Comparable antibacterial effects and action mechanisms of silver and iron oxide nanoparticles on *Escherichia coli* and *Salmonella typhimurium*

Lilit Gabrielyan1,2, Hamlet Badalyan3, Vladimir Gevorgyan4 & Armen Trchounian1,2*

The current research reports the antibacterial effects of silver (Ag) and citric acid coated iron oxide (Fe3O4) NPs on *Escherichia coli* wild type and kanamycin-resistant strains, as well as on *Salmonella typhimurium* MDC1759. NPs demonstrated significant antibacterial activity against these bacteria, but antibacterial effect of Ag NPs is more pronounced at low concentrations. Ag NPs inhibited 60–90% of *S. typhimurium* and drug-resistant *E. coli*. The latter is more sensitive to Fe3O4 NPs than wild type strain: the number of bacterial colonies is decreased ~4-fold. To explain possible mechanisms of NPs action, H⁺-fluxes through the bacterial membrane and the H⁺-translocating F0F1-ATPase activity of bacterial membrane vesicles were studied. N,N′-Dicyclohexylcarbodiimide (DCCD)-sensitive ATPase activity was increased up to ~1.5-fold in the presence of Fe3O4 NPs. ATPase activity was not detected by Ag NPs even in the presence of DCCD, which confirms the bactericidal effect of these NPs. The H⁺-fluxes were changed by NPs and by addition of DCCD. H2 yield was inhibited by NPs; the inhibition by Ag NPs is stronger than by Fe3O4 NPs. NPs showed antibacterial effect in bacteria studied in concentration-dependent manner by changing in membrane permeability and membrane-bound enzyme activity. The F0F1-ATPase is suggested might be a target for NPs.

In recent years, the increase of the bacterial infections and the development of antimicrobial resistance have been avowed as one of the key problems of the biomedicine of twenty-first century, which require a finding of novel antibacterial agents. Nanoparticles (NPs) are considered a great alternative to antibiotics, since they show a high potential to solve the problem of antibiotic resistance. Various NPs are used in biomedicine, pharmaceutics, cosmetics, food industry, textile coating, etc.1–5.

In particular, silver (Ag) NPs and iron oxide (Fe3O4) have attracted much attention in biomedical field4–9. Ag NPs show a pronounced antibacterial activity not only against various pathogens, but also against bacterial biofilms10–12. Effect of NPs depends on the particle size, shape, surface characteristics and magnetic properties1,2,13,14. For example, at the same concentration 10 nm Ag NPs are more effective than 100 nm Ag NPs against methicillin-resistant *Staphylococcus aureus*15. Moreover, Ag NPs demonstrate the stronger inhibitory effect than other NPs15,16. Ag NPs can physically interact with the cell surface of bacteria due to their large surface area, providing better interaction with bacteria17. Franchi et al.18 reported that Ag NPs can damage bacterial cell membranes, which provide to increasing of permeability of bacterial membrane. The Ag NPs can preferably affect the respiratory chain in bacterial cells19.

Iron oxide NPs such as Fe3O4 and γ-Fe2O3 NPs due to their super paramagnetic, high magnetic susceptibility and other properties are promising agents for biomedical applications. Nowadays, they have been used in drug delivery systems to deliver various compounds such as peptides, DNA molecules, chemotherapeutic, and hyper-thermic drugs1,17,18. These systems have a potential to minimize the side-effects and the required concentration

1Department of Medical Biochemistry and Biotechnology, Russian-Armenian University, 0051 Yerevan, Armenia. 2Department of Biochemistry, Microbiology and Biotechnology, Biology Faculty, Yerevan State University, 0025 Yerevan, Armenia. 3Department of General Physics and Astrophysics, Yerevan State University, 0025 Yerevan, Armenia. 4Department of Technology for Materials and Electronic Technique Structures, Russian-Armenian University, 0051 Yerevan, Armenia. 5email: Trchounian@ysu.am
of drugs, as well as decrease the damage of normal tissues. In these systems, magnetic targeting of drug delivery is considered as the most efficient way\textsuperscript{13}.

The antimicrobial action of NPs is probably a result of their interaction with bacterial membrane, which can lead to alterations of membrane-bound mechanisms, membrane damage and the bacterial death\textsuperscript{20,21}. Moreover, the antibacterial mechanisms of NPs action are not fully explored and in this case, the study of the effects of NPs on various bacteria is important for finding out of the NPs' possible action mechanisms.

It is known, that susceptibility of microorganisms to NPs depends not only on NPs' characteristics, but also on stabilizer type. Due to the tendency of NPs to aggregation, their use requires the stabilization. Stabilizer, which used for the magnetic NPs, can affect the properties of NPs\textsuperscript{22-27}. Oleic acid was suggested as a promising stabilizer for magnetic NPs\textsuperscript{22-24}. Oleic acid can form a protective monolayer around the NPs, which is necessary for monodispersed magnetic NPs formation\textsuperscript{25-24}. However, stabilization of magnetic NPs with oleic acid makes the NPs soluble only in organic solvents and therefore limits their use in biomedicine\textsuperscript{22,23}. By the way, for biomedical applications in aqueous media, the hydrophobic stabilizer needs to be replaced by a hydrophilic one\textsuperscript{25}.

In our previous studies oleic acid coated \( \text{Fe}_3\text{O}_4 \) NPs' effects on Gram-negative \textit{Escherichia coli} BW25113, ampicillin-resistant \textit{E. coli} DH5a-pUC18, kanamycin-resistant \textit{E. coli} pARG-25 stains and Gram-positive \textit{Enterococcus hirae} ATCC9790 growth and membrane-associated mechanisms have been investigated\textsuperscript{26,27}. Our results showed that the \( \text{Fe}_3\text{O}_4 \) NPs demonstrate different effects on Gram-negative and Gram-positive bacteria. Gram-positive \textit{E. hirae} displayed higher susceptibility to NPs than Gram-negative \textit{E. coli}, because the components of cell wall of Gram-positive and Gram-negative bacteria have different pathways for NPs adsorption\textsuperscript{28}. Stabilizer oleic acid had no any effect on growth properties of investigated bacteria.

