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Antibacterial activity of nitric oxide releasing silver nanoparticles

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Abstract. Silver nanoparticles (AgNPs) are well known potent antimicrobial agents. Similarly, the free radical nitric oxide (NO) has important antibacterial activity, and due to its instability, the combination of NO and nanomaterials has been applied in several biomedical applications. The aim of this work was to synthesize, characterize and evaluate the antibacterial activity of a new NO-releasing AgNPs. Herein, AgNPs were synthesized by the reduction of silver ions (Ag^+) by catechin, a natural polyphenol and potent antioxidant agent, derived from green tea extract. Catechin acts as a reducing agent and as a capping molecule on the surface of AgNPs, minimizing particle agglomeration. The as-synthesized nanoparticles were characterized by different techniques. The results showed the formation of AgNPs with average hydrodynamic size of 44 nm, polydispersity index of 0.21, and zeta potential of -35.9 mV. X-ray diffraction and Fourier transform infrared spectroscopy revealed the presence of the AgNP core and cathecin as capping agent. The low molecular weight mercaptosuccinic acid (MSA), which contain free thiol group, was added on the surface of catechin-AgNPs, leading to the formation of MSA-catechin-AgNPs (the NO precursor nanoparticle). Free thiol groups of MSA-catechin-AgNPs were nitrosated leading to the formation of S-nitroso-mercaptosuccinic acid (S-nitroso-MSA), the NO donor. The amount of 342 ± 16 μmol of NO was released per gram of S-nitroso-MSA-catechin-AgNPs. The antibacterial activities of catechin-AgNPs, MSA-catechin-AgNPs, and S-nitroso-MSA-catechin-AgNPs were evaluated towards different resistant bacterial strains. The results demonstrated an enhanced antibacterial activity of the NO-releasing AgNP. For instance, the minimal inhibitory concentration values for *Pseudomonas aeruginosa* (ATCC 27853) incubated with AgNPs-catechin, AgNPs-catechin-MSA, and AgNPs-catechin-S-nitroso-MSA were found to be 62, 125 and 3 µg/mL, respectively. While in the case of *Klebsiella pneumoniae* (ATCC 700603) the minimum bactericidal concentration values for treatments with AgNPs-catechin, AgNPs-catechin-MSA, and AgNPs-catechin-S-nitroso-MSA were found to be 1000, 500, and 125 µg/mL, respectively. The antibacterial

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actions of the NO-releasing nanoparticle were superior in comparison with the antibacterial effects of AgNPs, in most of the tested antibiotic resistant bacteria strains. These results highlight the promising uses of NO-releasing AgNPs against resistant bacteria in several biomedical applications.

1. Introduction

Silver nanoparticles (AgNPs) have been attracted the attention of the scientific community in the last decades due to their significant antimicrobial properties [1-3]. Traditional methods for the synthesis of AgNPs are based on the chemical route, which uses strong reducing agents, such as sodium borohydride [4,5]. Although chemical synthesis of AgNPs has a consider control over nanoparticle size distribution, this route involves the presence of toxic chemicals, yielding hazardous byproducts, leading to the environment contamination. In addition, traditional chemical methods are significant expensive since they demand high-energy input and manufacturing [6,7]. In contrast, "green chemistry" has been considered an interesting approach to overcome the main limitations to synthesize several classes of metallic nanoparticles, including AgNPs [8,9].Biogenic synthesis of metallic nanoparticles, including AgNPs, are considered clean, cost and eco effective route and non-toxic to the environment [6-9]. The biogenic (or green synthesis) of AgNPs can be performed at room temperature and at ambient conditions.

