Supplementary information

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

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1. Meaterial and methods

1.1. Checkerboard: determination of combined bactericidal activity

After incubation of microplates containing bacteria exposed to different combinations of silver and apramycin, results were interpreted by visually observing the well turbidity. Results were transferred to a schematic, indicating in each box if visible growth (well turbidity) was present or absent. Then, Fractional Inhibitory Concentration (FIC) for each antimicrobial was calculated. The sum of these FICs allows to obtain the Fractional Inhibitory Concentration Index (FICI) for each antimicrobial combination. FICI allows the determination of combined bacteriostatic effects, which transiently inhibit bacterial growth without resulting in bacterial death.

The calculation of FICs for each antimicrobial and FICI is carried out using the following equations:[1,2]

$$FIC\ for\ antimicrobial\ A = \frac{MIC\ of\ antimicrobial\ A\ in\ combination\ with\ B}{MIC\ of\ antimicrobial\ A\ alone} \quad (1)$$

$$FIC\ for\ antimicrobial\ B = \frac{MIC\ of\ antimicrobial\ B\ in\ combination\ with\ A}{MIC\ of\ antimicrobial\ B\ alone} \quad (2)$$

$$FICI = FIC_A + FIC_B \quad (3)$$

The interpretation of FICI is as follows:

- Synergism: FICI ≤ 0.5
- Indifference: 0.5 > FICI ≤ 4
- Antagonism: FICI > 4

In addition, Fractional Bactericidal Concentration Index (FBCI) was determined after seeding on plates aliquots from wells in which bacterial growth was inhibited. After incubation, results were interpreted by observing qualitative bacterial growth on the plates. Then, Fractional Bactericidal Concentration (FBC) for each antimicrobial was calculated. The sum of these FBCs allows to obtain the Fractional Bactericidal Concentration Index (FBCI) for each antimicrobial combination. FBCI allows the determination of combined bactericidal effects, that is, those that cause bacterial death. The interpretation of FBCI is the same as FICI (synergism, indifference and antagonism). To calculate FBC for each antimicrobial and FBCI, the same expressions (1) - (3) are used, but replacing MIC concentrations by MBC concentrations.[2,3]

1.2. Instrumentation

A Perkin-Elmer NexION 2000B mass spectrometer (Toronto, Canada) was used for ICP-MS measurements in conventional mode and in single cell mode (SC-ICP-MS). For the first mode, the sample introduction system consisted of a glass concentric nebulizer and a baffled cyclonic spray chamber (Meinhard, Colorado, USA). For single cell mode, the sample introduction system

consisted of an Asperon™ linear pass spray chamber (PerkinElmer, Toronto, Canada), equipped with a high efficiency glass concentric micronebuliser (Meinhard, Colorado, USA). For conventional mode, data acquisition was performed using a dwell time of 50 ms, 20 sweeps and 10 replicates per measurement. Default instrumental and data acquisition parameters for SC-ICP-MS are listed in Table S1.

Table S1. Default instrumental and data acquisition parameters for SC-ICP-MS

Instrumental parameters	
RF power	1600 W
Argon gas flow rate	
Plasma	15 L min^{-1}
Auxiliary	1.2 L min^{-1}
Nebulizer	0.3 L min^{-1}
Make-up	0.9 L min^{-1}
Sample flow rate	15 µL min^{-1}
Data acquisition parameters	
Dwell time	100 µs
Total acquisition time	60 s
Isotopes monitored	^{107}Ag, ^{197}Au

A Scanning Electron Microscope (JEOL JSM 6360-LV), working at 15 kV, was used for observation of bacterial samples. In-lens secondary electron detector of the same microscope was used to obtain higher resolution images. A Transmission Electron Microscope (JEOL 1010), working at 80 kV, and a Gatan Bioscan camera were also used.

