nature nanotechnology

SUPPLEMENTARY INFORMATION

https://doi.org/10.1038/s41565-017-0013-y

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Bacterial resistance to silver nanoparticles and a way how to overcome it

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Experimental methods

The influence of surface stabilizers on the bacterial growth and viability

The stock solutions of surface stabilizers (2% PAA, 2% gelatin, 2% PEG, and 2% TWEEN 80) and saline solution were diluted in MH cultivation broth in microtiter plate. The first well of plate contained mixture of tested surface stabilizers/saline diluted 1:1 with MH cultivation broth followed by dilution in other wells of microtiter plate by two-fold dilution system. The final volume of solution was 100 μL per well. The prepared microtiter plate was inoculated with "Ag-sensitive" and "Ag-resistant" *E. coli* on separate parts of plate. The final concentration of bacterial strain referred to 1×10^6 CFU mL^{-1}. The inoculated microtiter plate was covered with foil to prevent evaporation and placed in a spectrophotometer with a built-in incubator (BIO-TEK ELx808) for 24 hours at 37 °C. Optical density (630 nm) was measured every hour for 24 hours and readings were used to plot growth curves.

Viability test of "Ag-sensitive" and "Ag-resistant" *E. coli* cells with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was performed to evaluate their metabolic activity. First, the cells were diluted in the LBG medium to achieve an OD600 value of 0.110. The assay was conducted essentially according to Wang *et al.* (Wang, H., Cheng, H., Wang, F., Wei, D. & Wang, X. An improved 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay for evaluating the viability of *Escherichia coli* cells. *J. Microbiol. Methods* **82,** 330–333 (2010)). The water-insoluble formazan dye produced upon incubation of the cells with MTT (Cat. No. M2128, Sigma-Aldrich) at 37 °C for 20 min was recovered by centrifugation at 10,000 g for 10 min and dissolved in 2.5 mL of dimethyl sulfoxide. Then the colored samples were measured at 550 nm on a spectrophotometer (pure dimethyl sulfoxide was used as a blank). The assay was performed also in the presence of 1% TW, PEG, GEL, or PAA to check the possible influence.

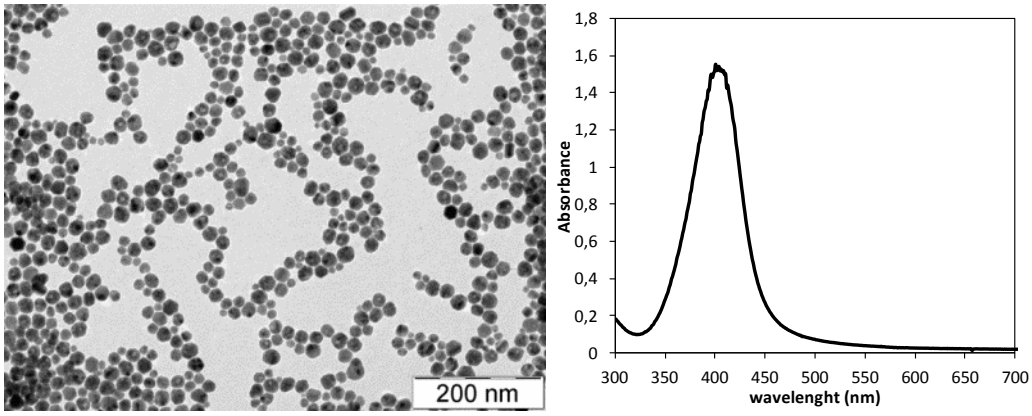
Revert to sensitive phenotype in cultivation without silver NPs

The "Ag-resistant" *E. coli* strain was continually incubated for 120 hours at 37 °C in 50 mL of silver NPs free MH broth. Appropriate culture conditions were ensured by transferring the bacterial suspension to fresh culture medium at defined time intervals (5, 24, 48, 72, and 96 hours). At the same time, 100 μL of bacterial suspension was inoculated on an MH agar plate. MH agar plates were incubated for 24 hours at 37 °C and grown bacterial colonies were used for silver NPs MIC determination.