The use of plant extract and/or compounds derived from plant extracts to obtain metallic nanoparticles have been gained considerable attention in recent years [10,11]. Catechin is a natural polyphenol and potent antioxidant molecule, which belongs to the group of flavanols, and it is the main constituent of the green tea extract [12]. In biogenic synthesis of AgNPs, catechin acts not only as powerful reducing agent of Ag^+ to Ag° (leading to the formation of AgNPs), but also as a capping agent, stabilizing the obtained nanoparticles. In this work, AgNPs were synthesized by green chemistry by the action of catechin, leading to the formation of catechin-AgNPs. The obtained nanoparticles were characterized by different techniques. The results demonstrated the successfully formation of AgNPs. The obtained nanoparticles were stabilized by the presence of catechin as capping agent.

In a further step, mercaptosuccinic acid (MSA), a low molecular weight thiol (SH) containing molecule was conjugated on the surface of catechin-AgNPs, leading to the formation of MSA-catechin-AgNPs. MSA was maintained on the surface of catechin-AgNPs by positive electrostatic interactions. The amount of free thiol groups on the surface of MSA-catechin-AgNPs was evaluated. It should be noted that the presence of free thiol groups on the surface of a nanoparticle represents a site for nanoparticle conjugation with important biological molecules [13].

Free thiol groups on the surface of MSA-catechin-AgNPs were nitrosated leading to the formation of S-nitroso-MSA-catechin-AgNPs, which act as spontaneous nitric oxide (NO)-releasing nanoparticle. The aim of this work was to synthesize, characterize and evaluate the antibacterial activity of a new NO-releasing AgNPs. NO is an endogenous found free radical that plays several physiological and pathophysiological roles, such as the cell defense against microbes [14]. The amount of NO release from S-nitroso-MSA-catechin-AgNPs was determined. The combination of NO and AgNPs might find important applications in the combat of resistant bacteria. In this direction, the antibacterial activities of S-nitroso-MSA-catechin-AgNPs, MSA-catechin-AgNPs and catechin-AgNPs were demonstrated towards different bacterial strains (*Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 29213), *Klebsiella pneumoniae* (ATCC 700603), *Salmonella enterica* (ATCC 14028), and *Escherichia coli* (ATCC 35218)). The Minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were obtained. All tested nanoparticles demonstrated antibacterial effects, and in most of the cases, the NO-releasing AgNPs (S-nitroso-MSA-catechin-AgNPs) demonstrated superior antibacterial effects, compared to the other groups.

Therefore, the results demonstrated the successful biogenic synthesis of AgNPs by the polyphenol catechin, the nanoparticle functionalization with NO group and the antibacterial activities of the nanoparticles. To our best knowledge, this is the first report to demonstrate the biogenic synthesis of NO-releasing AgNPs and their antibacterial effects. More studies are required for further characterization of the antimicrobial activities of the NO-releasing biogenic AgNPs.

2. Methods

2.1. Synthesis of AgNPs by catechin

A volume of 0.5 mL of aqueous solution of catechin (0.1 mol/L) was deposited in the Erlenmeyer, followed by the addition of 96.5 mL of deionized water. The final suspension was stirred at 80°C. Then, 3 mL of a stock solution (0.1 mol/L) of $AgNO_3$ were added to obtain a final concentration of 3 mmol/L and the pH was adjusted to 10 with NaOH. Aliquots of 2 mL were removed from the Erlenmeyr flaks after 4 h of reaction and their UV-visible spectra were obtained using a spectrophotometer (Genesys 10S) at the resolution of 1 nm from 200 to 800 nm for each sample. The final mixture was further stirred for 1 h, centrifuged and washed several times with water, followed by freezer-dryer. This procedure led to the formation of catechin-AgNPs.