1.3. Standards

Diluted suspensions of gold and silver nanoparticles were prepared from commercially available suspensions. An ultra-uniform gold nanoparticle (PEG-carboxil 0.8 kDa surface) suspension of 47.8 ± 1.8 nm diameter and a monodisperse citrate-stabilized silver nanoparticle suspension of 10.3 ± 2.1 nm diameter were obtained from NanoComposix (San Diego, CA, USA). Dilutions were prepared in ultrapure water (Milli-Q Advantage, Molsheim, France) by accurately weighing (± 0.1 mg) aliquots of the stock suspensions after 1 min sonication. After dilution and before each analysis, the suspensions were bath sonicated for 1 min. Longer sonication times were not used to avoid excessive heating of the suspensions. Aqueous gold and silver solutions were prepared from standard stock solutions of 1000 mg L^{-1} (Sigma Aldrich, Saint Louis, MO, USA) by dilution. Nitric acid (Baker Instranalyzed for Trace Metals Analysis, J.T. Baker, Holland), hydrogen peroxide (Scharlau, Scharlab. S.L., Barcelona, Spain) were also used. For SEM and TEM analysis, glutaraldehyde (4%), osmium tetroxide (Electron Microscopy Sciences, Fisher

Scientific, Thermo Fisher Scientific, Waltham, MA, USA), epoxy resin, uranyl acetate, lead citrate and ethanol (Sigma Aldrich, Saint Louis, MO, USA) were used.

For microbiological cultures, apramycin sulfate (Alfa Aesar, Karlsruhe, Germany), phosphate buffered saline (PBS) (Sigma-Aldrich, Saint Louis, MO, USA), sodium chloride physiological solution (0.9% NaCl) (Panreac, Barcelona, Spain), Mueller-Hinton broth (MHB) (Scharlau, Scharlab S.L., Barcelona, Spain), Mueller-Hinton (MH) agar (Bio-Rad, La Coquette, France), Tween 80 (Sigma-Aldrich, Saint Louis, MO, USA) and sodium phosphate monobasic (NaH_2PO_4) and sodium phosphate dibasic (Na_2PO_4) solutions (Sigma-Aldrich, Saint Louis, MO, USA) were used. The solutions of physiological saline solution and PBS and liquid and agar culture media were autoclaved for 20 min at 121ºC at 1 atm. A commercial McFarland turbidity standard (Fisher Scientific, Thermo Fisher Scientific, Waltham, MA, USA) was used as a reference for concentrations of bacterial suspensions.

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

Bacteriostatic effects for combinations of apramycin - silver (I) and apramycin-silver nanoparticles against *E. coli* bacteria were determined. After observing the presence or absence of turbidity in wells, FIC and FICI were calculated, according to equations (1) - (3), for each well where no visual bacterial growth was observed. Table S2 and Table S3 show the results obtained for combinations of apramycin and silver (I) or silver nanoparticles, respectively.

Table S2. Fractional Inhibitory Concentrations (FIC) of apramycin and silver (I), Fractional Inhibitory Concentration Indexes (FICI) and their interpretation for each combination of apramycin - silver (I) against *E. coli* ATCC 25922

Well	[Apramycin] µg mL^{-1}	FIC_{Apram}	[Ag(I)] µg mL^{-1}	$FIC_{Ag(I)}$	FICI	Interpretation
D4	8	0.50	4	0.50	1	Indifference
D5	4	0.25	4	0.50	0.75	Indifference
D6	2	0.125	4	0.50	0.63	Indifference
D8	0.50	0.031	4	0.50	0.53	Indifference
E4	8	0.50	2	0.25	0.75	Indifference
E5	4	0.25	2	0.25	0.50	Synergism
E6	2	0.125	2	0.25	0.38	Synergism
F4	0.50	0.031	1	0.125	0.63	Indifference
F5	8	0.50	1	0.125	0.38	Synergism
G4	4	0.25	0.50	0.063	0.56	Indifference
G5	8	0.50	0.50	0.063	0.31	Synergism
H4	4	0.25	0.25	0.031	0.53	Indifference

Table S3. Fractional Inhibitory Concentrations (FIC) of apramycin and silver nanoparticles, Fractional Inhibitory Concentration Indexes (FICI) and their interpretation for each combination of apramycin - silver nanoparticles against *E. coli* ATCC 25922

Well	[Apramycin] μg mL^{-1}	FIC_{Apram}	[AgNPs] μg mL^{-1}	FIC_{AgNPs}	FICI	Interpretation
B4	8	0.50	8	0.50	1	Indifference
B5	4	0.25	8	0.50	0.75	Indifference
B6	2	0.125	8	0.50	0.63	Indifference
C4	8	0.50	4	0.25	0.75	Indifference
C5	4	0.25	4	0.25	0.50	Synergism
D4	8	0.50	2	0.125	0.63	Indifference
D5	4	0.25	2	0.125	0.38	Synergism
E4	8	0.50	1	0.063	0.56	Indifference
E5	4	0.25	1	0.063	0.31	Synergism
F4	8	0.50	0.50	0.031	0.53	Indifference
G4	8	0.50	0.25	0.016	0.52	Indifference
H4	8	0.50	0.125	0.008	0.51	Indifference
H5	4	0.25	0.125	0.008	0.26	Synergism