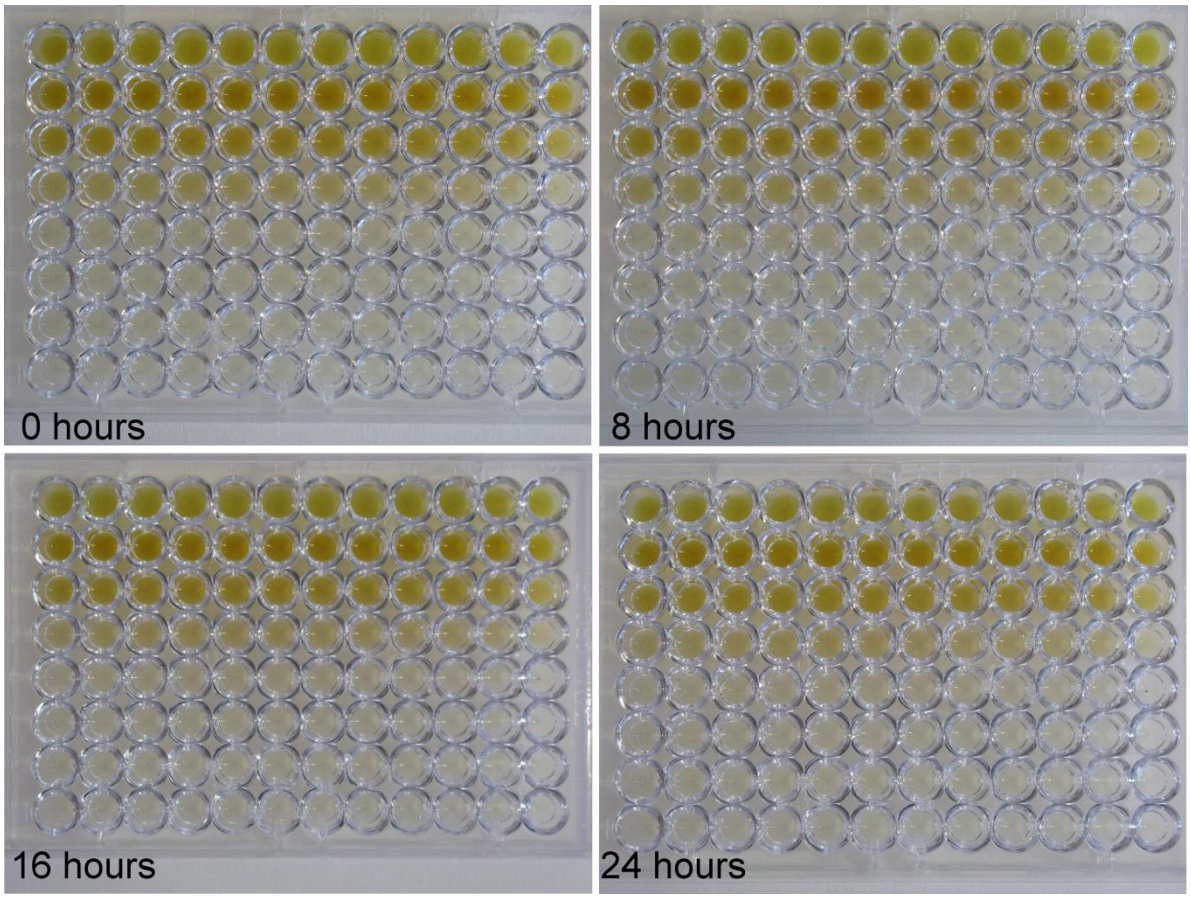
To confirm the stability of silver NPs resistance after pre-treatment in the pomegranate extract, the 24-hours cultivation of resistant bacterial strain in the highest sub-inhibitory concentration of pomegranate extracts was performed, followed by MIC silver NPs determination.