2.2. Caracterization of catechin-AgNPs

Catechin-AgNPs were characterized by through Fourier transformed infrared (FTIR) spectroscopy (CARY 630 FTIR Agilent Technologies) in the range 450-4000 cm^{-1} at a resolution of 4 cm^{-1}. X-ray diffraction (XRD) measurements were performed in reflection set-up, with a conventional X-ray generator, CuKα radiation of 1.5418 Å coupled to a scintillation detector. The morphology the nanoparticles was determined by Field-emission scanning electron microscopy (FEI Quanta FEG250, STEM) at 30 kV. The nanoparticle size distribution was estimated by using the software SigmaScan Pro 5.0. The hydrodynamic diameter and zeta potential were measured at 25°C by dynamic light scattering (DLS) using the Zetasizer Nano ZS90 System (Malvern Instruments, Malvern, UK). Prior to the DLS measurement, the aqueous suspensions of nanoparticles were passed through a 0.22 µm polyvinylidene fluoride (PVDF) membrane.

2.3. Functionalization of catechin-AgNPs with MSA leading to MSA-catechin-AgNPs

Catechin-AgNPs (20 mg) were suspended in 10 mL of deionized water in an ultrasound bath for 10 mim at room temperature. MSA (200 mg) is dissolved in 10 mL of deionized water. MSA solution was added to catechin-AgNPs suspension, and the final suspension was stirred for 14 h at room temperature. The MSA-catechin-AgNPs obtained were isolated by centrifugation, washed and dried.

2.4. Quantification of free thiol groups (SH) on the surface of MSA-catechin-AgNPs

The quantification of free thiol groups on the surface of MSA-catechin-AgNPs was performed by titration of SH presented in MSA with a thiol reagent 5,50-dithiobis-(2-nitrobenzoic acid) (DTNB). [13]. This quantification is based on the detection of the absorbance at 412 nm, which corresponds to the 2-nitro-5-thiobenzoate anion (TNB^{2-}) generated in the reaction of SH groups with DTNB. Appropriate amounts of thiolated nanoparticles were added to 3.0 mL of 0.01 mol L^{-1} DTNB in PBS buffer (pH 7.4) containing 1 mmol L^{-1} of ethylenediaminetetraacetic acid. After 5 min of incubation, the suspensions were filtered by centrifugal ultrafiltration using a Microcon centrifugal filter device containing ultrafiltration membranes (MWCO 10-kDa cutoff filter, Millipore). The supernatant was placed into a quartz cuvette, and the intensity of the absorption band at 412 nm was measured in an UV–vis spectrophotometer (Agilent 8453). The experiments were performed in triplicate.

2.5 *Nitrosation of MSA-catechin-AgNPs leading to the formation of S-nitroso-MSA-catechin-AgNPs*

MSA-catechin-AgNPs (10 mg) were dispersed in 1 mL of deionized water by using an ultrasound bath, and the pH of the suspension was adjusted to 4.0. Aliquot of 200 µL of sodium nitrite ($NaNO_2$), 60 mmol/L, was added to the MSA-catechin-AgNPs suspension under stirring for 30 min. The obtained S-nitroso-MSA-catechin-AgNPs were immediately used.

2.6. Quantification of NO release from S-nitroso-MSA-catechin-AgNPs
The amount of NO released from S-nitroso-MSA-catechin-AgNPs was measured by a NO electrode (2.0 mm ISO-NOP) connected to a TBR4100/1025 Free Radical Analyzer (World Precision Instruments). Aliquots of 100 µL of aqueous suspension of S-nitroso-MSA-catechin-AgNPs (5. mg/mL) were added to the sampling compartment, which contained 10 mL of 10 mL copper(II) chloride ($CuCl_2$) (0.1 mol L^{-1}). This condition allowed for the detection of free NO released from the NPs. The experiments were performed in duplicate with the standard error of the mean. Calibration curves were obtained with aqueous solutions of freshly prepared S-nitrosoglutathione (1–500 µmol L^{-1}) (data not shown).

