



Cite this: DOI: 10.1039/d5na00404g

Synergistic activity of silver nanoparticles and antibiotics: apramycin against *Escherichia coli*

Ana C. Gimenez-Ingalature, ^a Isabel Abad-Álvaro, ^{a*} Patricia Chueca,^b
Pilar Goñi ^b and Francisco Laborda ^a

The combination of silver nanoparticles and conventional antibiotics could be a promising alternative strategy to fight against antimicrobial resistance. The occurrence of synergistic bactericidal activity between these combinations is supported by a large number of studies, but there is still a great lack of information about the action mechanisms. In this study, a direct analytical method for the detection and quantification of silver content in cells based on single cell inductively coupled plasma mass spectrometry (SC-ICP-MS), in combination with microscopy and microbiological techniques, has been used. Quantification of silver accumulated in *Escherichia coli* bacteria exposed to combinations of silver(I) or silver nanoparticles and apramycin can help elucidate the synergistic mechanisms of silver–antibiotic combinations. The combination of silver with apramycin resulted in the occurrence of synergistic effects, allowing the reduction of silver(I), silver nanoparticle and apramycin concentrations (from 4- to 16-fold) while preserving the individual bactericidal effects of each antimicrobial. Severe damage in bacteria walls, including double membrane rupture and cytoplasm leakage, was observed when the combination of apramycin and silver was used. In general, the presence of apramycin has promoted silver uptake by bacteria.

Received 25th April 2025

Accepted 31st July 2025

DOI: 10.1039/d5na00404g

rsc.li/nanoscale-advances

1. Introduction

Despite the current era of technological advances, infectious diseases are still among the leading causes of death, mainly because of the development of resistance to antimicrobial agents by microorganisms responsible for diseases.¹ Antimicrobial resistance (AMR) is one of the greatest public health challenges worldwide and is considered one of the top ten health threats by the World Health Organization (WHO). AMR occurs when bacteria, viruses, fungi and parasites no longer respond to antimicrobials, which become ineffective. Some infectious diseases are caused by multidrug-resistant bacteria and become difficult or impossible to treat, increasing the risk of disease spread, severe illness, disability, and death. AMR is a natural process that happens over time through genetic changes in pathogens. Its emergence and spread are accelerated by human activity, mainly the misuse and overuse of antimicrobials used in humans or animals.² The first case of AMR dates back to 1967 when penicillin-resistant *Streptococcus pneumoniae* was detected in Australia.³ In recent decades, it is estimated that about 700 000 people die from the infection of multidrug-resistant bacteria worldwide every year and this

number could increase to 10 million people by 2050.⁴ To date, resistance is neither limited to a specific bacterial strain nor opposed to a specific antibiotic. According to the WHO, approximately 79% of bacteria have developed resistance to one or more antibiotics.² The development of new antibiotics often takes decades, making it impossible to stop the problem of resistance in the short term. In 2019, the WHO identified 32 antibiotics in the clinical development phase against priority pathogens, of which only 6 were considered innovative. Therefore, it is necessary to develop alternative antimicrobials that are economical and efficient.²

Some metals such as silver, copper, zinc or magnesium have been traditionally used to treat infectious diseases, long before the antibiotic revolution.⁵ Specifically, metallic silver has been used to prevent infections, although prolonged exposure could produce argyria, gastrointestinal disorders and even death. Nowadays, silver is used in water treatment systems and medical applications such as burn treatments, dentistry and catheters. Likewise, silver ions have been proposed as an effective antimicrobial alternative to antibiotics due to their low cytotoxicity.^{6,7} On the other hand, nanotechnology offers a useful platform to modify and control the physicochemical properties of metallic materials in order to produce antimicrobial nanomaterials that are effective and non-toxic for humans.⁸ In recent years, a new type of nanoparticle-based antimicrobial is being investigated to fight against AMR, including iron oxide, zinc oxide, copper oxide, titanium dioxide, silver, gold or

^aGroup of Analytical Spectroscopy and Sensors (GEAS), Institute of Environmental Sciences (IUCA), University of Zaragoza, Pedro Cerbuna 12, 50009 Zaragoza, Spain. E-mail: iabad@unizar.es; Tel: +34 876553329

^bGroup of Water and Environmental Health, Institute of Environmental Sciences (IUCA), Domingo Miral s/n, 50009 Zaragoza, Spain



graphene oxide nanoparticles.¹ Their physicochemical properties (size, shape, chemical surface, concentration, and coating) and their high surface area-to-volume ratio play a relevant role and their effectiveness has been demonstrated in *in vivo* and *in vitro* studies.^{9,10}

Thus, the combination of nanoparticles and conventional antibiotics emerges as an alternative strategy to fight against AMR. Several authors have investigated the bactericidal efficacy of these combinations, those with silver nanoparticles being the most studied.¹¹ One of the first studies was performed by Li *et al.* in 2005, who studied the amoxicillin–silver nanoparticle combination against *Escherichia coli* (*E. coli*). They demonstrated a significant decrease in cell growth in the presence of the antibiotic–nanoparticle combination whereas individually, the bactericidal activity was low.¹² Other combinations using silver nanoparticles and antibiotics with different action mechanisms (β -lactam, aminoglycosides, quinolones, glycopeptides, chloramphenicol, tetracyclines, quinolones, polymyxin, rifampicin...) were tested against different bacteria (*E. coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Clostridium difficile*...) and showed an increase in inhibitory and bactericidal effects compared to the individual bactericidal effect of each antimicrobial.^{7,13–16} The combination of silver nanoparticles and aminoglycoside antibiotics is one of the most studied. These antibiotics present a structure composed of amino sugars attached by glycosidic bonds to an aminocyclitol ring, most commonly 2-deoxystreptamine. This group includes kanamycin, tobramycin, gentamycin, neomycin and apramycin, among others.¹⁷ Apramycin is a structurally unique aminoglycoside, which is able to elude almost all mechanisms of bacterial resistance.^{17,18} Although apramycin is a widely used antibiotic in veterinary medicine, this unique feature makes it particularly attractive as a candidate for next-generation antibiotics for clinical use in humans to combat antimicrobial resistance.^{17,19}

Some authors have used different microbiological methods in order to suggest synergistic mechanisms for silver–aminoglycoside combinations. These methods studied the production of reactive oxygen species (ROS), antibiotic uptake in the presence/absence of silver and bacterial survival rate.^{7,20} Microscopy techniques such as transmission electron microscopy (TEM) or field emission scanning electron microscopy (FESEM) allow the detection of structural alterations, irregularities in membrane content distributions or even, cytosol leakage in bacteria.^{21–24} On the other hand, flow cytometry is used for evaluating membrane permeability and Fourier Transform Infrared (FT-IR), UV-visible and Raman spectroscopy allow one to assess chemical interactions of silver nanoparticles and antibiotics.^{21,25} However, there are other useful analytical techniques to corroborate the bactericidal mechanisms. Single cell inductively coupled plasma mass spectrometry (SC-ICP-MS) allows the detection and quantification of silver content in cells and is used to obtain information on the mass distribution of silver per cell,²⁶ which could help to elucidate the synergistic mechanisms of silver–antibiotic combinations. To the best of authors' knowledge, this work is the first to use SC-ICP-MS to

directly determine the silver species involved in the synergistic bactericidal activity of combinations of silver and conventional antibiotics and to help elucidate the mechanisms of action related to this bactericidal activity. This work develops a methodology combining analytical and microbiological techniques to detect and quantify the silver species present in cultures of *E. coli* bacteria previously exposed to combinations of silver(I) or silver nanoparticles and apramycin, a conventional antibiotic used in veterinary medicine. In addition, the chemical information provided by this technology is used to elucidate the possible synergistic action mechanisms of different antimicrobial combinations. The analytical platform is based on the use of SC-ICP-MS and microscopy techniques, in combination with microbiological techniques for determining antimicrobial susceptibility to antimicrobials and this is going to allow: (i) the evaluation of individual bactericidal activities of antimicrobials (silver(I), 10 nm silver nanoparticles and apramycin) and silver–apramycin combinations, (ii) the identification of possible synergistic effects after the combination of silver and apramycin, (iii) the determination of total silver accumulated in bacteria and its distribution per bacteria, (iv) the detection of possible structural alterations in bacteria after exposure to antimicrobials, and (v) the evaluation of the relationship between silver detected in bacteria, the possible structural alterations and the possible mechanisms of bactericidal activity of silver–apramycin combinations.