Determination of minimum duration for killing

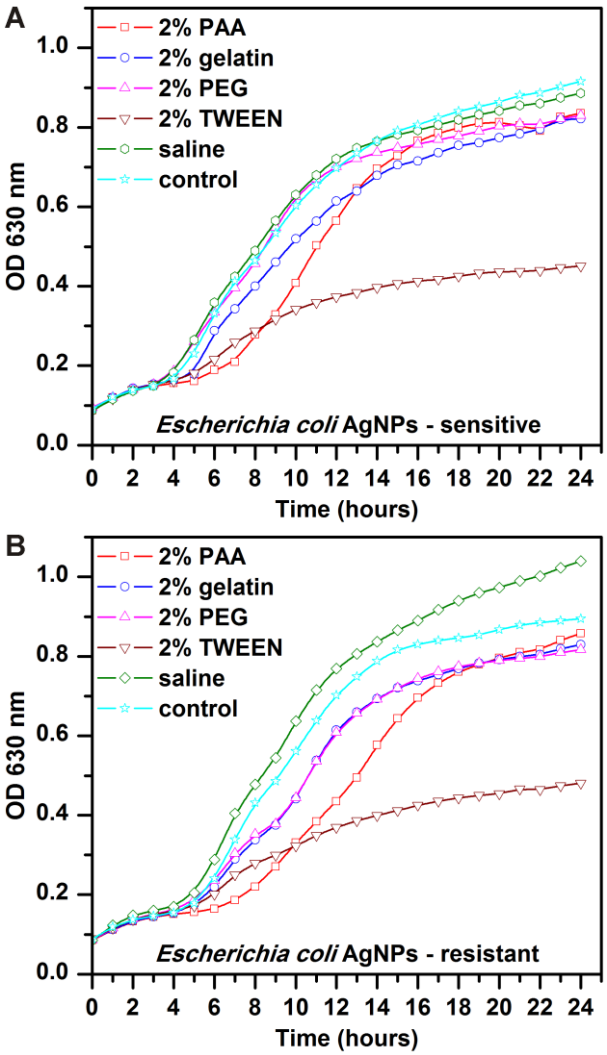
Silver NPs were diluted in MH cultivation broth in microtiter plate. The final volume of solution was 100 μL per well and concentration of silver NPs ranged from 54 to 0 mg L^{-1}. The prepared microtiter plate was inoculated with "Ag-sensitive" *E. coli* (MIC = 6.75 mg L^{-1}). The final concentration of bacterial strain referred to 1×10^6 CFU mL^{-1}. 10 μL of bacterial suspensions were inoculated and spread on MH agar plates from concentrations of silver NPs (54, 27, 13.5, 6.75, and 0 mg L^{-1} silver NPs) every hour within 20-hour time frame. After incubation for 24 h at 37 °C, the numbers of bacteria within inoculum were determined semi-quantitatively.



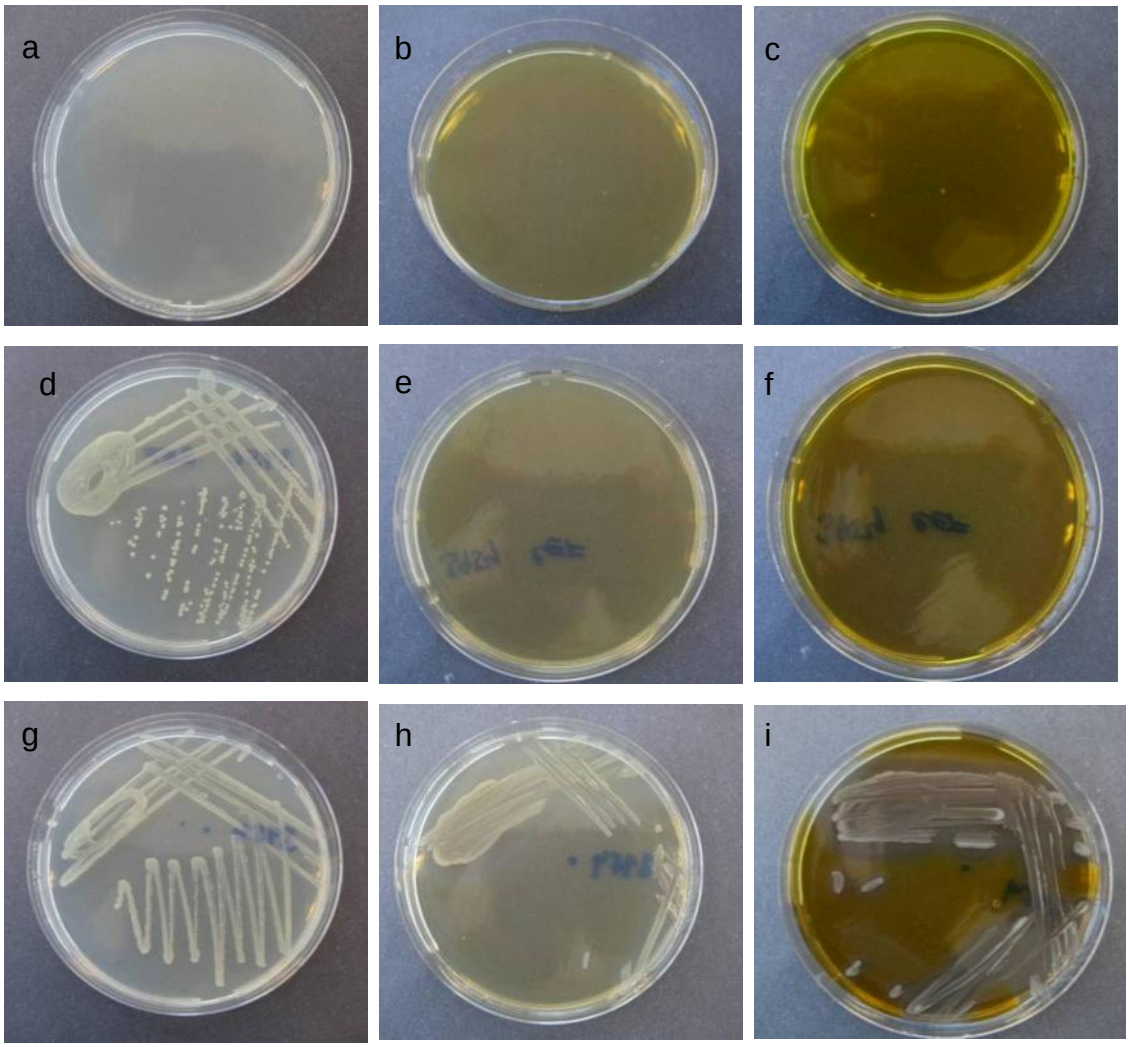
Supplementary Fig. 1 | TEM image and UV/Vis spectra of the original dispersion of silver NPs with diameters of 28 nm prepared by the modified Tollens process and used in cultivation experiments with various Gram-negative bacteria.