Citric acid is another stabilizer, which able to stabilize magnetic NPs, but there are few data in literature about citric acid coated NPs antibacterial properties. It has been shown that treating NPs by citric acid in aqueous solution stabilizes the NPs surfaces\textsuperscript{25-27}. The citric acid coated NPs remained stable in aqueous solution even in a week, and this stability is sustained under an electric field in aqueous media\textsuperscript{27}. Study of citric acid stabilized NPs antibacterial properties is very important for the regulation of various bacteria growth in biomedicine and biotechnology.

In the present work, the antibacterial effects and possible mechanisms of Ag NPs and citric acid coated \( \text{Fe}_3\text{O}_4 \) NPs on \textit{E. coli} wild type and drug-resistant strains as well as in comparison with \textit{Salmonella typhimurium} MDC1759, have been studied for the first time. Additional characteristics of NPs were determined. In order to analyze the role of different membrane-bound systems and to find out probable targets \( \text{H}^+\)-translocating, \( \text{F}_0\text{F}_1 \)-ATPase activity, \( \text{H}^+\)-fluxes through the bacterial membrane and \( \text{H}_2 \) yield were also investigated.

## Results

### Growth characteristics of \textit{E. coli} wild type and drug-resistant strains, and \textit{S. typhimurium} in the presence of Ag NPs and citric acid coated \( \text{Fe}_3\text{O}_4 \) NPs.

\textit{Escherichia} and \textit{Salmonella} species are the most common foodborne human pathogens causing to various types of illnesses\textsuperscript{3,28-30}. In this case, it is important to study the effect of Ag and \( \text{Fe}_3\text{O}_4 \) NPs (coated by citric acid) on \textit{E. coli} wild type and antibiotic-resistant strains, as well as \textit{S. typhimurium} MDC1759 strain, for revealing the action mechanisms. The growth parameters of \textit{E. coli} wild type K-12, kanamycin-resistant pARG-25, and \textit{S. typhimurium} MDC1759 strains in the presence of Ag and \( \text{Fe}_3\text{O}_4 \) NPs have been investigated. Bacteria grown in the presence of kanamycin (50 μg mL\(^{-1}\)) were used as positive control. The negative controls are the strains, cultivated without antibiotic.

The results obtained show antibacterial effects of Ag and \( \text{Fe}_3\text{O}_4 \) NPs. The intensity of NPs effects depends on the type of NPs, their concentrations and bacterial strains. Both NPs showed inhibitory effect on \textit{E. coli} growth (Fig. 1). Moreover, the effect of \( \text{Fe}_3\text{O}_4 \) NPs also depends on the type of stabilizer. Stabilization of NPs by various compounds enhances an antimicrobial activity of NPs\textsuperscript{24,26,31}.

In the presence of citric acid coated \( \text{Fe}_3\text{O}_4 \) NPs (100–250 μg mL\(^{-1}\)) inhibition of \textit{E. coli} K-12 growth was observed (Fig. 1A). NPs demonstrated concentration dependent effect on \textit{E. coli} growth (Fig. 3). The specific growth rate of \textit{E. coli} K-12 at NPs' concentration 50 μg mL\(^{-1}\) was similar to the control, and decreased ~ 1.2-fold at 100 μg mL\(^{-1}\) NPs (Fig. 3). The maximal inhibitory effect has been observed at the concentration 250 μg mL\(^{-1}\), which led to the decrease in bacterial specific growth rate by ~ 2.0-fold, indicating the bacteriostatic effect of the \( \text{Fe}_3\text{O}_4 \) NPs. The antibacterial effect of \( \text{Fe}_3\text{O}_4 \) NPs may be due to several mechanisms. ROS together with superoxide radicals (\( \text{O}_2^- \)), hydroxide radical (\( \text{OH}^- \)) and singlet oxygen formed by \( \text{Fe}_3\text{O}_4 \) NPs could be the reason of inhibition. Similar results were obtained in other studies showing the antibacterial activity of \( \text{Fe}_3\text{O}_4 \) NPs against \textit{E. coli}\textsuperscript{25}. In our previous study oleic acid coated \( \text{Fe}_3\text{O}_4 \) NPs show antibacterial activity against \textit{E. coli} BW25113 strain\textsuperscript{26}. However, the citric acid coated \( \text{Fe}_3\text{O}_4 \) NPs demonstrate more pronounced antibacterial activity than \( \text{Fe}_3\text{O}_4 \) NPs coated by oleic acid at the same concentration. The maximal inhibitory effect of oleic acid coated \( \text{Fe}_3\text{O}_4 \) NPs has been observed at the 500 μg mL\(^{-1}\) concentration\textsuperscript{27}. Moreover, citric acid does not show any effect on growth rates of investigated bacteria (not shown).

Ag NPs show more pronounced bactericidal effect than \( \text{Fe}_3\text{O}_4 \) NPs (Fig. 1). Moreover, Ag NPs display more expressed antibacterial effect at low concentrations. In the presence of 10 μg mL\(^{-1}\) Ag NPs a 2.6-fold suppression of growth of \textit{E. coli} K-12 was observed (see Fig. 1A). The 20–30 μg mL\(^{-1}\) Ag NPs led to 4.0–6.5-fold decrease in bacterial growth rate, indicating the bactericidal effect of these concentrations.