2. Materials and methods

2.1. Bacterial cultures with silver and apramycin

Escherichia coli ATCC 25922 strain was used in all experiments. The strain was cultured in Müller Hinton (MH) agar and grown overnight at 37 °C and then inoculated in Müller Hinton Broth (MHB) + 2% Tween 80. The culture medium combining MH and 2% Tween 80 was optimized to prevent the aggregation of nanoparticles, while preserving the viability of *E. coli* bacteria.²⁷ Bacterial suspensions of *E. coli* ATCC were exposed to silver(I), 10 nm silver nanoparticles, apramycin and two different combinations of apramycin–Ag(I) and apramycin–silver nanoparticles (Table 1) for 24 h at 37 °C and 100 rpm. Details of standards and reagents are included in the SI. Bacterial cells

Table 1 Bacterial suspensions of *E. coli* ATCC 25922 exposed to silver(I), 10 nm silver nanoparticles and/or apramycin

Sample	Concentrations (mg L ⁻¹)		
	Ag(I)	10 nm AgNPs	Apramycin
<i>E. coli</i> + 0.25 mg L ⁻¹ apramycin	—	—	0.25
<i>E. coli</i> + 2.0 mg L ⁻¹ apramycin	—	—	2.0
<i>E. coli</i> + Ag(I)	0.5	—	—
<i>E. coli</i> + Ag(I) + 0.25 mg L ⁻¹ apramycin	0.5	—	0.25
<i>E. coli</i> + Ag(I) + 2.0 mg L ⁻¹ apramycin	0.5	—	2.0
<i>E. coli</i> + AgNPs	—	2.0	—
<i>E. coli</i> + AgNPs + 0.25 mg L ⁻¹ apramycin	—	2.0	0.25
<i>E. coli</i> + AgNPs + 2.0 mg L ⁻¹ apramycin	—	2.0	2.0



non-incubated with antimicrobials and culture medium without bacteria or antimicrobials were used as control. After exposure time, bacterial cultures were centrifuged at 4600g 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. The evaluation of the effectiveness of the PBS washing process in removing remaining silver that had not interacted with bacteria was previously demonstrated.²⁸ 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, the optical density of bacterial cultures was measured with a UV-vis spectrophotometer (UV-2004 Lan Optics) at 600 nm.

2.2. Antibacterial assays: determination of MIC and MBC

Determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of silver and apramycin was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.²⁹ *Escherichia coli* suspensions were prepared in tubes containing physiological solution (0.9% NaCl) at a concentration corresponding to a 0.5 McFarland standard (1.5×10^8 CFU mL⁻¹). Bacteria were exposed to two-fold serial concentrations of silver(I) (0.06–64 mg L⁻¹), 10 nm silver nanoparticles (0.06–16 mg L⁻¹) or apramycin (4–1024 mg L⁻¹) in MHB + 2% Tween 80, in a 96-well microdilution plate. The microplates were incubated at 37 °C for 24 h. Bacteria without exposure to antimicrobials were used as positive controls, whereas incubation medium MHB + 2% Tween 80 and suspensions of silver or antibiotic (without exposure to bacteria) were used as negative controls. The experiments were performed in triplicate. MIC was defined as the minimum concentration that inhibited bacterial growth and corresponded to the absence of turbidity in the wells after incubation. To determine MBCs, aliquots from wells in which bacterial growth was inhibited were collected, seeded on a MH plate and incubated at 37 °C for 24 h. After incubation, MBC results were interpreted by observing qualitative bacterial growth on the plates. MBC is the lowest concentration of an antimicrobial agent that results in killing 99.9% of the bacteria, corresponding to the absence of bacterial growth on the plates.

2.3. Checkerboard: determination of combined bactericidal activity

The checkerboard procedure was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines^{29,30} to evaluate the bactericidal activity of apramycin and silver antimicrobial combinations and the possible occurrence of synergistic effects. Inoculum of *E. coli* bacteria was prepared as previously described. Using a 96-well plate, two-fold serial dilutions of silver(I) (0.25–32 mg L⁻¹) or silver nanoparticles (0.125–16 mg L⁻¹) were prepared in horizontal rows, and two-fold serial dilutions of apramycin (0.25–64 mg L⁻¹) were prepared in vertical rows. All antimicrobials were diluted in MHB + 2% Tween 80 medium. The plates were prepared well-by-well to obtain a single plate in which both antimicrobial agents were cross-diluted. Afterwards, bacterial inoculum was added to

each well. A positive control (bacteria without exposure to antimicrobials) and negative controls (MHB + 2% Tween 80, silver or antibiotic) were evaluated. The microplates were incubated at 37 °C for 24 h. After incubation, results were interpreted by visually observing the well turbidity and the Fractional Inhibitory Concentration Index (FICI) was determined. Details of determination and interpretation of FICI are included in the SI. The Fractional Bactericidal Concentration Index (FBCI) was determined from the FICI results. Aliquots from wells in which bacterial growth was inhibited were collected, seeded on an MH plate and incubated at 37 °C for 24 h. After incubation, results were interpreted by observing qualitative bacterial growth on the plates and the FBCI was determined (details in the SI).

2.4. Acid digestion of bacterial samples

Aliquots of 100 µL of bacterial suspensions were subjected to centrifugation (Thermo Heraeus Multifuge X1R, equipped with a swigging bucket rotor, Waltham, USA) for 20 min at 10 000 g to remove the PBS medium. 500 µL of HNO₃ (69–70% w/v) and 100 µL of H₂O₂ (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% HNO₃ (v/v) and the content of total silver was quantified by ICP-MS. Five replicates of each sample were analysed.

2.5. SC-ICP-MS measurements and data processing

Bacterial cell suspensions obtained from the exposure experiments with silver and/or antibiotic were analysed by SC-ICP-MS (instrument and data acquisition details included in Table S1) to detect and quantify silver in bacteria. 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 obtain a cell number concentration of approximately 10⁸ cells L⁻¹. Suspensions were measured in the 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.^{31,32} Nebulization efficiency was calculated according to the methods developed by Pace *et al.*³³ by using the ultra-uniform gold nanoparticle standard described in the SI. Similar results were obtained for the frequency and the size methods (60.5 ± 0.3% and 57.1 ± 0.3%, respectively, *n* = 6). The sample flow rate was measured gravimetrically.

2.6. Detection of possible structural alterations in bacteria by SEM and TEM

Bacterial cell suspensions from the exposure experiments with silver and/or antibiotic were observed by SEM and TEM (instrument details included in the SI). Bacterial samples were subjected to centrifugation for 20 min at 10 000 g to remove the PBS medium. The obtained pellet was washed under the same conditions with 0.1 M phosphate buffer (PB), which was prepared from 0.2 M NaH₂PO₄ and 0.2 M Na₂PO₄. Afterwards, the cells were collected, fixed with 2.5% glutaraldehyde in 0.1 M



Table 2 Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) for different antimicrobials against *E. coli* ATCC 25922

Antimicrobial	MIC	MBC
	$\mu\text{g mL}^{-1}$	$\mu\text{g mL}^{-1}$
Ag(I)	8	8
10 nm AgNPs	16	16
Apramycin	16	16

PB (pH = 7.4) for 24 h, washed with 0.1 M PB and post-fixed with 2% osmium tetroxide in 0.1 M PB for 1 h. Finally, the samples were dehydrated with ethanol. For SEM observation, samples were carbon-coated. For TEM observation, uranyl acetate staining was performed, followed by embedding in epoxy resin, cutting to ultra-thin sections and a final lead citrate staining.

3. Results

3.1. Evaluation of individual bactericidal activity of silver antimicrobials and apramycin

The bacteriostatic and bactericidal activity (MIC and MBC, respectively) of silver(I), silver nanoparticles and apramycin was determined by the dilution method. Table 2 shows the MIC and MBC results obtained against *E. coli* ATCC 25922. In general, the MBC is the same or at most one or two dilutions higher than the MIC. The obtained results showed this behaviour. It was observed that the MIC and MBC of silver(I) were lower than those obtained for silver nanoparticles and apramycin.