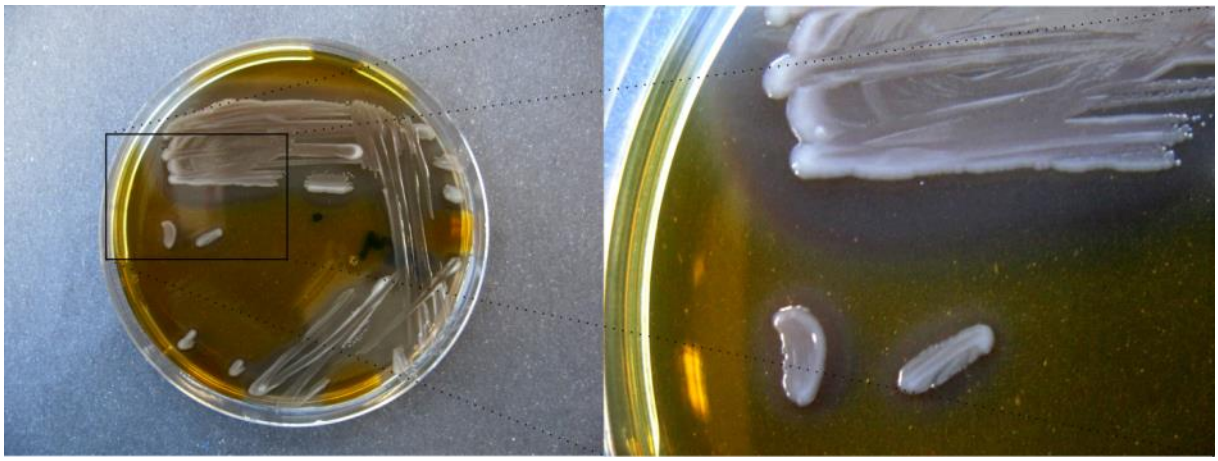
Supplementary Fig. 2 | Photographs showing the absence of aggregation or precipitation of silver NPs in microplates containing "Ag-susceptible" *E. coli* CCM 3954 after 0, 8, 16, and 24 hours of cultivation. The concentration of silver NPs decreases gradually from 54 mg L^{-1} to 0.84 mg L^{-1} on going from the second to the eighth row. The upper row contains pristine dispersions of silver NPs (108 mg L^{-1}) without any bacteria or culture media.