Moreover, kanamycin-resistant \textit{E. coli} pARG-25 strain had more susceptibility to \( \text{Fe}_3\text{O}_4 \) NPs than \textit{E. coli} wild type strain (Fig. 1B). In the presence of 100–250 μg mL\(^{-1}\) \( \text{Fe}_3\text{O}_4 \) NPs a 1.5–3-fold inhibition of bacterial growth was observed (see Fig. 1B). Similar results were obtained with ampicillin-resistant \textit{E. coli} DH5a-pUC18 strain (not shown). Citric acid coated \( \text{Fe}_3\text{O}_4 \) NPs demonstrated more noticeable antibacterial effect than \( \text{Fe}_3\text{O}_4 \) NPs stabilized by oleic acid\textsuperscript{28}. In the case of Ag NPs, as seen in Fig. 1B, there were no obvious differences between antibacterial effect of these NPs on wild type and drug-resistant bacteria.
The effect of Fe₃O₄ NPs on S. typhimurium MDC1759 growth rate is the same with the action of iron oxide NPs on E. coli K-12 strain (Fig. 1C). Antibacterial effect of Ag NPs on S. typhimurium MDC1759 was less pronounced than on E. coli both strains (Fig. 1C). At the same time, S. typhimurium demonstrated susceptibility to the high concentrations of Ag NPs: 20 μg mL⁻¹ Ag NPs led to ~2.5-fold decrease in growth specific rate (Fig. 1C).

Figures 2 and 3 display the number of viable colonies of E. coli K-12 and pARG-25, and S. typhimurium MDC1759, grown in the absence and presence of 100 μg mL⁻¹ Fe₃O₄ NPs. CFU of E. coli K-12 was decreased 1.3-fold in the presence of Fe₃O₄ NPs (Fig. 2A,B). At the same time, S. typhimurium MDC1759 and drug-resistant E. coli strain has more susceptibility to Fe₃O₄ NPs (stabilized by citric acid) than wild type strain: CFU was decreased 2.5–4-fold, respectively (see Fig. 2A). In the case of kanamycin-resistant E. coli strains Fe₃O₄ NPs
coated by citric acid showed noticeable antibacterial effect in comparison with oleic acid stabilized Fe3O4 NPs20. Ag NPs inhibited ~ 60 to 90% of S. typhimurium MDC1759 and drug-resistant E. coli viable colonies (Fig. 2C,D). Thus, NPs show significant antibacterial activity against used bacteria. Different effects of Fe3O4 and Ag NPs on bacterial growth may be due to the difference in the interaction between the bacterial cells and NPs2,11. Small size of NPs can contribute to their antibacterial activity7,11. NPs can interact closely with bacterial membranes and the inactivation of bacteria could be due to their penetration into the bacterial cell1,9,11.

Figure 2. The number of viable colonies of E. coli K-12, kanamycin-resistant E. coli pARG-25, and S. typhimurium MDC1759 strains grown in the presence of citric acid coated Fe3O4 NPs (100 μg mL⁻¹) (A). The colonies of E. coli K-12 grown in the absence and presence of Fe3O4 NPs (100 μg mL⁻¹) (B). The colonies of E. coli pARG-25 grown in the absence and presence of Fe3O4 NPs (100 μg mL⁻¹) and Ag NPs (10 μg mL⁻¹) (C). The colonies of S. typhimurium MDC1759 cultivated in the absence and presence of Ag NPs (10 μg mL⁻¹) (D).
H⁺-coupled membrane transport has been determined in *E. coli* wild type, drug-resistant strains, and *S. typhimurium* in the absence and presence of citric acid stabilized Fe₃O₄ and Ag NPs (Table 1). Fe₃O₄ NPs suppressed energy-dependent H⁺-efflux in *E. coli* K-12 and pARG-25 strains ~3.0 to ~1.3-folds, respectively (see Table 1). H⁺-fluxes also decreased in the presence of DCCD, an inhibitor of the H⁺-translocating ATPases (see Table 1). The presence of Ag NPs in the assay medium led to the increase in H⁺ fluxes, in comparison with Fe₃O₄ NPs. A more noticeable effect was observed in *E. coli* K-12: H⁺-fluxes were increased ~1.2-fold. The effect of Ag NPs on *E. coli* pARG-25 was weaker in comparison with *E. coli* wild type strain (see Table 1). At the same time, in the presence of Ag NPs the H⁺-fluxes were increased even in the presence of DCCD, indicating that Ag NPs affected the bacterial membrane leading to changes in membrane structure and permeability. The same results were obtained with *E. coli* BW 251131. In case of *S. typhimurium* both NPs suppressed energy-dependent H⁺-efflux even by addition of DCCD (Table 1).

Table 1. The changes of H⁺-fluxes across the bacterial membranes of *E. coli* K-12, drug-resistant *E. coli* pARG-25 and *S. typhimurium* MDC1759 strains in the presence of citric acid coated Fe₃O₄ (100 μg mL⁻¹) stabilized by citric acid, and Ag NPs (10 μg mL⁻¹). *The bacteria were washed and transferred into Tris–phosphate buffer; bacterial cells were treated with NPs for 10 min. **The bacterial cells were treated with 0.2 mM DCCD for 10 min. ***P is difference between the values of experimental simples and appropriate control.*