2.7. Antibacterial activities of the synthesized NPs
The antibacterial activities of catechin-AgNPs, MSA-catechin-AgNPs and S-nitroso-MSA-catechin-AgNPs, at different concentrations, were evaluated against *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 29213), *Klebsiella pneumoniae* (ATCC 700603), *Salmonella enterica* (ATCC 14028), and *Escherichia coli* (ATCC 35218), all samples kindly provided by Oswaldo Cruz Foundation (Fiocruz, Rio de Janeiro, Brazil). Bacterial strains were incubated with the different concentrations of nanoparticles for 24 h. Minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were obtained, using micro-dilution assays in 96- well plates, as previous described [15], and according to the Clinical and Laboratory Standards Institute (CLSI) [16].

3. Results and Discussion
The main objective of this study was to to synthesize, characterize and evaluate the antibacterial activity of a new NO-releasing AgNPs. To this end, AgNPs were synthesized by catechin, in a green synthetic route, followed by the coating of the obtained nanoparticles with S-nitroso-MSA, as a spontaneous NO releasing molecule. It is expected a superior antimicrobial activity of the NO-releasing-AgNPs. The following sections describe the obtained results.

3.1. Synthesis and characterization of catechin-AgNPs
Uv-visible spectrophotometry is a simple and direct method to confirm the formation of AgNPs from $AgNO_3$ [17]. The reduction of Ag^+ to Ag° occurs immediately upon the pH adjustment, which is accompanied by a change in the suspension color from pale yellow to brown, indicating the formation of AgNPs. Figure 1A shows the Uv-visible spectra for catechin-AgNPs at different pHs.

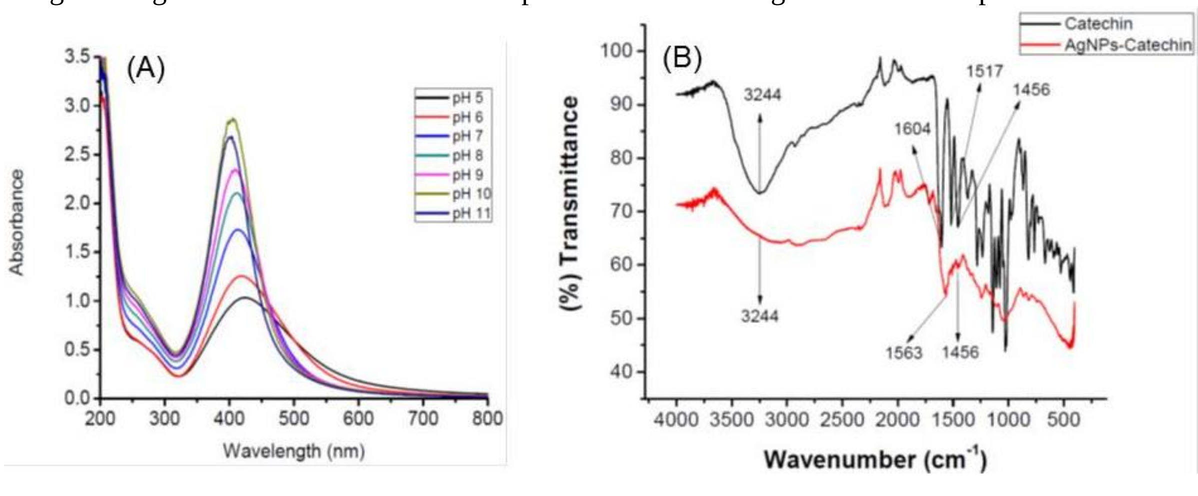

Figure 1. Plasmonic absorbantion bands of catechin-AgNPs at different pHs (A). FTIR spectra of catechin and catechin-AgNPs (B).

As can be observed, with the increase of the pH, the intensity of the plasmonic band increases, band becomes narrower, and the bands shift to lower wavelength values [20]. These peaks are due to the plasmonic band of $AgNO_3$ [18,19].