3.2. Evaluation of bactericidal activity of silver antimicrobial and apramycin combinations and identification of synergistic effects

After evaluating the individual bacteriostatic and bactericidal activity of each antimicrobial agent, apramycin was combined

with silver(I) or silver nanoparticles to evaluate the bactericidal activity of each combination and the possible occurrence of synergistic effects. Checkerboard is a method based on the method for MIC and MBC determination, but in this case, two-fold serial concentrations of each antimicrobial were combined. First, bacteriostatic effects for the combinations of apramycin–silver(I) and apramycin–silver nanoparticles against *E. coli* bacteria were determined (Fig. 1). Wells with no turbidity and therefore, no visible bacterial growth, are shown in grey, whereas white cells indicate the presence of visual bacterial growth and so, these combinations have no bacteriostatic effects. Calculations of FICs and FICI are detailed in the SI. For the apramycin–silver(I) combination (Fig. 1a), the results showed that indifferent bacteriostatic effects were obtained in many of the cases studied, which means that the activity of two antimicrobials does not differ from the activity of the most effective individual antimicrobial. On the other hand, the combination of certain concentrations of apramycin with silver(I) resulted in the occurrence of synergistic bacteriostatic effects, meaning that the activity of two antimicrobials is significantly higher than the most effective individual antimicrobial. As a result, a decrease in apramycin concentration up to 8-fold and up to 16-fold for silver(I) concentration was observed. Likewise, the combination of certain concentrations of apramycin with silver nanoparticles (Fig. 1b) produced synergism and up to 4- and 128-fold reduction in the concentration of apramycin and silver nanoparticles, respectively.

In addition, bactericidal effects for the same antimicrobial combinations against *E. coli* were determined (Fig. 2) by observing the presence of bacterial growth on the plates. The bacterial growth is indicated with an asterisk. Calculations of FBCs and FBCI are detailed in the SI. In this study, the occurrence of synergistic bactericidal effects was also demonstrated for both antimicrobial combinations. For the apramycin–silver(I) combination (Fig. 2a), it allowed to reduce up to 8 and 16 times the concentration of apramycin and silver(I), respectively.

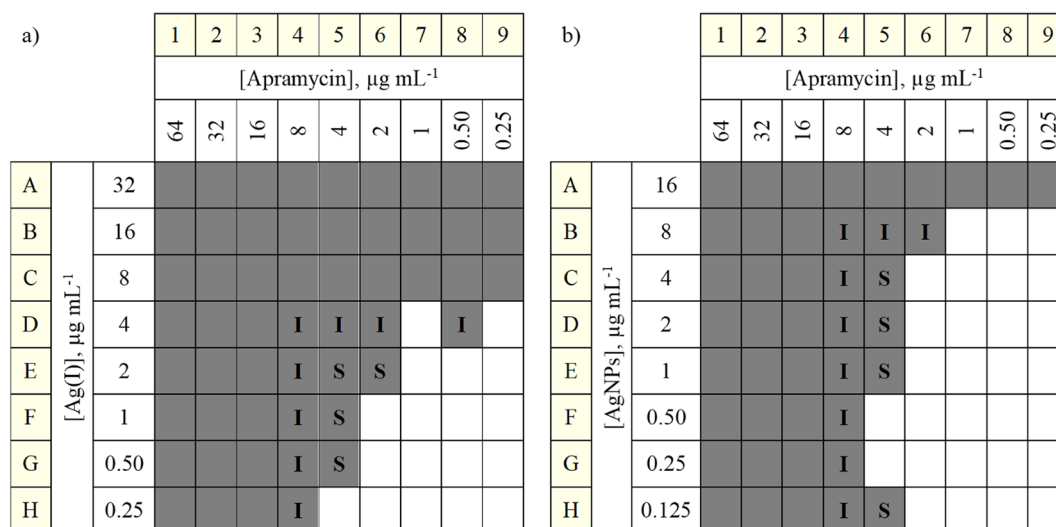


Fig. 1 Bacteriostatic effects of antimicrobial combinations against *E. coli* ATCC 25922: (a) apramycin–silver(I) and (b) apramycin–silver nanoparticles. Absence of turbidity: grey. Presence of turbidity: white. Synergism: S. Indifference: I.



		[Apramycin], $\mu\text{g mL}^{-1}$								
		64	32	16	8	4	2	1	0.50	0.25
[Ag(I)], $\mu\text{g mL}^{-1}$	A	32								
	B	16								
	C	8								
	D	4			I	I	I	*	*	*
	E	2			I	S	S	*	*	
	F	1			I	S	*	*		
	G	0.50			I	S	*	*		
	H	0.25			I	*	*			

		[Apramycin], $\mu\text{g mL}^{-1}$								
		64	32	16	8	4	2	1	0.50	0.25
[AgNPs], $\mu\text{g mL}^{-1}$	A	16								
	B	8			I	I	I	*		
	C	4			I	S	*			
	D	2			I	S	*			
	E	1			I	*	*			
	F	0.50			I	*	*			
	G	0.25			I	*	*			
	H	0.125			I	*	*			

Fig. 2 Bactericidal effects of antimicrobial combinations against *E. coli* ATCC 25922: (a) apramycin–silver(I) and (b) apramycin–silver nanoparticles. Absence of turbidity: grey. Presence of turbidity: white. Synergism: S. Indifference: I. *: bacterial growth.

For the apramycin–silver nanoparticle combination (Fig. 2b), the reduction was up to 4 and 8 times for apramycin and silver nanoparticle concentration, respectively.

3.3. Detection and quantification of silver in bacteria by SC-ICP-MS

In order to help elucidate the bactericidal role played by apramycin and silver in the bactericidal mechanism, it is necessary to quantify silver accumulated by bacteria during the incubation process. The uptake of silver(I) and silver nanoparticles by *E. coli* bacteria was quantified after their exposure to different apramycin–silver combinations, as well as to individual antimicrobials alone, following the conditions described previously. To avoid bacterial death, bacteria were exposed to lower concentrations than those that produced synergistic bacteriostatic and bactericidal effects. In order to study if apramycin concentration contributes to a higher accumulation of silver in bacteria, two different combinations of apramycin–silver(I) and apramycin–silver nanoparticles were studied. In these combinations, Ag(I) and silver nanoparticle concentrations remained

constant (0.5 and 2.0 mg L^{-1} , respectively), while apramycin concentration was varied (0.25 and 2.0 mg L^{-1}).

Bacterial samples previously diluted with ultrapure water were analysed by SC-ICP-MS to detect the accumulated silver in individual bacteria. In addition, bacterial and incubation medium control samples were measured. Fig. 3 shows the silver mass per bacteria distributions from *E. coli* bacteria exposed to silver(I) and apramycin–silver(I) combinations. Under the corresponding measurement conditions, the element mass per particle limit of detection was 13 ag of silver. For bacteria exposed to silver(I) (Fig. 3a), a first large distribution of bacteria accumulating small amounts of silver and a second small distribution of bacteria that accumulated higher amounts of silver were observed. In the presence of apramycin, the first distribution corresponding to bacteria accumulating small amounts of silver disappeared (Fig. 3b and c). On the other hand, the magnitude of the second distribution corresponding to bacteria accumulating higher amounts of silver increased when the apramycin concentration was lower (Fig. 3b) whereas the magnitude decreased for the highest apramycin concentration (Fig. 3c). These results demonstrated the influence of apramycin concentration in relation to accumulated silver.

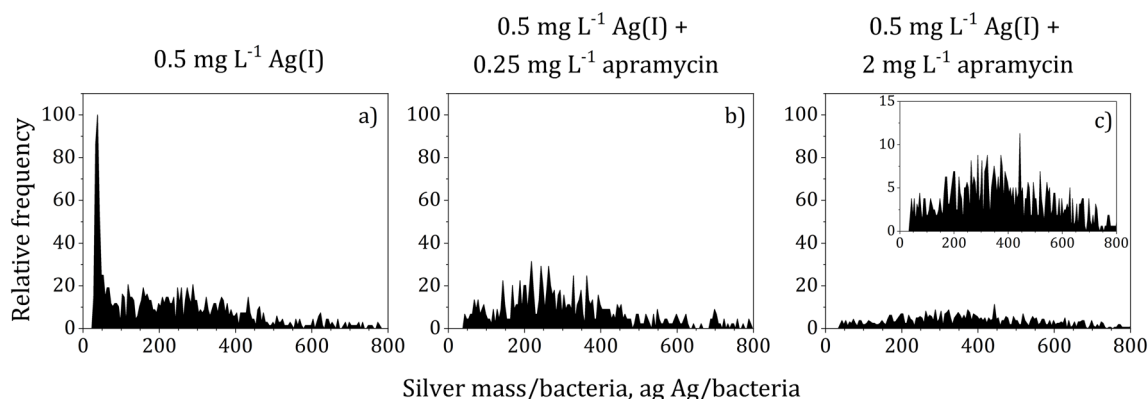


Fig. 3 Silver mass per bacteria distributions analysed by SC-ICP-MS for samples of *E. coli* ATCC 25922 bacteria exposed to: (a) $0.50 \text{ mg L}^{-1} \text{ Ag(I)}$, (b) $0.50 \text{ mg L}^{-1} \text{ Ag(I)}$ + 0.25 mg L^{-1} apramycin and (c) $0.50 \text{ mg L}^{-1} \text{ Ag(I)}$ + 2 mg L^{-1} apramycin.