| Bacteria and conditions* | H⁺-fluxes (mmol H⁺ min⁻¹ (10¹⁰ cells)⁻¹) | H⁺-fluxes+DCCD** (mmol H⁺ min⁻¹ (10¹⁰ cells)⁻¹) |
|--------------------------|----------------------------------------|-----------------------------------------------|
| *E. coli* K-12 (without NPs) | 2.50 ± 0.02 | 1.10 ± 0.02 |
| *E. coli* K-12 + Fe₃O₄ NPs | 0.75 ± 0.01 | 0.68 ± 0.01 |
| P<0.01                   | P<0.01 |
| *E. coli* K-12 + Ag NPs | 2.87 ± 0.02 | 1.25 ± 0.02 |
| P<0.05                   | P<0.05 |
| *E. coli* pARG-25 (without NPs) | 2.32 ± 0.02 | 1.05 ± 0.02 |
| *E. coli* pARG-25 + Fe₃O₄ NPs | 1.80 ± 0.02 | 0.98 ± 0.01 |
| P<0.01                   | P<0.01 |
| *E. coli* pARG-25 + Ag NPs | 2.55 ± 0.02 | 1.15 ± 0.01 |
| P<0.05                   | P<0.01 |
| *S. typhimurium* MDC1759 (without NPs) | 1.50 ± 0.02 | 0.83 ± 0.01 |
| *S. typhimurium* MDC1759 + Fe₃O₄ NPs | 1.16 ± 0.02 | 0.27 ± 0.02 |
| P<0.01                   | P<0.01 |
| *S. typhimurium* MDC1759 + Ag NPs | 1.32 ± 0.02 | 0.34 ± 0.01 |
| P<0.01                   | P<0.01 |

H⁺-fluxes through the bacterial membrane and H⁺-translocating ATPase activity in *E. coli* wild type and drug-resistant strains in the presence of Ag NPs and citric acid coated Fe₃O₄ NPs. In order to explain the possible mechanisms and find out probable targets of the effect of NPs changes in H⁺-fluxes through the bacterial membrane and H⁺-translocating F₀F₁-ATPase activity in *E. coli* wild type and drug-resistant strains were investigated.

The membrane-bound H⁺-translocating F₀F₁-ATPase activity of *E. coli* membrane vesicles was analyzed in the presence of citric acid coated Fe₃O₄ and Ag NPs to reveal their effects on the ATPase. In facultative anaerobic
bacteria, such as *E. coli*, the H⁺-translocating ATPase is reversible depending on bacterial growth conditions. During the preparation of membrane vesicles, spheroplasts from Gram-negative *E. coli* were obtained.

Fe₃O₄ NPs led to a ~ 1.3 to 1.5-fold increase in total FOF1-ATPase activity in membrane vesicles of *E. coli* K-12 and pARG-25, respectively (Fig. 3). DCCD-sensitive ATPase activity was increased ~ 1.2- and 1.5-folds in *E. coli* K-12 and pARG-25, respectively (see Fig. 3). In the case of Ag NPs, ATPase activity was not detected even in the presence of DCCD (no shown), which confirms the bactericidal effect of these NPs. Therefore, the effect of NPs on the ATPase can be responsible for the antibacterial effect.

NPs can directly affect the FOF1-ATPase, because ATPase activity was changed even in the absence of DCCD, an inhibitor of H⁺-translocating systems; or this effect can be intermediated by membrane-associated formate hydrogen lyase (FHL) complexes, which are responsible for H₂ production in *E. coli*. The bacterial membrane permeability can be changed during the bacterial growth in the presence of NPs.

The redox potential changes and H₂ production ability in *E. coli* wild type and drug-resistant strains in the presence of Ag NPs and citric acid coated Fe₃O₄ NPs. The redox potential (E₝) is an important factor, which characterizes the metabolic activity of bacteria under various growth conditions. To reveal the action mechanisms of Ag and Fe₃O₄ NPs on both *E. coli* strains, the kinetics of E₝ during the anaerobic growth has been studied. The anaerobic growth (6 h) of bacteria was accompanied by a drop in the value of E₝ from a positive value (+ 120 ± 10 mV) at the beginning of the growth lag phase to a negative value (∼ 580 ± 15 mV) for *E. coli* K-12 and (∼ 550 ± 10 mV) for *E. coli* pARG-25 (Fig. 4A). This decrease indicates an enhancement of reduction processes that characterizes bacterial metabolism under anaerobic conditions and the generation of H₂. The addition of NPs resulted in a delayed redox potential drop (Fig. 4A). In the presence of Fe₃O₄ NPs value of the E₝ decreased up to (∼ 540 ± 10 mV) for *E. coli* K-12 and (∼ 500 ± 10 mV) for *E. coli* pARG-25 (see Fig. 4A). Ag NPs exhibited more pronounced effect: in the presence of 10 μg mL⁻¹ Ag NPs, the E₝
values of *E. coli* K-12 and pARG-25 cells were decreased up to (~410 ± 5 mV) and (~400 ± 5 mV), respectively (see Fig. 4A). The inhibition of bacterial growth in the presence of NPs can be coupled with *E. coli* or with a direct effect of NPs on the bacterial membrane.

A correlation between the decrease of *Eh* and production of *H2* is shown for *E. coli*, which can generate *H2* under the action of FHL complexes23,23. During glucose fermentation, *H2* production was determined during 6 h anaerobic growth in both strains: 3.62 and 2.20 mmol *H2* L⁻¹ in *E. coli* K-12 and pARG-25 cells, respectively (Fig. 4B). The *H2* production by *E. coli* coupled with the activity of membrane-associated *H*-producing enzymes—hydrogenases, which are involved in *H2* metabolism in *E. coli*21,23,23. Ag and Fe3O4 NPs inhibited *H2* yield in both *E. coli* strains (see Fig. 4B). *H2* production by both strains in the presence of 100 μg mL⁻¹ citric acid coated Fe3O4 NPs was ~1.5 to 1.7-fold lower in comparison with control cells (see Fig. 4B). Citric acid coated Fe3O4 NPs demonstrated more noticeable effect on *H2* yield than NPs stabilized by oleic acid20,21. In the presence of 100 μg mL⁻¹ oleic acid coated NPs *H2* yield in *E. coli* pARG-25 was decreased 1.2-fold in comparison with control, and was not changed significantly in *E. coli* BW2511320,21. In the presence of Ag NPs *H2* yield in *E. coli* K-12 was ~5.0-fold lower than *H2* yield in control cells (see Fig. 4B).