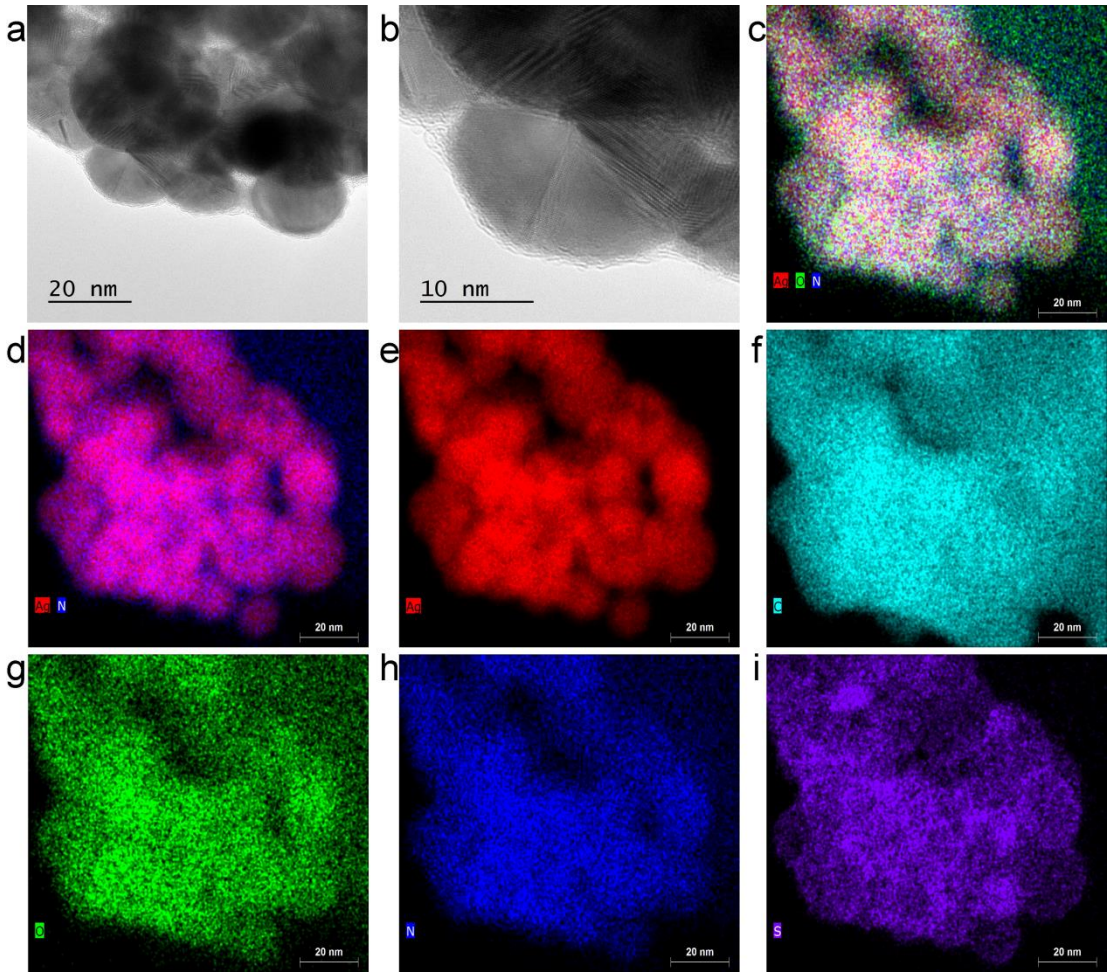
Supplementary Fig. 3 | Growth curves of "Ag-sensitive" (A) and "Ag-resistant" (B) *E. coli* CCM 3954 in the presence of stabilizers, showing that the stabilizers have no (PEG, PAA, GEL) or only very minor (TW) negative effects on bacterial growth.