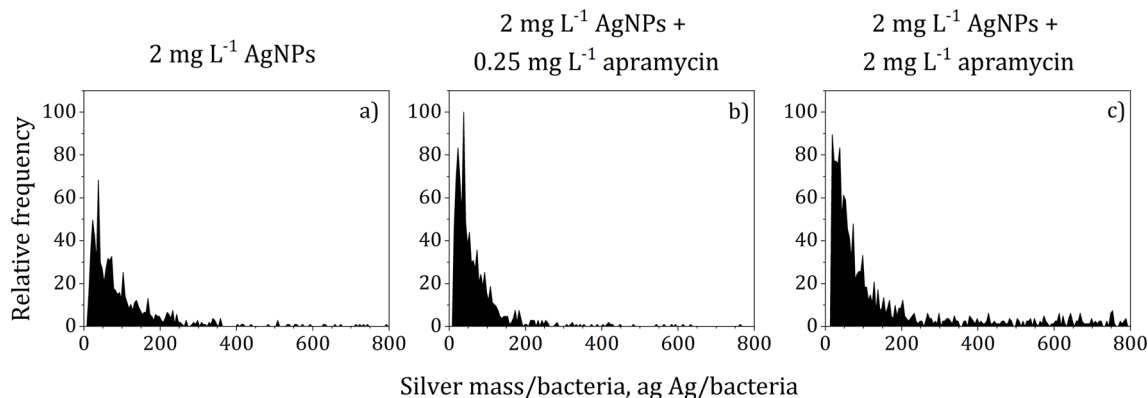


Fig. 4 Silver mass per bacteria distributions analysed by SC-ICP-MS for samples of *E. coli* ATCC 25922 bacteria exposed to: (a) 2 mg L⁻¹ AgNPs, (b) 2 mg L⁻¹ AgNPs + 0.25 mg L⁻¹ apramycin and (c) 2 mg L⁻¹ AgNPs + 2 mg L⁻¹ apramycin.

Likewise, Fig. 4 shows the silver mass per bacteria distributions from *E. coli* bacteria exposed to silver nanoparticles and their combinations with apramycin. Distribution with a greater magnitude was observed in both apramycin-containing samples (Fig. 4b and c) compared to bacteria exposed only to silver nanoparticles (Fig. 4a). In these cases, the influence of apramycin on silver accumulation was also demonstrated.

In addition, the percentage of bacteria that accumulated silver at detectable levels in comparison to total bacteria present in the suspension was determined. Fig. 5 shows the fraction of bacteria containing silver above 13 ag (mass per particle limit of detection). When apramycin is added at the lowest concentration (0.25 mg L⁻¹) to silver(I) and silver nanoparticles, it was observed that the fraction of silver-containing bacteria was similar to those obtained in the absence of apramycin (approximately 3–5%). On the contrary, when apramycin concentration was increased to 2 mg L⁻¹, the fraction of silver-containing bacteria increased significantly up to approximately

15 and 30% in the case of the combination with silver nanoparticles and silver(I), respectively.

Furthermore, from direct SC-ICP-MS analysis, total silver accumulated by bacteria was determined. The results are expressed as silver concentration in the original bacterial suspension. The results obtained by SC-ICP-MS were compared with those obtained by ICP-MS after acid digestion (Table 3). The percentage of silver uptake by bacteria in relation to the initial silver concentration exposed during incubation was also obtained. The results showed once again the influence of apramycin presence on silver uptake by bacteria, except for the combination of silver nanoparticles with the lowest apramycin concentration (0.25 mg L⁻¹), where silver uptake was similar to that obtained in the absence of apramycin. Application of a *t*-test (95% confidence level) showed that the direct analysis by SC-ICP-MS provided similar results to those obtained by ICP-MS after acid digestion of the samples. No significant differences were found between both methods (*p* > 0.05).

3.4. Detection of possible structural alterations in bacteria by SEM and TEM

In order to detect the possible bacterial wall alterations as a consequence of apramycin or silver presence, samples were observed by SEM and TEM. Fig. 6 shows the SEM images of bacterial control and bacteria exposed to apramycin and silver nanoparticles, individually and combined. Control bacteria presented a normal bacillus shape, smooth wall surface and cellular integrity (Fig. 6a). On the contrary, in the presence of apramycin (Fig. 6b), although bacteria preserved their bacillus shape, their walls were damaged in most of them because of the antibiotic bactericidal effect. The wall surface became rough and presented holes and gaps (marked with red circles). Likewise, in the presence of silver nanoparticles (Fig. 6c), bacterial walls were damaged and become rough. Finally, after exposure to the combination of apramycin–silver nanoparticles (Fig. 6d), bacteria were severely damaged and rough and fragmented walls and the occurrence of holes and gaps were observed.

Fig. 7 shows the TEM images for samples discussed above. Moreover, TEM images for bacteria exposed to ionic silver individually can be found in the SI (Fig. S1). Control bacteria

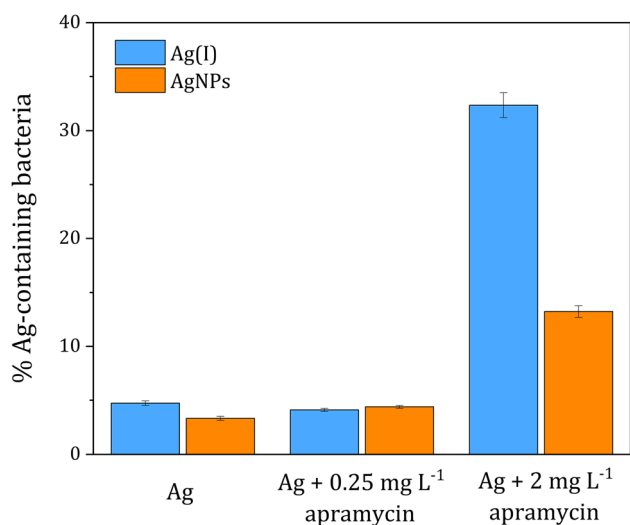


Fig. 5 Percentage of silver-containing bacteria *E. coli* ATCC 25922 in relation to total bacteria. Exposed silver concentration: 0.50 mg L⁻¹ Ag(I) and 2 mg L⁻¹ 10 nm AgNPs.



Table 3 Silver mass concentration of *E. coli* bacteria samples analysed by SC-ICP-MS and ICP-MS after acid digestion of samples and percentage of silver uptake (from silver mass concentration obtained by SC-ICP-MS and initial silver concentration added during the incubation process). Mean \pm standard deviation ($n = 5$)

Sample	Silver mass concentration $\mu\text{g L}^{-1}$		Silver uptake by bacteria %
	SC-ICP-MS	Acid digestion-ICP-MS	
Incubation medium control	2.2 ± 0.2	<0.5	—
Bacterial control	<0.1	0.3 ± 0.1	—
0.25 mg L^{-1} apramycin	<0.1	0.5 ± 0.1	—
2 mg L^{-1} apramycin	<0.1	0.2 ± 0.1	—
Ag(i)	72.0 ± 3.0	73.3 ± 6.5	21.3 ± 0.9
Ag(i) + 0.25 mg L^{-1} apramycin	88.9 ± 0.9	85.6 ± 2.0	32.0 ± 0.3
Ag(i) + 2 mg L^{-1} apramycin	44.8 ± 3.1	40.6 ± 0.6	10.1 ± 0.7
AgNPs	26.5 ± 1.4	25.3 ± 0.9	1.6 ± 0.1
AgNPs + 0.25 mg L^{-1} apramycin	21.9 ± 2.1	21.1 ± 1.3	1.2 ± 0.1
AgNPs + 2 mg L^{-1} apramycin	393.6 ± 22.9	424.3 ± 50.2	21.1 ± 1.3