The results point out that NPs show antibacterial effect in both bacteria in concentration-dependent manner by changing membrane-bound enzyme activity.

**Discussion**

Nowadays, due to the development of drug-resistant bacteria, the search of new more effective antibacterial agents required. Various NPs are suggested as novel antimicrobial agents against different pathogens owing to their unique physicochemical properties1,4–7. As it was shown in our previous work, oleic acid coated Fe3O4 NPs display different effect on the growth properties and membrane activity of Gram-negative *Escherichia coli* BW 25113 and Gram-positive *Enterococcus hirae* ATCC 979021. Iron oxide NPs demonstrate better antibacterial effect on Gram-negative, than on Gram-positive bacteria21. This effect can be coupled with the components of cell wall of Gram-positive and Gram-negative bacteria. Gram-positive bacteria have a thick peptidoglycan layer, teichoic acids and pores, which allow penetration of external molecules, including NPs7. In contrast, the cell wall of Gram-negative bacteria has a thin peptidoglycan layer between the cytoplasmic membrane and the outer membrane, which forms a penetration barrier for external molecules.

Stabilizer such as oleic acid can be used not only to prevent aggregation of magnetic NPs, but also for protection of NPs22–24. Citric acid also can be used to stabilize the magnetic NPs for biomedical application25–27.

In this study, antimicrobial effects and possible mechanisms of Fe3O4 NPs (coated by citric acid) and Ag NPs on *E. coli* K-12 wild type and pARG-25 kanamycin-resistant strains have been investigated. Citric acid coated Fe3O4 NPs exhibit significant antibacterial activity against *E. coli* both strains, but kanamycin-resistant *E. coli* pARG-25 strain is more susceptible to Fe3O4 NPs than wild type strain. Moreover, the effect of Fe3O4 NPs depended of stabilizer type. Citric acid coated Fe3O4 NPs demonstrated more noticeable action against drug-resistant bacteria than oleic acid stabilized NPs26. In the presence of 250 μg mL⁻¹ citric acid coated Fe3O4 NPs a ~3fold inhibition of bacterial growth was observed, whereas the same concentration of oleic acid coated NPs suppressed growth rate ~2-fold20.

Ag NPs exhibit a more pronounced bactericidal effect in comparison with Fe3O4 NPs. Moreover, Ag NPs have a more expressed antibacterial effect at low concentrations. The low concentration effect of Ag NPs has been reported by various researchers10–12,34. There were no obvious differences in bactericidal activity between *E. coli* wild type and drug-resistant strains, confirming that Ag NPs have a broad spectrum of action. Ag NPs are assumed to affect not only the growth of both *E. coli* strains, but also the energy-dependent *H*⁺-coupled membrane transport and ATPase activity in bacteria. The increase of *H*⁺-fluxes in the presence of DCCD indicated the significant effect of Ag NPs on the bacterial membrane structure and permeability. Probably Ag NPs change the permeability of the bacterial membrane and inhibit the cell respiration by penetrating via cell wall9,10,14. These studies confirm that the Fe3F3-ATPase, which plays a crucial role in bacterial metabolism, can be a sensitive target for metals NPs action. The effect of NPs used on the Fe3F3-ATPase can be responsible for the antibacterial effect, and this ATPase can be a target for NPs. Similar data were obtained in mammalian cells, where Ag NPs inhibited mitochondrial ATPase activity of rat liver cells15.

Different effects of Fe3O4 and Ag NPs on *E. coli* ATPase activity may be coupled to the differences in the interaction of NPs with bacterial cell wall. The antimicrobial activity of metal NPs is a result of NPs interaction with bacterial membranes and their penetration into the bacterial cell, causing membrane damage and bacterial death25–27.

Thus, metal NPs studied exhibited antibacterial activity against bacteria, including drug-resistant strains, and they can be applied in biomedicine for the treatment of various infections and in biotechnology and the food industry for controlling bacterial growth.

**Materials and methods**

**Bacterial strains, cultivation conditions and growth determination.** This study was performed with *S. typhimurium* MDC1759, *E. coli* K-12 wild type and kanamycin-resistant pARG-25 strains (Microbial Depository Center, National Academy of Science, Yerevan, Armenia). Bacteria grown in the presence of kanamycin (50 μg mL⁻¹) were used as positive control. The negative controls are the strains, cultivated without antibiotic. These strains were cultivated in peptone medium at 37 °C and pH 7.520,21. Anaerobic conditions, favorable for intestine microorganisms, including pathogenic ones, were maintained20. For creation of anaerobic
conditions O₂ was bubbled out from media by autoclaving at 120 °C for 20 min, and then bottles were closed by press caps. To reach anaerobic conditions the bottles were kept sealed to maintain anoxic conditions and all experiments were performed under strict absence of O₂.

The growth of bacteria was determined by measuring the optical density at 600 nm using Spectro UV–Vis Auto spectrophotometer (Labomed, Los Angeles, USA). The concentration of initial inoculum was 10⁸ of number of colony forming units (CFU) mL⁻¹. Specific growth rate was determined as the quotient of ln2 division on doubling time of absorbance over the interval, when the logarithm of absorbance of the culture at 600 nm increased with time linearly (logarithmic growth phase), and it was expressed as h⁻¹ as described. Colloidal Ag NPs ("Silverton", "Tonus-Less", Armenia) in the concentration range from 5 to 30 μg mL⁻¹ and Fe₃O₄ NPs (stabilized by citric acid) from 50 to 250 μg mL⁻¹ were added to growth medium with inoculum.