Figure 1B shows the FTIR spectra of pure catechin and catechin-AgNPs, as indicated in the Figure. Band at 3244 cm^{-1} is associated with O-H stretching vibrations of the phenolic group of catechin. Vibrations at 1604 cm^{-1} corresponds to the stretching vibration of C=C present in aromatic and aliphatic compounds, while vibrations at 1517 cm^{-1} corresponds to the vibrations of C-O of esters, ethers, and phenols, and vibrations at 1456 cm^{-1} correspond to the C-O of ethers [21,22]. Upon catechin conjugation with AgNPs, there is a decrease in the intensity of the band at 3244 cm^{-1}, which corresponds to the decrease in the OH. This can be explained since OH groups participate in the reduction of Ag^+ to Ag°. Bands at 1563 and 1456 cm^{-1} correspond to aromatic C=C in the modified catechin.

Figure 2 shows the XRD pattern of catechin-AgNPs. Peak values of around 38.10°, 44.47°, 64.63°, 77.44°, and 81.33° correspond to the XRD pattern of indexed [111], [200], [220], [311], and [222] facets of Ag° NPs [2]. These results confirm the reduction of Ag^+ to Ag° by catechin, which acts as reducing and capping agent.

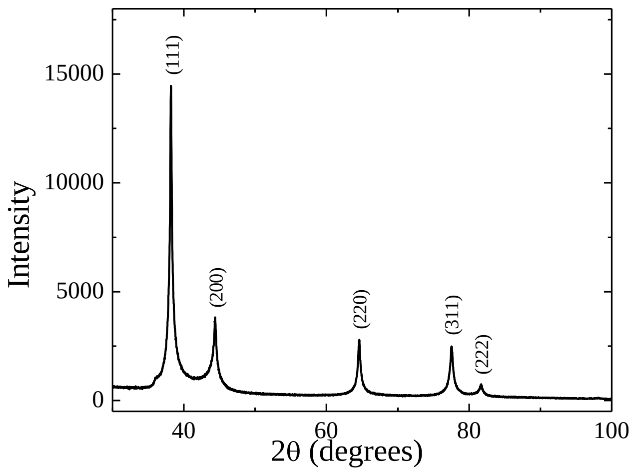

Figure 2. XDR patter of catechin-AgNPs.

Figure 3A shows the morphology of quasi spherical catechin-AgNPs with the presence of agglomerates. Figure 3B shows that the size distribution of the nanoparticles was found to be between 10 and 40 nm with average size of 23.4 ± 8.4 nm at solid state. The agglomeration observed in Figure 3A might be due to the drying process prior microscopy analysis.

DLS measurements revealed that the average hydrodynamic size of catechin-AgNPs was 44 nm, with PDI value of 0.21 and zeta potential of – 35.9 mV. The hydrodynamic size of the nanoparticles was found to be higher in comparison to the average size of the nanoparticles assayed by STEM. As expected, higher hydrodynamic sizes of NPs measured by DLS, compared with the sizes obtained by TEM, are attributed to the presence of extra hydrate layers in aqueous environments [23]. The results indicate the formation of catechin-AgNPs at the nanosize scale in aqueous suspension, and the PDI value indicates that the size distribution is moderate polydispersive. The negative value of zeta potential is due to the presence of catechin on the surface of AgNPs, since negative charge is expected for polyphenols [10]. This result indicates the presence of catechin on nanoparticle surface. Moreover, the magnitude of this zeta potential demonstrates the stability of the nanoparticles in aqueous suspension, avoiding nanoparticle agglomeration.

