm nanoparticles. Therefore, a partial distribution of nanoparticles above 14.6 nm was detected. In order to complement this information obtained by SP-ICP-MS, it was necessary to use other complementary techniques such as HDC-ICP-MS.

3.3.3. Detection of silver nanoparticles in bacteria after alkaline digestion by HDC-ICP-MS

Due to the limitations in detection of 10 nm nanoparticles by SP-ICP-MS, *E. coli* ATCC 25922 and *E. coli* J62 bacteria exposed to 10 nm silver nanoparticles, previously digested with TMAH and $CaCl_2$, were analyzed by HDC-ICP-MS. Fig. 5 shows the chromatograms obtained for digested *E. coli* ATCC 25922 and *E. coli* J62 bacteria exposed to 10 nm silver nanoparticles. Two main peaks were observed in both chromatograms, the first at retention times of 8.50 and 8.60 nm and the second one at 9.58 and 9.50 min. The first peak corresponded to the presence of 10 nm nanoparticles, while the second one was due to the presence of dissolved silver. Therefore, these results demonstrated that, although 10 nm silver nanoparticles were not significantly oxidized in the culture medium, bacteria contributed to their oxidation since ionic silver was found associated to bacteria (internalized and/or adsorbed).

Thus, HDC-ICP-MS allowed the separation and detection of silver nanoparticles and their dissolved forms in digested bacterial samples. These results agreed with those obtained by SP-ICP-MS and confirmed that, in the case of bacteria exposed to 10 nm silver nanoparticles, silver accumulated in bacteria was found as particulate and dissolved form, with a higher proportion of the latter. This behavior is different from those observed for 60 nm silver nanoparticles, where no nanoparticle uptake by bacteria was observed.

3.4. Bactericidal activity of silver (I) and silver nanoparticles

In this study, a strategy based on different analytical methods that allows the detection and quantification of silver nanoparticles and released ions by direct procedures, such as SP-ICP-MS or HDC-ICP-MS, which do not require previous separation steps has been developed. First, these analyses have allowed the direct detection and identification of the different silver species resulting from the incubation process in the culture medium. On the other hand, these techniques, in combination with an alkaline digestion with TMAH, have allowed to detect and identify directly the different silver species accumulated by the *E. coli* bacteria (internally or adsorbed in their cell wall) after the incubation process. In addition, the presence of silver-containing bacteria has been demonstrated by SC-ICP-MS. Finally, a relationship between the analytical results obtained and the bactericidal activity of these silver nanoparticles can be established. As discussed in the introduction, the three possible ways of bactericidal activity are: 1) ion-only action; 2) particle-only action; 3) combined ion-particle action.

After analyzing the results obtained from these studies for both silver nanoparticles exposed to bacteria, it was concluded that the bactericidal activity of 60 nm silver nanoparticles would be related to ion-only action. The analysis of nanoparticle incubation in the culture medium confirmed the presence of silver ions and silver nanoparticles.