Figure 5. TEM image (A) and atomic force microscopy of Ag NPs using appropriate microscope (B). Ag NPs distribution in the colloid solution depending of their size (C). For details, see "Materials and methods".
Characterization of Ag and Fe₃O₄ nanoparticles. The structure, form and size of Ag NPs (synthesized by electrochemical method²⁶) were investigated using TEM image and atomic force microscopy method by appropriate microscope (NT-MDT Nanoeducator 2, Russia). The data showed that Ag NPs have spherical form with average size of ~ 30 nm (Fig. 5).

Fe₃O₄ NPs coated with citric were synthesized by co-precipitation method²⁰,²⁷. Fe₃O₄ NPs have round form and average size of 10 nm (Fig. 6). The synthesized Fe₃O₄ NPs were characterized also by X-ray powder diffraction (XRD) to determine the sample phases and average particle size of the dried powder. The XRD pattern of the sample was recorded on diffractometer system EMPYREAN using CuKα (λ = 1.5406 Å) radiation at room temperature in the range of 10° to 120° in the 2θ scale, Generator Settings 40 mA, 45 kV. The XRD patterns of the dried sample of Fe₃O₄ NPs and diffraction peaks parameters are shown in Fig. 6 and Table 2. XRD data can be used to distinguish the crystallinity and the average size of nanoparticles. The strongest reflection from the (311) diffraction peak indicate of a cubic spinel structure. The reflection from other planes (022), (040), (151) and (044) also correspond with a cubic unit cell²⁸.

### Table 2. Peak list of Fe₃O₄ NPs. All the peaks of XRD patterns were analyzed and indexed using ICDD data base. It can be noticed from XRD pattern and Table 2 that the 2θ position, d-spacing and intensity of the diffraction peaks are in good agreement with the standard pattern for Ref. Code 96-900-5841.

| Pos (°2θ) | Height (cts) | FWHM left (°2θ) | d-spacing (Å) | Rel. Int (%) | Tip width | Matched by  |
|-----------|--------------|-----------------|---------------|--------------|-----------|-------------|
| 30.2360   | 123.24       | 0.5117          | 2.95996       | 47.15        | 0.6140    | Fe₂₄O₃₂   |
| 35.5989   | 261.39       | 0.3582          | 2.52199       | 100.00       | 0.4298    | Fe₂₄O₃₂   |
| 43.2865   | 68.51        | 0.4093          | 2.09024       | 26.21        | 0.4912    | Fe₂₄O₃₂   |
| 57.2379   | 63.41        | 0.7364          | 1.60953       | 24.26        | 0.8596    | Fe₂₄O₃₂   |
| 62.8740   | 79.50        | 0.5117          | 1.47813       | 30.41        | 0.6140    | Fe₂₄O₃₂   |

Figure 6. TEM image of synthesized Fe₃O₄ NP (A). The sizes of some nanoparticles indicated immediately in the figure. XRD pattern of the Fe₃O₄ NPs (B).
Testing of bacterial sensitivity to NPs. To determine the sensitivity of E. coli strains to used NPs, bacteria were grown in the presence of Fe₃O₄ NPs (100 μg mL⁻¹) and Ag NPs (10 μg mL⁻¹), after which various dilutions (10⁻¹⁻¹⁰ folds) of bacterial suspension were applied. Then 100 μL of each sample was transferred on nutrient agar plates, which were incubated at 37 °C. Nutrient agar plates contained peptone media with 1.5% bacteriological agar. The numbers of bacterial viable colonies were counted after 24 h of incubation for determination of CFUs presented in each sample by: \( T = n \times 10^m \), where \( n \) is the number of bacterial viable colonies, \( m \) is the number of dilution. NPs free plates incubated under the same conditions were used as control.

Determination of H⁺-fluxes through bacterial membrane. The H⁺-flux through the bacterial membrane was determined using appropriate selective electrode (HJ1131B, HANNA Instruments, Portugal), as described. Bacteria were cultivated in the presence of Fe₃O₄ NPs (100 μg mL⁻¹) or Ag NPs (10 μg mL⁻¹). Bacterial cells were transferred into the 150 mM Tris–phosphate buffer (pH 7.5), and then energy source—glucose (11 mM) was added. The H⁺-flux was expressed as a change in the ion external activity in mmol H⁺ per min per 10⁹ cells. The bacterial cultures were incubated with 0.2 mM N,N’-dicycleyxylcarbodiimide (DCCD), an inhibitor of the F₉F₁-ATPase, for 10 min.

ATPase activity assay. Bacterial membrane vesicles were obtained by the Kaback method, as described. ATPase activity was determined by amount of inorganic phosphate (Pi), liberated after adding 3 mM ATP to membrane vesicles, as measured by the Tauskki and Shorr method, as described. ATPase activity was expressed in nmol Pi per µg protein per min. Bacteria were cultivated in the presence of Fe₃O₄ NPs (100 μg mL⁻¹) or Ag NPs (10 μg mL⁻¹). For DCCD studies, membrane vesicles were incubated with 0.2 mM DCCD for 10 min.