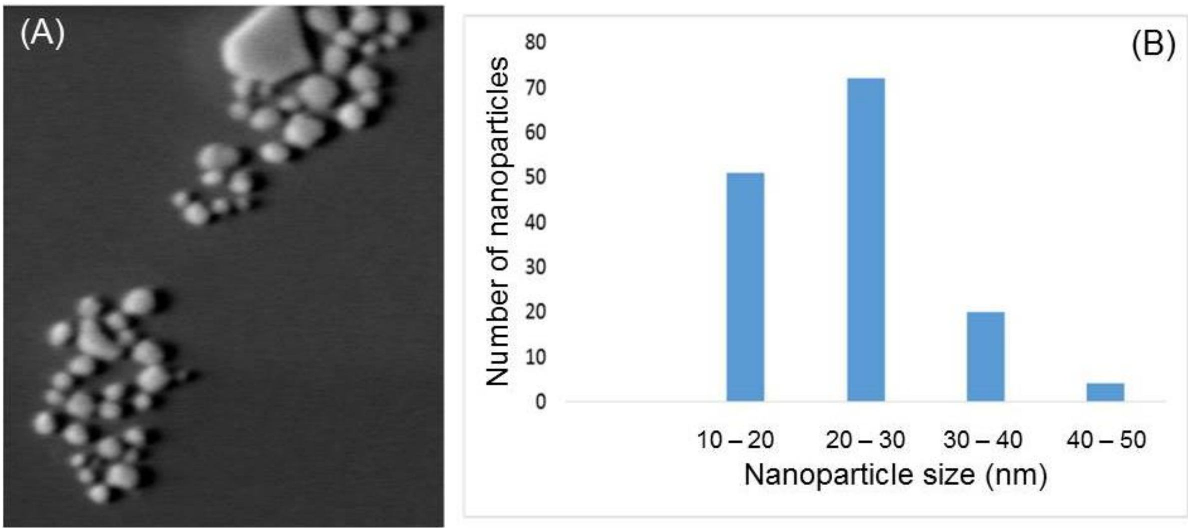

Figure 3. Field-emission scanning electron microscopy (STEM) of catechin-AgNPs (A) and their corresponding size distribution at solid state (B).

3.2. Functionalization of catechin-AgNPs with MSA leading to MSA-catechin-AgNPs

MSA, a low molecular weight thiol-containing molecule, was conjugated on the surface of catechin-AgNPs leading to the formation of MSA-catechin-AgNPs, which contain free thiol (SH) groups on their surface (Figure 4).

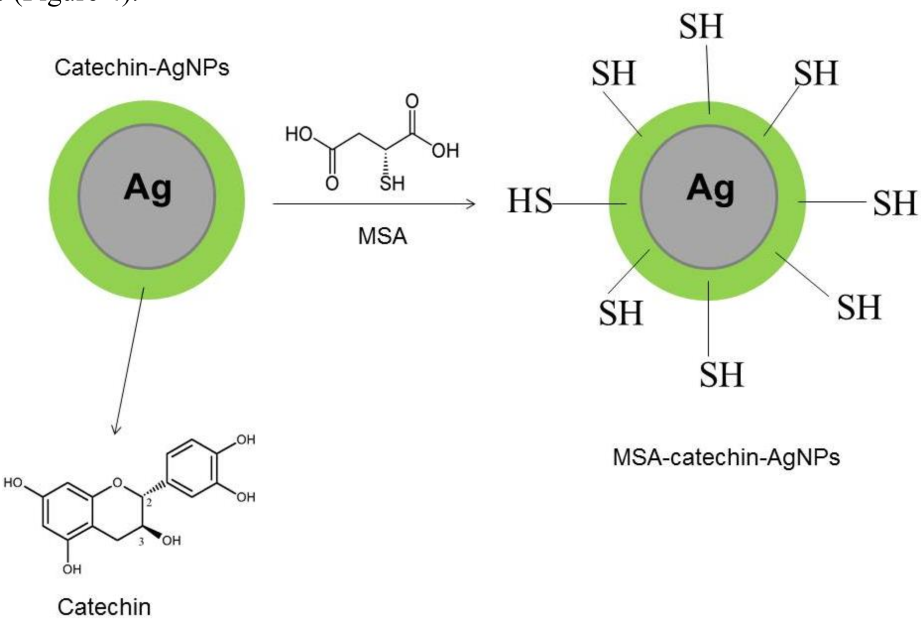

Figure 4. Schematic representation of the functionalization of catechin-AgNPs with mercaptossunic acid (MSA), a thiol containing-molecule, leading to the formation of MSA-catechin-AgNPs.