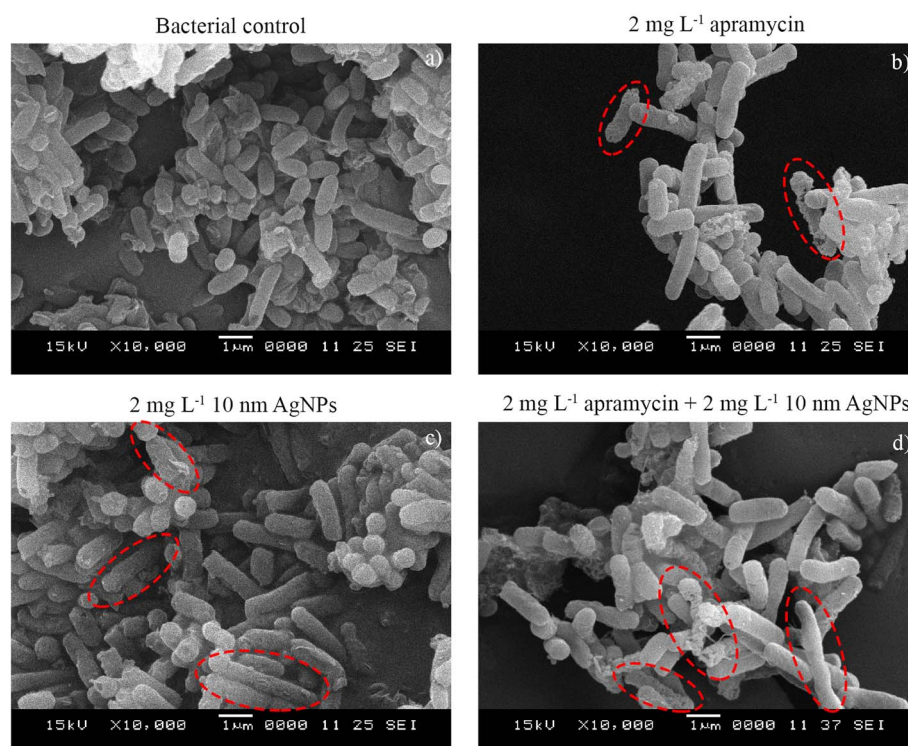


Fig. 6 SEM images (10000 \times) of *E. coli* ATCC 25922: (a) control and exposed to (b) 2 mg L^{-1} apramycin, (c) 2 mg L^{-1} 10 nm AgNPs and (d) 2 mg L^{-1} apramycin + 2 mg L^{-1} 10 nm AgNPs.

had an intact double membrane and a homogeneous cytoplasm inside bacteria, as well as their bacillus shape, as was observed by SEM. On the contrary, in the presence of apramycin (Fig. 7b), a significant structural damage was observed. The double membrane was completely broken and the cytoplasm had been released. Alterations of the bacterial shape or irregularities in the membrane structure were observed. Likewise, in the presence of ionic silver and silver nanoparticles (Fig. S1, 7c and d), double membrane rupture and membrane irregularities were also observed, as well as cytoplasm shrinkage and heterogeneity (indicated by arrows). Finally, after exposure to the combination of apramycin–silver nanoparticles (Fig. 7e and f), same damage

and alterations described for individual antimicrobial exposure were observed, as well as double membrane rupture and cytoplasm leakage through membrane ruptured areas (marked with red circles).

4. Discussion

In this study, it was showed that silver(i), 10 nm silver nanoparticles and apramycin have individual antibacterial activity against *E. coli* ATCC 25922 with MIC and MBC values of $8 \mu\text{g mL}^{-1}$ for silver(i) and $16 \mu\text{g mL}^{-1}$ for silver nanoparticles and apramycin. These results indicate that silver(i) has a higher



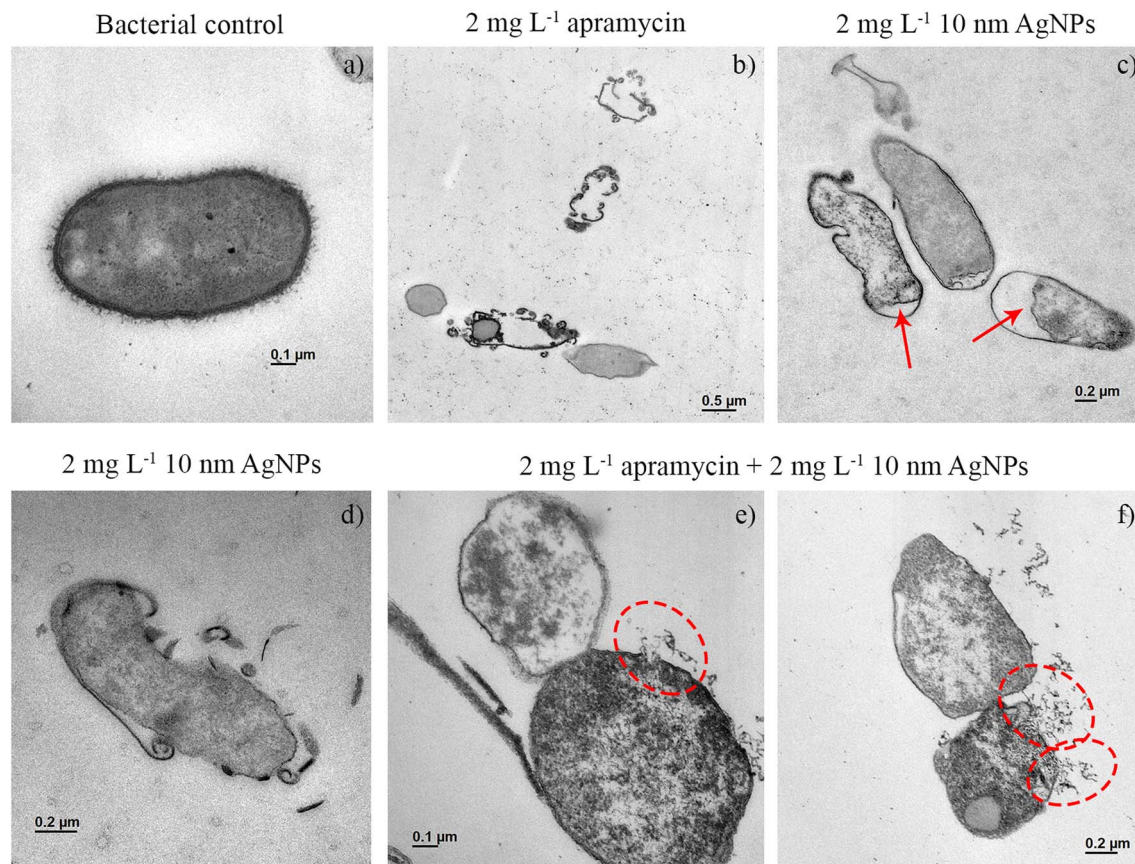


Fig. 7 TEM images ($250\,00\times$ – $100\,000\times$) of bacteria *E. coli* ATCC 25922: (a) control and exposed to (b) 2 mg L^{-1} apramycin, (c and d) 2 mg L^{-1} 10 nm AgNPs, and (e and f) 2 mg L^{-1} 10 nm AgNPs + 2 mg L^{-1} apramycin. Red arrows: shrinkage and heterogeneity of the cytoplasm. Red circles: membrane rupture and cytoplasm leakage.

bacteriostatic and bactericidal activity against *E. coli* bacteria. Because the antimicrobial bactericidal effect depends on factors such as culture medium, bacterial strain, inoculum initial concentration, as well as nanoparticle concentration, size or coating, different MIC and MBC values for silver(I), silver nanoparticles and apramycin are reported in the literature. Silver(I) values against *E. coli* ATCC 25922 in MH medium range from 1 to $31\text{ }\mu\text{g mL}^{-1}$,^{34–36} which are consistent with those obtained in this study. Likewise, MIC values for silver nanoparticles with different size and coating against *E. coli* vary between 0.25 and $100\text{ }\mu\text{g mL}^{-1}$,^{37,38} which are in agreement with our results. Finally, in relation to apramycin, there are no clinical MIC and MBC criteria established by the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUROCAST). Some authors have evaluated apramycin bactericidal activity against a large number of *E. coli* strains, generally non-reference strains, and compared it with other aminoglycosides. They obtained MIC values between 1 and $256\text{ }\mu\text{g mL}^{-1}$,^{18,39–41} suggesting an epidemiological cut-off value of $16\text{--}32\text{ }\mu\text{g mL}^{-1}$, which agrees with our results. Comparing apramycin with other aminoglycosides (amikacin, gentamicin and tobramycin), Juhas *et al.* found that apramycin had higher bactericidal effects against the same strains than other aminoglycosides.¹⁸ These

results could make the combination of apramycin with silver based materials an attractive alternative to fight against AMR.