The medium pH, redox potential and H₂ yield determinations. The pH of the medium was measured during bacterial growth at certain time intervals (from 0 to 6 h) by a pH-meter (HANNA Instruments, Portugal) with pH-selective electrode (HJ1131B), as described. The initial pH was adjusted at 7.5 ± 0.1 by 0.1 M NaOH or 0.1 M HCl. The medium redox potential (\( E_h \)) was measured during bacterial growth using a pair of redox [platinum (Pt) and titanium–silicate (Ti–Si)] electrodes, as described. \( E_h \) kinetics determined using the pair of redox electrodes during culture growth gives information about main redox processes and also H₂ evaluation. The H₂ yield in E. coli, cultivated in the presence of Fe₃O₄ NPs (100 μg mL⁻¹) or Ag NPs (10 μg mL⁻¹), was calculated by the decrease of \( E_h \) to low negative values during bacterial growth, as described, and expressed in mmol H₂ per L.

Reagents, data processing and others. Yeast extract, peptone, Tris (aminomethane), Agar–Agar Kobe from Carl Roth GmbH (Germany); glucose, DCCD from Sigma Aldrich (USA), and other reagents of analytical grade were used. The average data are presented from 3 independent experiments; error bars are presented on figures. The validity of the differences between different series of experiments was evaluated by Student criteria (P)²⁰.²¹

Received: 9 February 2020; Accepted: 24 July 2020
Published online: 04 August 2020

References
1. Trchounian, A., Gabrielyan, L. & Mnatsakanyan, N. Nanoparticles of various transition metals and their applications as antimicrobial agents. In Metal Nanoparticles: Properties, Synthesis and Applications (eds Saylor, Y. & Irby, V.) 161–211 (Nova Science Publishers, New York, 2018).
2. Wang, L., Hu, Ch. & Shao, L. The antimicrobial activity of nanoparticles: Present situation and prospects for the future. Int. J. Nanomed. 12, 1227–1249. https://doi.org/10.2147/IJN.S121956 (2017).
3. Raghunath, A. & Perumal, E. Metal oxide nanoparticles as antimicrobial agents: A promise for the future. Int. J. Antimicrob. Agents 49, 137–152. https://doi.org/10.1016/j.ijantimicag.2016.11.011 (2017).
4. Arias, L. S. et al. Iron oxide nanoparticles for biomedical applications: A perspective on synthesis, drugs, antimicrobial activity, and toxicity. Antibiotics 7, 46. https://doi.org/10.3390/antibiotics7020046 (2018).
5. Lee, S. H. & Jun, B.-H. Silver nanoparticles: Synthesis and application for nanomedicine. Int. J. Mol. Sci. 20, 865. https://doi.org/10.3390/ijms20040865 (2019).
6. Burdușel, A. C. et al. Biomedical applications of silver nanoparticles: An up-to-date overview. Nanomaterials 8, 681. https://doi.org/10.3390/nano8090681 (2018).
7. Gabrielyan, L. & Trchounian, A. Antibacterial activities of transient metals nanoparticles and membranous mechanisms of action. World J. Microbiol. Biotechnol. 35, 162. https://doi.org/10.1007/s11274-019-2742-6 (2019).
8. Khatoon, N. et al. Ampicillin silver nanoformulations against multidrug resistant bacteria. Sci. Rep. 9, 6848. https://doi.org/10.1038/s41598-019-43309-0 (2019).
9. Park, S. B. et al. Silver-coated magnetic nanocomposites induce growth inhibition and protein changes in foodborne bacteria. Sci. Rep. 9, 17499. https://doi.org/10.1038/s41598-019-53080-x (2019).
10. Franci, G. et al. Silver nanoparticles as potential antibacterial agents. Molecules 20, 8856–8874. https://doi.org/10.3390/molecules20380856 (2015).
11. Vardanyan, Z., Gevorkyan, V., Ananyan, M., Vardapetyan, H. & Trchounian, A. Effects of various heavy metal nanoparticles on Enterococcus hirae and Escherichia coli growth and proton-coupled membrane transport. J. Nanobiotechnol. 13, 69. https://doi.org/10.1186/s12951-015-0131-3 (2015).
12. Gurunathan, S., Han, J. W., Kwon, D. N. & Kim, J. H. Enhanced antibacterial and anti-biofilm activities of silver nanoparticles against Gram-negative and Gram-positive bacteria. Nanose. Res. Lett. 9, 373. https://doi.org/10.1186/1556-276X-9-373 (2014).
13. Hong, X., Wen, J., Xiong, X. & Hu, Y. Shape effect on the antibacterial activity of silver nanoparticles synthesized via a microwave-assisted method. Environ. Sci. Pollut. Res. Int. 23, 4489–4497. https://doi.org/10.1007/s11356-015-5668-z (2016).
14. Pal, S., Tak, Y. K. & Song, J. M. Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium Escherichia coli. Appl. Environ. Microbiol. 73, 1712–1720 (2007).

15. Ayala-Núñez, N. V., Lara Villegas, H. H., del Carmen Ixtepan Turrent, L. & Rodriguez Padilla, C. Silver nanoparticles toxicity and bactericidal effect against methicillin-resistant Staphylococcus aureus: Nanoscale does matter. NanoBiotechnology 5, 2. https://doi.org/10.1007/s12030-009-9029-1 (2009).

16. Rai, M., Yadav, A. & Gade, A. Silver nanoparticles as a new generation of antimicrobials. Biotechnol. Adv. 27, 76–83. https://doi.org/10.1016/j.biotechadv.2008.09.002 (2009).

17. Chen, Y. & Chen, B. A. Application and development of magnetic iron oxide nanoparticles in tumor targeted therapy. Chin. J. Cancer 29, 118–122 (2010).

18. Mody, V. V. et al. Magnetic nanoparticle drug delivery systems for targeting tumor. Appl. Nanosci. 4, 385–392 (2014).

19. Chatterjee, S., Sanyopadhyay, A. & Sarkar, K. Effect of iron oxide and gold nanoparticles on bacterial growth leading towards biological application. J. Nanobiotechnol. 9, 34. https://doi.org/10.1186/s11680-011-0055-4 (2011).