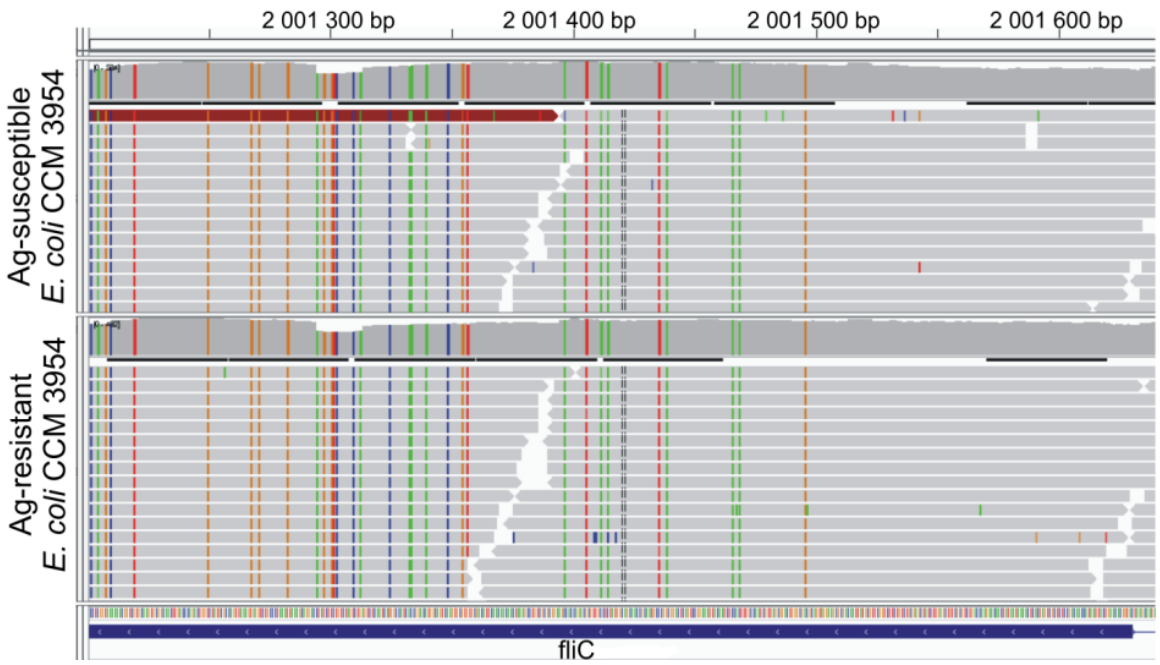
Supplementary Fig. 4 | Pure agar plate without any bacteria or silver NPs (A). Agar plates containing 20 mg L^{-1} (B) and 40 mg L^{-1} (C) of silver NPs. Growth of "Ag-susceptible" *Escherichia coli* CCM 3954 on an agar plate without silver NPs (D) and growth inhibition of "Ag-susceptible" *Escherichia coli* CCM 3954 on agar plates containing 20 mg L^{-1} (E) and 40 mg L^{-1} (F) of silver NPs. Growth of "Ag-resistant" *Escherichia coli* CCM 3954 on agar plates without silver NPs (G) and with 20 mg L^{-1} (H) and 40 mg L^{-1} (I) of silver NPs.



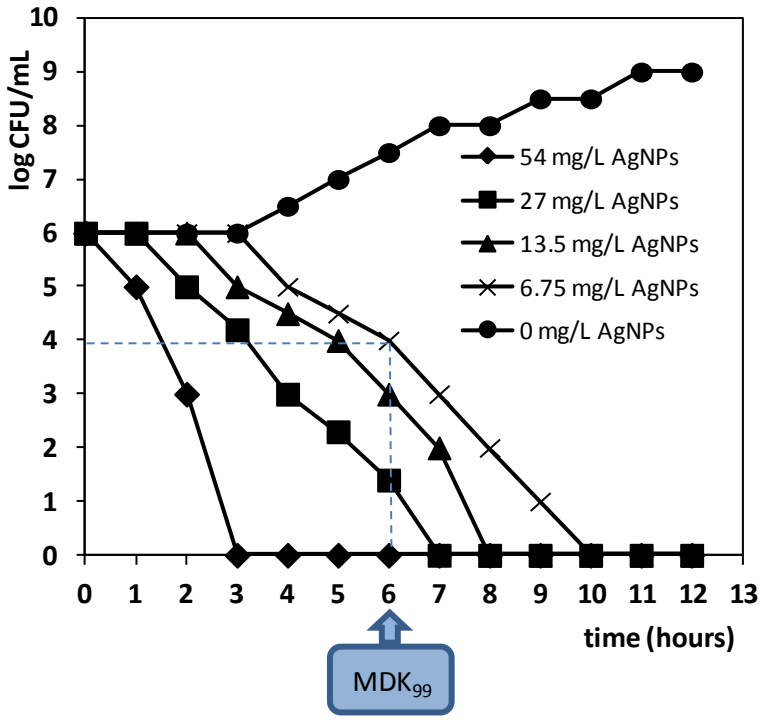
Supplementary Fig. 5 | Grey areas surrounding grown "Ag-resistant" *E. coli* CCM 3954 resulting from the aggregation of silver NPs after 24 hours of cultivation.