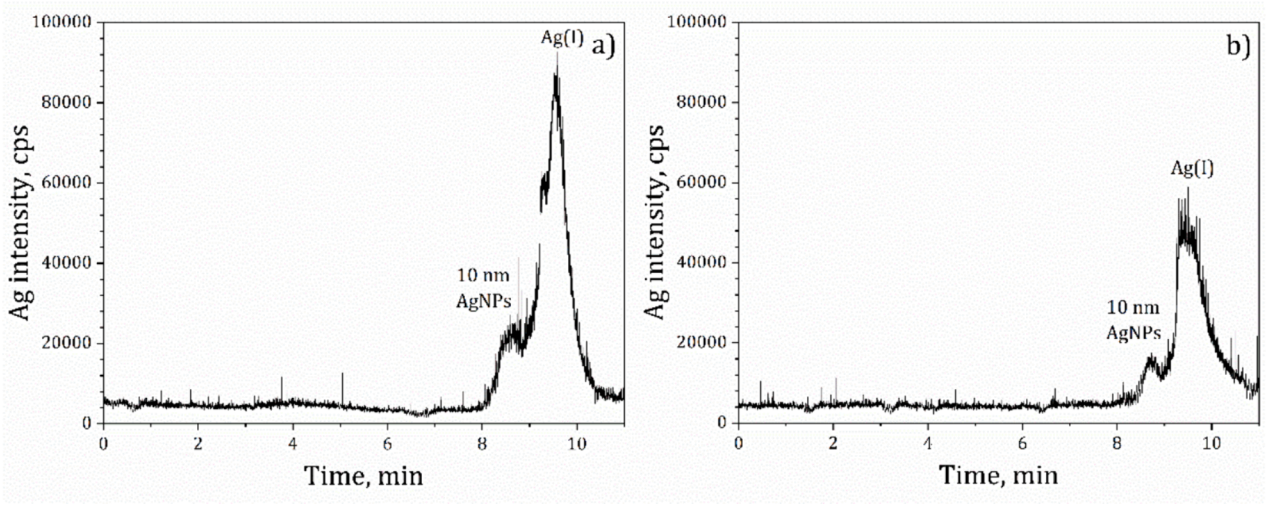

Fig. 5. Hydrodynamic chromatograms of samples digested with TMAH and $CaCl_2$ for a) *E. coli* ATCC 25922 and b) *E. coli* J62 bacteria exposed to 10 nm silver nanoparticles.

Furthermore, after the analysis of *E. coli* bacteria exposed to silver nanoparticles and digested with TMAH by SP-ICP-MS, the absence of 60 nm nanoparticles was demonstrated, indicating that these nanoparticles had not been accumulated in bacteria. This fact could be explained by the large size of nanoparticles and their negative surface charge, which is in agreement with the literature [21,28,33,70]. On the other hand, the presence of silver-containing bacteria was detected by SC-ICP-MS, which indicated that bacteria had taken up silver. This silver would be as ionic silver, as demonstrated by SP-ICP-MS, which would come from the partial oxidation of nanoparticles in the culture medium, justifying the ion-only action.

In the case of 10 nm silver nanoparticles, the bactericidal activity would be associated with a combined ion-particle action. This is justified by the analysis in the culture medium, which demonstrated that nanoparticles remained in their original form, with no relevant oxidation and release of silver ions. The small size of these nanoparticles would allow their interaction with bacteria, facilitating their adsorption on the bacterial wall, its disruption and/or their internalization into the bacteria. This fact was demonstrated from the results obtained after alkaline digestion of bacteria and analysis by SP-ICP-MS and HDC-ICP-MS, where the presence of 10 nm silver nanoparticles was demonstrated. Furthermore, silver (I) from the partial oxidation of the nanoparticles was detected by HDC-ICP-MS in the digested bacteria. When nanoparticles come into contact with the bacterial wall, an oxidation process could occur and silver ions would be released from their surface, which could be adsorbed on the bacterial wall or internalized through membrane porins or the altered membrane. Alternatively, nanoparticles could be internalized into bacteria after contact with bacterial wall, and once inside, they could be oxidized to silver (I). These results are in agreement with literature [21,28,31,33,37,70], where authors provided a series of analytical evidence that corroborates the interaction of nanoparticles with the bacterial surface by electron microscopy or the presence of silver (I) in bacteria by ICP-MS techniques.

4. Conclusion

The use of an analytical platform based on SC-ICP-MS, SP-ICP-MS and HDC-ICP-MS has allowed the detection of silver-containing bacteria, as well as the direct detection and identification of silver species present in *E. coli* bacteria exposed to silver (I) and silver nanoparticles. This has allowed the study of the silver species involved in the bactericidal activity of silver nanoparticles. In the case of 10 nm silver nanoparticles, their bactericidal effect could be associated with a combined ion-particle action. This is justified by the demonstration that nanoparticles were found in their original form in the culture medium, without observation of the presence of silver ions, which indicates that nanoparticles were not oxidized. Their small size favors contact with bacteria, which could cause a membrane alteration and favors their adsorption on cell wall and/or their introduction into bacteria. This fact was justified by the presence of 10 nm silver nanoparticles after alkaline digestion of bacteria. This contact between nanoparticles and bacteria could produce an oxidation of adsorbed or internalized nanoparticles, justifying the presence of silver ions associated with bacteria.

In contrast, the bactericidal activity of 60 nm silver nanoparticles has been associated with ions only. This activity is justified by the presence of silver ions and silver nanoparticles after incubation in the culture medium. The analysis of digested *E. coli* bacteria with TMAH confirmed the absence of 60 nm silver nanoparticles, indicating that these nanoparticles were not taken up by bacteria. Therefore, ions would be responsible of bactericidal activity of 60 nm silver nanoparticles.

CRediT authorship contribution statement

Ana C. Gimenez-Ingalaturre: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Isabel Abad-Álvaro:** Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing. **Mariam Bakir:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Patricia Chueca:** Formal analysis, Methodology, Writing – review & editing. **Pilar Goñi:** Methodology, Supervision, Writing – review & editing. **Francisco Laborda:** Conceptualization, Data curation, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2024.111296.

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