Subsequently, two different combinations of apramycin and silver(I) or silver nanoparticles were evaluated to determine the bactericidal activity of each combination. The occurrence of synergistic bacteriostatic and bactericidal effects was observed for all antimicrobial combinations. This fact allowed us to decrease the antimicrobial individual concentrations, while keeping the individual bactericidal effects of each antimicrobial. In this sense, the presence of silver(I) produced a greater reduction in apramycin and silver(I) concentrations (8- and 16-fold, respectively). On the other hand, the presence of silver nanoparticles allowed for a slightly lower reduction of concentrations (4- and 8- fold reduction for apramycin and silver nanoparticles, respectively). This fact is in agreement with the behaviour observed in a previous study, where higher bactericidal effects were obtained for silver(I).²⁸ To the best of authors' knowledge, this is the first study to evaluate the combination of apramycin with silver(I) and silver nanoparticles against *E. coli* ATCC 25922. Other studies with aminoglycosides, such as gentamicin, ampicillin, kanamycin or tobramycin, evaluated their combination with silver nanoparticles against *E. coli* and *Pseudomonas aeruginosa* and demonstrated synergistic bacteriostatic effects,^{22,24,42,43} but none of them was based on the use



of apramycin. Habash *et al.* showed the influence of nanoparticle size on bactericidal activity because nanoparticle sizes lower than 20 nm produced synergism, whereas higher nanoparticle sizes resulted in only additive effects. In addition, they suggested that the synergy produced by small silver nanoparticles differs for each antibiotic and could be influenced by the antibiotic mechanism.^{22,24}

To improve the knowledge about the mechanism of action associated with the bactericidal activity of antimicrobial combinations and the individual role played by each antimicrobial, an analytical methodology based on SC-ICP-MS was applied in this work. Silver accumulated by bacteria during the incubation process was quantified, and silver mass per bacteria distributions and percentage of bacteria containing silver were obtained. In addition, bacterial structural alterations as a consequence of apramycin or silver presence were observed by SEM and TEM. Taking into account the results obtained through this study, the following conclusions were reached.

After combining silver(i) with a lower apramycin concentration, bacteria accumulated 32% of the silver, whereas in its absence, silver uptake was 21% (Table 3). Regarding the fraction of bacteria accumulating silver (Fig. 5), no differences were observed in the presence or absence of apramycin (4–5%). These facts would indicate that the presence of apramycin promotes the uptake of higher amounts of silver per bacteria. This deduction would be in agreement with silver mass per bacteria distributions, where a decrease in the first distribution consisting of bacteria accumulating small amounts of silver was observed. Likewise, an increase in the second one, corresponding to bacteria accumulating higher amounts of silver (Fig. 3b), was observed in comparison with apramycin absence (Fig. 3a).

On the other hand, for the combination of silver(i) with a higher apramycin concentration, silver uptake decreased significantly from 21% to 10% (Table 3), whereas the fraction of bacteria accumulating silver increased from 5% to, approximately, 30% (Fig. 5). In both combinations of silver(i) and apramycin, as seen in TEM and SEM images, apramycin and silver(i) produce wall alterations and holes which would facilitate the internalization of silver and other molecules of apramycin, justifying the increase in the fraction of bacteria accumulating silver and silver accumulation. This mechanism of wall disruption has been previously observed by other authors after the evaluation of aminoglycosides (gentamicin and tobramycin) against *Pseudomonas aeruginosa*.^{22,24,44,45} On the other hand, when bacterial wall damage is too large, cytoplasm leakage could occur, and therefore, a loss of the previously accumulated silver could take place. This fact would justify the decrease in silver uptake obtained by SC-ICP-MS, because released silver would be lost during centrifugation in the sample preparation step, and consequently, the silver quantification would be underestimated.

After analysing these results, an action mechanism associated with the synergistic bactericidal effects of silver(i) and apramycin combination can be proposed. Initially, silver ions could interact with bacterial walls and produce the alteration of the outer membrane, promoting the uptake of apramycin

molecules and silver ions into bacteria. The biodistribution of silver(i) in *E. coli* was determined in a previous study, where silver was mainly internalized,²⁸ which would support the hypothesis of silver internalization through the membrane. Once inside, both apramycin and silver ions would bind to ribosomes, leading to the production of misfolded proteins that could be incorporated and alter membrane organization.⁴⁶ This fact, in turn, could promote the massive introduction of more apramycin molecules and silver ions.²⁰ These ions would also act by their mechanisms and bind to biomolecules such as proteins or DNA and inhibit some processes such as respiratory chain or ATP synthesis.^{10,47} The combination of these bactericidal effects of apramycin and silver ions would result in severe damage in biological processes and bacterial wall disruption, leading to bacterial death.⁶

In the case of the combination of 10 nm silver nanoparticles and a lower apramycin concentration, silver uptake by bacteria was similar (1.2–1.6%) (Table 3) to that in the absence of apramycin. Likewise, the fraction of bacteria accumulating silver was also similar in both cases (3–4%) (Fig. 5). This would indicate that the studied apramycin concentration would not significantly affect silver uptake by bacteria. On the contrary, after combining silver nanoparticles with a higher apramycin concentration, silver uptake increased to 21% (Table 3), whereas the fraction of bacteria accumulating silver increased to 13% (Fig. 5). As in the case of silver(i), TEM and SEM images show that both antimicrobials produce extensive bacterial wall alteration and hole formation, which would allow a higher silver uptake. These alterations produced by silver nanoparticles in *E. coli* bacteria have been previously observed by other authors.^{7,48,49}

After analysing these results, an action mechanism associated with the synergistic bactericidal effects of silver nanoparticles and apramycin combination can be proposed. Initially, silver nanoparticles could interact with bacterial wall and were adsorbed on it, resulting in membrane alteration and greater permeability. During nanoparticle-wall interaction, nanoparticles could release silver ions, which would, in turn, lead to further wall disruption. In a previous study, silver accumulated by bacteria was found to be dissolved and particulate silver when using 10 nm AgNPs as the ones used here, which would support the hypothesis of silver released from nanoparticles.²⁶ In addition, in the mechanism proposed above, the wall alteration would allow the introduction of apramycin and silver, which would bind to ribosomes, inhibit protein synthesis and produce misfolded proteins.^{20,46,50} This, in turn, would promote further apramycin and silver introduction, and the individual action mechanisms would be activated, leading to severe damage and bacterial death. Davis B. D⁵¹ demonstrated how the action of aminoglycoside antibiotics is concentration-dependent, supporting the mechanism of action described here. This indicates that at low concentrations, apramycin could only cause mistranslation of mRNA, resulting in a small amount of defective proteins that would not be sufficient to contribute any effect to the antibiotic silver combination. In a previous study, it was shown that a silver fraction was internalized by bacteria after their exposure to



silver nanoparticles, supporting the hypothesis of silver internalization through membranes.²⁸ However, kinetic studies should be considered to establish the sequence of events. Likewise, Vazquez-Muñoz *et al.* suggested that the antimicrobial activity of silver nanoparticle–antibiotic combination is based, mainly, on cell structure alteration instead of direct nanoparticle–antibiotic interaction,²¹ which is also in concordance with that suggested in the present study.

5. Conclusions

In conclusion, in this study, a combination of SC-ICP-MS with microscopy and microbiological techniques is applied for the first time to quantify the silver accumulated in bacteria exposed to different combinations of silver(I), silver nanoparticles and apramycin, as well as to determine the damage caused to bacteria. This methodology has allowed the evaluation and determination of the synergistic bactericidal effect of silver–apramycin combinations, as well as the action mechanisms related to these bactericidal effects. The combination of silver with apramycin has resulted in the occurrence of synergistic effects, allowing the reduction of silver(I), silver nanoparticle and apramycin concentrations while preserving the individual bactericidal effects of each antimicrobial. The silver(I)–apramycin combination has allowed a greater reduction in both antimicrobials when compared to the combination with silver nanoparticles. On the other hand, generally the presence of apramycin has promoted the silver uptake by bacteria.