A value of 355 ± 19 µmol of free SH group per gram of MSA-catechin-AgNPs was obtained. MSA was conjugated with catechin-AgNPs by positive electrostatic interactions. The quantification of free thiol (SH) groups on the surface of MSA-catechin-AgNPs was determined by the reaction with a thiol specific reagent, DTNB, as previous described [10,23]. In drug delivery applications, the presence of free SH groups on the surface of nanoparticles represents a site for nanoparticle conjugation with important therapeutic molecules. In this present work, NO was loaded on MSA-catechin-AgNPs through SH groups.

3.3. Nitrosation of MSA-catechin-AgNPs leading to the formation of S-nitroso-MSA-catechin-AgNPs

Free thiol groups (SH) on the surface of MSA-catechin-AgNPs were nitrosated by the addition of sodium nitrite ($NaNO_2$) in slight acidified solution [10,23], leading to the formation of S-nitroso-MSA-catechin-AgNPs, which act as spontaneous NO donor due to the cleavage of S-N bound, as represented in Figure 5.

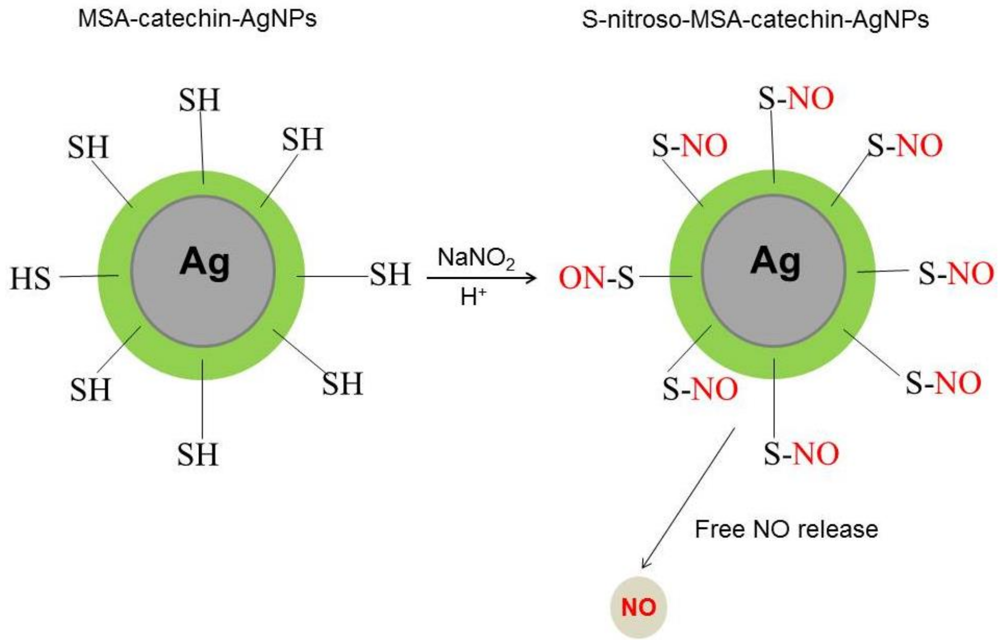

Figure 5. Schematic representation of nitrosation of free thiol groups on the surface of MSA-catechin-AgNPs by sodium nitrite ($NaNO_2$) leading to the formation of S-nitroso-MSA-AgNPs, which act as spontaneous NO donor.