20. Gabrielyan, L., Hovhannisyan, A. & Trchounian, A. Effects of iron oxide (Fe₃O₄) nanoparticles on Escherichia coli antibiotic-resistant strains. J. Appl. Microbiol. 126, 1108–1116. https://doi.org/10.1111/jam.14214 (2019).

21. Gabrielyan, L., Hovhannisyan, A., Gevorgyan, V., Ananyan, M. & Trchounian, A. Antibacterial effects of iron oxide (Fe₃O₄) nanoparticles: Distinguishing concentration-dependent effects with different bacterial cells growth and membrane-associated mechanisms. Appl. Microbiol. Biotechnol. 103, 2773–2782. https://doi.org/10.1007/s00253-019-09653-x (2019).

22. Lai, C. W., Low, F. W., Ta, M. F. & Hamid, Sh. D. A. Iron oxide nanoparticles decorated oleic acid for high colloidal stability. Adv. Polym. Technol. 37, 1712–1721. https://doi.org/10.1002/adp2.21829 (2018).

23. Patil, R. M. et al. Non- aqueous to aqueous phase transfer of oleic acid coated iron oxide nanoparticles for hyperthermia application. RSC Adv. 4, 4515–4522 (2014).

24. Gelbrich, T., Feyen, M. & Schmidt, A. M. Magnetic thermoresponsive core-shell nanoparticles. Macromolecules 39, 3469–3472. https://doi.org/10.1021/ma060060u (2006).

25. Răcuciu, M., Creangă, D. E. & Airinei, A. Citric-acid–coated magnetite nanoparticles for biological applications. Eur. Phys. J. https://doi.org/10.1140/epje/i2006-10051-y (2006).

26. Li, L. et al. Effect of synthesis conditions on the properties of citric-acid coated iron oxide nanoparticles. Microelectr. Eng. 110, 329–334. https://doi.org/10.1016/j.mee.2013.02.045 (2013).

27. Yokoyama, S., Suzuki, I., Motomiya, K., Takahashi, H. & Tohji, K. Aqueous electrophotothermal deposition of citric-acid-stabilized copper nanoparticles. Coll. Surf. A Physicochem. Eng. Aspects 545, 93–100 (2018).

28. Clements, A., Young, J. C., Constantinou, N. & Frankel, G. Infection strategies of enteric pathogenic Escherichia coli. Gut Microbes 3, 71–87. https://doi.org/10.4161/gmic.19182 (2012).

29. Foulquié Moreno, M. R., Saranimalopoulos, P., Tsakalidou, E. & De Vuyst, L. The role and application of enterococci in food and health. Int. J. Food Microbiol. 108, 1–24 (2006).

30. Akbar, A. et al. Synthesis and antimicrobial activity of zinc oxide nanoparticles against foodborne pathogens Salmonella typhimurium and Staphylococcus aureus. Biocatal. Agric. Biotechnol. 17, 36–42 (2019).

31. Espinosa-Cristóbal, L. F. et al. Bovine serum albumin and chitosan coated silver nanoparticles and its antimicrobial activity against oral and esophageal bacteria. J. Nanomater. https://doi.org/10.1155/2015/420853 (2015).

32. Blubulyan, S. & Trchounian, A. Impact of membrane-associated hydrogenases on the F₄,F₄-ATPase in Escherichia coli during glycerol and mixed carbon fermentation: ATPase activity and its inhibition by N, N′-dicyclohexylcarbodiimide in the mutants lacking hydrogenases. Arch. Biochem. Biophys. 579, 67–72. https://doi.org/10.1016/j.abb.2015.05.015 (2015).

33. Trchounian, K., Poladyan, A. & Trchounian, A. Enhancement of Escherichia coli bacterial biomass and hydrogen production by some heavy metal ions and their mixtures during glycerol vs glucose fermentation at a relatively wide range of pH. Int. J. Hydrogen Energy 42, 6596–6597. https://doi.org/10.1016/j.jhydene.2017.02.003 (2017).

34. Kim, J. S. et al. Antimicrobial effects of silver nanoparticles. Nanomedicine 3, 95–101. https://doi.org/10.1016/j.nano.2006.12.001 (2007).

35. Chichova, M. et al. Influence of silver nanoparticles on the activity of rat liver mitochondrial ATPase. J. Nanopart. Res. 16, 2243. https://doi.org/10.1007/s11051-014-2243-3 (2014).

36. Khaydarov, R. A., Khaydarov, R. R., Gapurova, O., Estrin, Y. & Scheper, Th. Electrochemical method for the synthesis of silver nanoparticles. J. Nanopart. Res. 11, 1193–1200. https://doi.org/10.1007/s11051-008-9513-x (2009).

37. Keikutia, Sh. et al. A new method for the synthesis of nanoparticles for biomedical applications. Eur. Chem. Bull. 4, 33–36 (2015).

38. Vaidyanathan, G., Sendhilnathan, S. & Arulmurugan, R. Structural and magnetic properties of Co₁− xZnxFe₂O₄ nanoparticles by co-precipitation method. J. Magnet. Magn. Materials 313, 293–299 (2007).

Acknowledgements
The authors thank Narine Mnatsakanyan (“Tonus-Leś’, Armenia) for supplying Ag NPs. This work was supported by Basic support from Russian-Armenian University and Committee of Science, Ministry of Education, Science, Culture and Sport of Armenia.

Author contributions
L.G. performed the main part of experimental work and data processing; H.B. provided atomic force microscopy of NPs; V.G. supplied Fe₃O₄ NPs and provided XRD characterization; L.G. and A.T. wrote the manuscript; A.T. supervised and coordinated the research, designed the study, edited the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Correspondence
Request for materials should be addressed to A.T.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