Supplementary Fig. 6 | HRTEM images of silver aggregates (a, b) showing the compact organic layer on the surface of the NPs. Combined EDS chemical mapping of silver, oxygen and nitrogen (c), and silver with nitrogen (d); and individual chemical maps of silver (e), carbon (f), oxygen (g), nitrogen (h), and sulfur (i).



Supplementary Fig. 7 | Part of the *fliC* gene including first 428 bp of the gene sequence.



Supplementary Fig. 8 | Time-kill assay for silver NPs at different concentrations, and MDK99 determination.

Supplementary Table 1 | Minimum inhibitory concentrations (MIC) of silver NPs as determined for "Ag-susceptible" (reference pristine bacteria not previously treated with silver NPs) and "Ag-resistant" bacteria (after twenty steps of culturing with silver NPs).

	MIC (mg L^{-1})	
	Ag-susceptible	Ag-resistant
E. coli CCM 3954	3.38	108
P. aeruginosa CCM 3955	1.69	54
E. coli 013	13.5	108

Note. The starting concentration of silver NPs used in the dilution method was 216 mg/L.

Supplementary Table 2 | Formazan dye absorbance values in five individual measurements, mean absorbances, and calculated bacterial viability (%) with and without stabilizers.

Sample	**Values per measurement**					**Mean value**	**Standard deviation**	**Relative standard deviation (%)**	**Viability (%)**
Ag-sensitive *E. coli*									
Control	0.44	0.47	0.49	0.44	0.49	0.46	0.024	5.28	**100**
TW	0.35	0.44	0.39	0.40	0.41	0.4	0.030	7.65	**86**
PEG	0.42	0.45	0.44	0.47	0.46	0.45	0.020	4.67	**97**
GEL	0.45	0.42	0.47	0.45	0.46	0.45	0.020	4.53	**98**
PAA	0.59	0.58	0.60	0.59	0.60	0.59	0.008	1.49	**128***
Ag-resistant *E. coli*									
Control	0.35	0.33	0.35	0.31	0.34	0.34	0.018	5.56	**100**
TW	0.29	0.27	0.28	0.26	0.26	0.27	0.014	5.44	**81**
PEG	0.31	0.32	0.28	0.29	0.32	0.30	0.014	4.93	**90**
GEL	0.30	0.32	0.32	0.33	0.32	0.32	0.008	2.83	**94**
PAA	0.58	0.51	0.55	0.49	0.50	0.52	0.035	6.78	**156***

*Higher values are related to increased permeability due to the acidic environment (Grela, E., Ząbek, A. & Grabowiecka, A. Interferences in the Optimization of the MTT Assay for Viability Estimation of *Proteus mirabilis*. *Avicenna J. Med. Biotechnol.* **7(4) Oct-Dec,** 159–167 (2015))

Supplementary Table 3 | MICs of non-stabilized and stabilized silver NPs towards "Ag-susceptible" *Escherichia coli* CCM 3954 and "Ag-resistant" *Escherichia coli* CCM 3954. The data demonstrate that the surface stabilization does not overcome the developed resistance.

	MIC (mg L^{-1})				
	Non-stabilized silver NPs	Stabilized silver NPs			
		PEG	PAA	GEL	TW
Ag-susceptible *Escherichia coli* CCM 3954	3.38	3.38	1.69	1.69	3.38
Ag-resistant *Escherichia coli* CCM 3954	108	54	54	54	54

PEG – polyethylene glycol; PAA – sodium salt of polyacrylic acid; GEL – gelatin; TW – polyoxyethylene sorbitan monooleate (Tween 80)