These results confirm that the combination of silver-based materials and conventional antibiotics could be a promising alternative strategy to fight against AMR. Specifically, the combination of apramycin and silver based materials could be applied to reduce the high amounts of antibiotics used nowadays. Likewise, this study model could also be applied to the determination of bactericidal action mechanisms of combinations of nanoparticles with other agents, as conventional antibiotics or disinfectants.

Author contributions

A. C. G.: formal analysis, investigation, data curation, methodology, visualization, writing – original draft. I. A.: formal analysis, investigation, data curation, methodology, supervision, writing – review & editing. P. C.: formal analysis, supervision. P. G.: methodology, supervision, writing – review & editing. F. L.: conceptualization, data curation, funding acquisition, methodology, resources, supervision, validation, writing – review & editing. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare that they have no conflict of 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

The data supporting this article have been included as part of the SI. The SI contains additional experimental data, detailed methodology and extended tables and figures supporting the findings of this study. Supplementary information is available online alongside the published article. See DOI: <https://doi.org/10.1039/d5na00404g>.

Acknowledgements

This work was supported by the project PID2021-123203OB-I00 funded by MCIN/AEI/10.13039/501100011033 and by “ERDF A way of making Europe” and the Government of Aragon (E29_23R and B43_23R). A. C. G. thanks the Government of Aragón for a predoctoral contract. I. A. thanks the European Union-Next Generation EU and the Spanish Ministry of Universities for funding under the María Zambrano Grant (MZ-240621). The authors would like to acknowledge the use of Servicio General de Apoyo a la Investigación-SAI, Universidad de Zaragoza.

References

- 1 A. J. Huh and Y. J. Kwon, ‘Nanoantibiotics’: A new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era, *J. Controlled Release*, 2011, **156**, 128–145.
- 2 World Health Organization, *Antimicrobial Resistance*, accessed 20 June 2024.
- 3 G. V. Doern, K. P. Heilmann, H. K. Huynh, P. R. Rhomberg, S. L. Coffman and A. B. Brueggemann, Antimicrobial resistance among clinical isolates of *Streptococcus pneumoniae* in the United States during 1999–2000, including a comparison of resistance rates since 1994–1995, *Antimicrob. Agents Chemother.*, 2001, **45**, 1721–1729.
- 4 J. O'Neill, *Tackling Drug-Resistant Infections Globally: Final Report and Recommendations. Review on Antimicrobial Resistance*, Wellcome Trust, 2016, p. 84.
- 5 J. A. Lemire, J. J. Harrison and R. J. Turner, Antimicrobial activity of metals: Mechanisms, molecular targets and applications, *Nat. Rev. Microbiol.*, 2013, **11**, 371–384, DOI: [10.1038/nrmicro3028](https://doi.org/10.1038/nrmicro3028).
- 6 F. Barras, L. Aussel and B. Ezraty, Silver and Antibiotic, New Facts to an Old Story, *Antibiotics*, 2018, **7**, 79.
- 7 J. R. Morones-Ramirez, J. A. Winkler, C. S. Spina and J. J. Collins, Silver Enhances Antibiotic Activity Against Gram-negative Bacteria, *Sci. Transl. Med.*, 2013, **5**, 190ra81, DOI: [10.1126/scitranslmed.3006276](https://doi.org/10.1126/scitranslmed.3006276).
- 8 J. T. Seil and T. J. Webster, Antimicrobial applications of nanotechnology Methods and literature, *Int. J. Nanomed.*, 2012, **7**, 2767–2781, DOI: [10.2147/IJN.S24805](https://doi.org/10.2147/IJN.S24805).
- 9 M. F. Gómez-Núñez, M. Castillo-López, F. Sevilla-Castillo, O. J. Roque-Reyes, F. Romero-Lechuga, D. I. Medina-Santos, R. Martínez-Daniel and A. N. Peón, Nanoparticle-Based Devices in the Control of Antibiotic Resistant



- Bacteria, *Front. Microbiol.*, 2020, **11**, 563821, DOI: [10.3389/fmicb.2020.563821](https://doi.org/10.3389/fmicb.2020.563821).
- 10 A. Hamad, K. S. Khashan and A. Hadi, Silver Nanoparticles and Silver Ions as Potential Antibacterial Agents, *J. Inorg. Organomet. Polym.*, 2020, **30**, 4811–4828, DOI: [10.1007/s10904-020-01744-x](https://doi.org/10.1007/s10904-020-01744-x).
 - 11 A. M. Allahverdiyev, K. V. Kon, E. S. Abamor, M. Bagirova and M. Rafailovich, Coping with antibiotic resistance: Combining nanoparticles with antibiotics and other antimicrobial agents, *Expert Rev. Anti-Infect. Ther.*, 2011, **9**, 1035–1052, DOI: [10.1586/eri.11.121](https://doi.org/10.1586/eri.11.121).
 - 12 P. Li, J. Li, C. Wu, Q. Wu and J. Li, Synergistic antibacterial effects of β -lactam antibiotic combined with silver nanoparticles, *Nanotechnology*, 2005, **16**, 1912, DOI: [10.1088/0957-4484/16/9/082](https://doi.org/10.1088/0957-4484/16/9/082).
 - 13 A. R. Shahverdi, A. Fakhimi, H. R. Shahverdi and S. Minaian, Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against *Staphylococcus aureus* and *Escherichia coli*, *Nanomedicine*, 2007, **3**, 168–171.
 - 14 A. M. Fayaz, K. Balaji, M. Girilal, R. Yadav, P. T. Kalaichelvan and R. Venketesan, Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: a study against gram-positive and gram-negative bacteria, *Nanomedicine*, 2010, **6**, 103–109.
 - 15 S. Baker, A. Pasha and S. Satish, Biogenic nanoparticles bearing antibacterial activity and their synergistic effect with broad spectrum antibiotics: Emerging strategy to combat drug resistant pathogens, *Saudi Pharm. J.*, 2017, **25**, 44–51.
 - 16 S. Gurunathan, J. W. Han, D. N. Kwon and J. H. Kim, Enhanced antibacterial and anti-biofilm activities of silver nanoparticles against Gram-negative and Gram-positive bacteria, *Nanoscale Res. Lett.*, 2014, **9**, 1–17.
 - 17 E. C. Böttger and D. Crich, Aminoglycosides: Time for the Resurrection of a Neglected Class of Antibacterials?, *ACS Infect. Dis.*, 2020, **6**, 168–172.
 - 18 M. Juhas, E. Widlake, J. Teo, D. L. Huseby, J. M. Tyrrell, Y. S. Polikanov, O. Ercan, A. Petersson, S. Cao, A. F. Aboklaish, A. Rominski, D. Crich, E. C. Böttger, T. R. Walsh, D. Hughes and S. N. Hobbie, In vitro activity of apramycin against multidrug-, carbapenem- And aminoglycoside-resistant enterobacteriaceae and *acinetobacter baumannii*, *J. Antimicrob. Chemother.*, 2019, **74**, 944–952.
 - 19 E. Bordeleau, P. J. Stogios, E. Evdokimova, K. Koteva, A. Savchenko and G. D. Wright, ApmA is a unique aminoglycoside antibiotic acetyltransferase that inactivates apramycin, *mBio*, 2021, **12**, 1–11.
 - 20 M. Herisse, Y. Duverger, I. Martin-Verstraete, F. Barras and B. Ezraty, Silver potentiates aminoglycoside toxicity by enhancing their uptake, *Mol. Microbiol.*, 2017, **105**, 115–126.
 - 21 R. Vazquez-Muñoz, A. Meza-Villezcás, P. G. J. Fournier, E. Soria-Castro, K. Juárez-Moreno, A. L. Gallego-Hernández, N. Bogdanchikova, R. Vazquez-Duhalt and A. Huerta-Saquero, Enhancement of antibiotics antimicrobial activity due to the silver nanoparticles impact on the cell membrane, *PLoS One*, 2019, **14**(11), e0224904, DOI: [10.1371/journal.pone.0224904](https://doi.org/10.1371/journal.pone.0224904).
 - 22 M. B. Habash, M. C. Goodyear, A. J. Park, M. D. Surette, E. C. Vis, R. J. Harris and C. M. Khursigara, Potentiation of Tobramycin by Silver Nanoparticles against *Pseudomonas aeruginosa* Biofilms, *Antimicrob. Agents Chemother.*, 2017, **61**(11), DOI: [10.1128/AAC.00415-17](https://doi.org/10.1128/AAC.00415-17).
 - 23 A. Kaur, S. Preet, V. Kumar, R. Kumar and R. Kumar, Synergetic effect of vancomycin loaded silver nanoparticles for enhanced antibacterial activity, *Colloids Surf., B*, 2019, **176**, 62–69.
 - 24 M. B. Habash, A. J. Park, E. C. Vis, R. J. Harris and C. M. Khursigara, Synergy of silver nanoparticles and aztreonam against *Pseudomonas aeruginosa* PAO1 biofilms, *Antimicrob. Agents Chemother.*, 2014, **58**, 5818–5830.
 - 25 H. Deng, D. McShan, Y. Zhang, S. S. Sinha, Z. Arslan, P. C. Ray and H. Yu, Mechanistic Study of the Synergistic Antibacterial Activity of Combined Silver Nanoparticles and Common Antibiotics, *Environ. Sci. Technol.*, 2016, **50**, 8840–8848.
 - 26 A. C. Gimenez-Ingalaturre, I. Abad-Álvaro, M. Bakir, P. Chueca, P. Goñi and F. Laborda, 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, *Microchem. J.*, 2024, **205**, 111296.
 - 27 A. C. Gimenez-Ingalaturre, E. Rubio, P. Chueca, F. Laborda and P. Goñi, Contribution to optimization and standardization of antibacterial assays with silver nanoparticles: the culture medium and their aggregation, *J. Microbiol. Methods*, 2022, **203**, 106618.
 - 28 A. C. Gimenez-Ingalaturre, I. Abad-Álvaro, P. Goñi, K. Billimoria, H. Goenaga-Infante and F. Laborda, Performance of single-cell ICP-MS for quantitative biodistribution studies of silver interactions with bacteria, *J. Anal. At. Spectrom.*, 2024, **39**, 743–753.
 - 29 A. L. Leber, *Clinical Microbiology Procedures Handbook*, Washington, DC, USA, 2016.
 - 30 P. Verma, in *Antimicrobial Susceptibility Testing Protocols*, CRC Press, 2007, pp. 275–298.
 - 31 F. Laborda, A. C. Gimenez-Ingalaturre, E. Bolea and J. R. Castillo, Single particle inductively coupled plasma mass spectrometry as screening tool for detection of particles, *Spectrochim. Acta, Part B*, 2019, **159**, 105654.
 - 32 F. Laborda, A. C. Gimenez-Ingalaturre, E. Bolea and J. R. Castillo, About detectability and limits of detection in single particle inductively coupled plasma mass spectrometry, *Spectrochim. Acta, Part B*, 2020, **169**, 105883.
 - 33 H. E. Pace, N. J. Rogers, C. Jarolimek, V. A. Coleman, C. P. Higgins and J. F. Ranville, Determining Transport Efficiency for the Purpose of Counting and Sizing Nanoparticles via Single Particle Inductively Coupled Plasma Mass Spectrometry, *Anal. Chem.*, 2011, **83**, 9361–9369.
 - 34 A. Pormohammad and R. J. Turner, Silver antibacterial synergism activities with eight other metal(Loid)-based antimicrobials against *Escherichia coli*, *Pseudomonas*