Likewise, bactericidal effects for combinations of apramycin - silver (I) and apramycin-silver nanoparticles against *E. coli* bacteria were determined. After observing the presence or absence of bacterial growth on plates, FBC and FBCI were calculated for each well where no bacterial growth was observed and its bordering wells with bacterial growth. Table S4 and Table S5 show the results obtained for combinations of apramycin and silver (I) or silver nanoparticles, respectively.

Table S4. Fractional Bactericidal Concentrations (FBC) of apramycin and silver (I), Fractional Bactericidal Concentration Indexes (FBCI) and their interpretation for each combination of apramycin - silver (I) against *E. coli* ATCC 25922

Well	[Apramycin] μg mL^{-1}	FBC_{Apram}	[Ag(I)] μg mL^{-1}	$FBC_{Ag(I)}$	FBCI	Interpretation
D4	8	0.50	4	0.50	1	Indifference
D5	4	0.25	4	0.50	0.75	Indifference
D6	2	0.125	4	0.50	0.63	Indifference
E4	8	0.50	2	0.25	0.75	Indifference
E5	4	0.25	2	0.25	0.50	Synergism
E6	2	0.125	2	0.25	0.38	Synergism
F4	0.50	0.031	1	0.125	0.63	Indifference
F5	8	0.50	1	0.125	0.38	Synergism

G4	4	0.25	0.50	0.063	0.56	Indifference
G5	8	0.50	0.50	0.063	0.31	Synergism
H4	4	0.25	0.25	0.031	0.53	Indifference

Table S5. Fractional Bactericidal Concentrations (FBC) of apramycin and silver nanoparticles, Fractional Bactericidal Concentration Indexes (FBCI) and their interpretation for each combination of apramycin - silver nanoparticles against *E. coli* ATCC 25922

Well	[Apramycin] μg mL^{-1}	FBC_{Apram}	[AgNPs] μg mL^{-1}	FBC_{AgNPs}	FBCI	Interpretation
B4	8	0.50	8	0.50	1	Indifference
B5	4	0.25	8	0.50	0.75	Indifference
B6	2	0.125	8	0.50	0.63	Indifference
C4	8	0.50	4	0.25	0.75	Indifference
C5	4	0.25	4	0.25	0.50	Synergism
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H4	8	0.50	0.125	0.008	0.51	Indifference

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

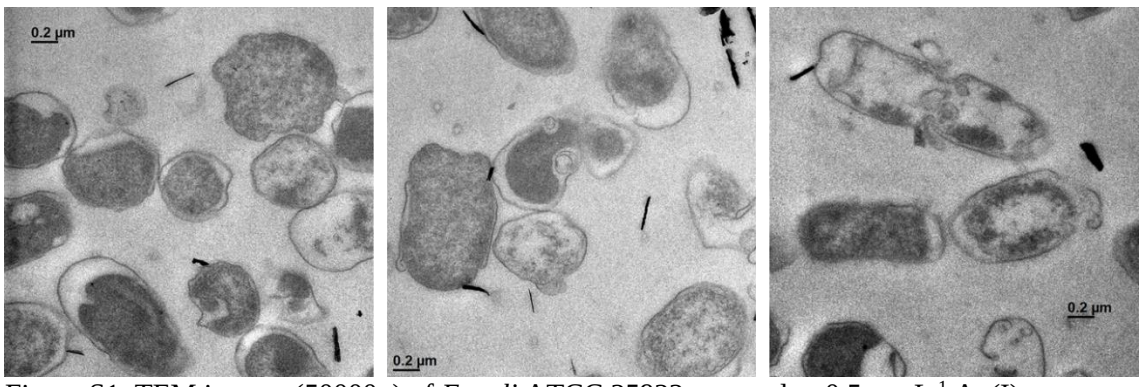

Figure S1. TEM images (50000x) of *E. coli* ATCC 25922 exposed to 0.5 mg L^{-1} Ag(I).

References

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