- aeruginosa*, and *Staphylococcus aureus*, *Antibiotics*, 2020, **9**, 1–19.
- 35 H. Acay, M. F. Baran and A. Eren, Investigating antimicrobial activity of silver nanoparticles produced through green synthesis using leaf extract of common grape (*Vitis Vinifera*), *Appl. Ecol. Environ. Res.*, 2019, **17**, 4539–4546.
 - 36 K. O. Lima, A. A. Vasconcelos, J. J. V. de Sousa Júnior, S. K. S. Escher, G. Nakazato and P. S. Taube Júnior, Green synthesis of silver nanoparticles using Amazon fruits, *Int. J. Nanosci. Nanotechnol.*, 2019, **15**, 179–188.
 - 37 S. Agnihotri, S. Mukherji and S. Mukherji, Size-controlled silver nanoparticles synthesized over the range 5–100 nm using the same protocol and their antibacterial efficacy, *RSC Adv.*, 2014, **4**, 3974–3983.
 - 38 G. Franci, A. Falanga, S. Galdiero, L. Palomba, M. Rai, G. Morelli and M. Galdiero, Silver nanoparticles as potential antibacterial agents, *Molecules*, 2015, **20**, 8856–8874.
 - 39 Y. Yang, T. Xiao, J. Li, P. Cheng, F. Li, H. Yu, R. Liu, I. Muhammad and X. Zhang, Wild-type cutoff for Apramycin against *Escherichia coli*, *BMC Vet. Res.*, 2020, **16**, 309.
 - 40 E. Tian, I. Muhammad, W. Hu, Z. Wu, R. Li, X. Lu, C. Chen and J. Li, Tentative epidemiologic cut-off value and resistant characteristic detection of apramycin against *Escherichia coli* from chickens, *FEMS Microbiol. Lett.*, 2019, **366**, 196.
 - 41 A. Kobe, A. Ebrecht and R. Fries, Minimum inhibitory concentrations of intestinal *Escherichia coli* from broiler chickens after oral administration of apramycin, *J. Appl. Bacteriol.*, 1996, **80**, 26–30.
 - 42 M. Smekalova, V. Aragon, A. Panacek, R. Prucek, R. Zboril and L. Kvitek, Enhanced antibacterial effect of antibiotics in combination with silver nanoparticles against animal pathogens, *Vet. J.*, 2016, **209**, 174–179.
 - 43 I. sok Hwang, J. H. Hwang, H. Choi, K. J. Kim and D. G. Lee, Synergistic effects between silver nanoparticles and antibiotics and the mechanisms involved, *J. Med. Microbiol.*, 2012, **61**, 1719–1726.
 - 44 J. L. Kadurugamuwa, A. J. Clarke and T. J. Beveridge, Surface action of gentamicin on *Pseudomonas aeruginosa*, *J. Bacteriol.*, 1993, **175**, 5798–5805.
 - 45 J. B. Bulitta, N. S. Ly, C. B. Landersdorfer, N. A. Wanigaratne, T. Velkov, R. Yadav, A. Oliver, L. Martin, B. S. Shin, A. Forrest and B. T. Tsuji, Two mechanisms of killing of *pseudomonas aeruginosa* by tobramycin assessed at multiple inocula via mechanism-based modeling, *Antimicrob. Agents Chemother.*, 2015, **59**, 2315–2327.
 - 46 J. Molina, E. Cordero, J. Palomino and J. Pachón, Aminoglucósidos y polimixinas, *Enferm. Infecc. Microbiol. Clín.*, 2009, **27**, 178–188.
 - 47 M. Godoy-Gallardo, U. Eckhard, L. M. Delgado, Y. J. D. de Roo Puente, M. Hoyos-Nogués, F. J. Gil and R. A. Perez, Antibacterial approaches in tissue engineering using metal ions and nanoparticles: From mechanisms to applications, *Bioact. Mater.*, 2021, **12**, 4470–4490, DOI: [10.1016/j.bioactmat.2021.04.033](https://doi.org/10.1016/j.bioactmat.2021.04.033).
 - 48 X. Li, J. J. Lenhart and H. W. Walker, Dissolution-accompanied aggregation kinetics of silver nanoparticles, *Langmuir*, 2010, **26**, 16690–16698.
 - 49 R. Zhao, M. Lv, Y. Li, M. Sun, W. Kong, L. Wang, S. Song, C. Fan, L. Jia, S. Qiu, Y. Sun, H. Song and R. Hao, Stable Nanocomposite Based on PEGylated and Silver Nanoparticles Loaded Graphene Oxide for Long-Term Antibacterial Activity, *ACS Appl. Mater. Interfaces*, 2017, **9**, 15328–15341.
 - 50 H. R. Ali, A. N. Emam, E. G. Hefny, N. F. Koraney, A. S. Mansour, A. M. Salama, S. F. Ali, S. H. Aboolo and M. A. Shahein, Silver nanoparticles enhance the effectiveness of traditional antibiotics against *S. aureus* causing bovine mastitis within the safety limit, *J. Nanopart. Res.*, 2021, **23**, 1–18.
 - 51 B. D. Davis, Mechanism of bacterial action of aminoglycosides, *Microbiol. Rev.*, 1987, **51**, 341–350.