The quantification of NO loading on the surface of S-nitroso-MSA-catechin-AgNPs was evaluated by electrochemical analysis with a specific NO sensor. The amount of 342 ± 16 μmol of NO was released per gram of S-nitroso-MSA-catechin-AgNP. This amount of NO release from nanoparticles is in the same range as reported for S-nitroso-MSA-Fe_3O_4 magnetic nanoparticles [24]. At this concentration range, NO is expected to have biological activities such as the antimicrobial effects [25]. To our best knowledge, this is the first report to describe the synthesis of NO-releasing AgNPs.

3.4. Antibacterial activities of the synthesized NPs

The antibacterial activities of catechin-AgNPs, MSA-catechin-AgNPs and S-nitroso-MSA-AgNPs were evaluated against different resistant bacterial strains. Table 1 and 2 show the minimal inhibitory concentration (MIC) and MBC values, respectively. As can be observed, the antibacterial effect is dependent on the bacteria strain and the nature of the AgNPs. For *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumoniae* lower values of MIC were found for the bacteria incubation with the NO-releasing nanoparticle (S-nitroso-MSA-catechin-AgNPs). For instance, MIC value of 3 μg/mL was observed for *Pseudomonas aeruginosa* treated with S-nitroso-MSA-catechin-AgNPs. In contrast, lower MIC values were found for catechin-AgNPs incubated with *Salmonella enterica* and *Escherichia coli.*

Table 1. MIC values (µg/mL) for different bacterial strains incubated for 24 h with catechin-AgNPs, MSA-catechin-AgNPs and S-nitroso-MSA-catechin-AgNPs.

Bacterial strain	Catechin-AgNPs	MSA-catechin-AgNPs	S-nitroso-MSA-catechin-AgNPs
Pseudomonas aeruginosa	62	125	3
Staphylococcus aureus	500	250	125
Klebsiella pneumoniae	1000	250	125
Salmonella enterica	62	250	125
Escherichia coli	62	250	125

Table 2. Minimum bactericidal concentration (MBC) values (µg/mL) for different bacteria strains incubated for 24 h with catechin-AgNPs, MSA-catechin-AgNPs and S-nitroso-MSA-catechin-AgNPs.

Bacterial strain	Catechin-AgNPs	MSA-catechin-AgNPs	S-nitroso-MSA-catechin-AgNPs
Pseudomonas aeruginosa	62	250	6
Staphylococcus aureus	500	500	125
Klebsiella pneumoniae	1000	500	125
Salmonella enterica	125	500	125
Escherichia coli	125	500	125

Table 2 shows that MBC values depend on bacterial strain and the nanoparticle. In all tested bacterial strains, MBC values decreased for S-nitroso-MSA-AgNPs in comparison with catechin-AgNPs, indicating that the NO releasing nanoparticles are more effective as antibacterial agent. Indeed, for *Pseudomonas aeruginosa* a MBC value of 6 µg/mL was found upon incubation with S-nitroso-MSA-AgNPs. Taking together the results demonstrated the all tested nanoparticles have antibacterial activity towards different bacterial strains. In most of the cases, the presence of NO on the nanoparticle surface enhanced the antibacterial effect due to a synergist effect of the NO donor and the AgNP.

4. Conclusions

This work describes the successful synthesis of AgNPs by catechin, the main product of green tea extract. Catechin acts as efficient reducing agent of Ag^+ to Ag° leading to the formation of catechin-AgNPs. Moreover, catechin acts a capping agent on the surface of AgNP, avoiding nanoparticle oxidation and/or aggregation. The obtained nanoparticles were characterized by different techniques, which indicate the formation of AgNP core coated with catechin. The surface of catechin-AgNPs was functionalized with MSA, a low molecular weight thiol containing molecule, leading to the formation of MSA-catechin-AgNPs. Free thiol groups on the surface of MSA-catechin-AgNPs were nitrosated by the addition of sodium nitrite leading to the formation of S-nitroso-MSA-catechin-AgNPs, which act as spontaneous NO donor. The antibacterial activities of catechin-AgNPs, MSA-catechin-AgNPs and S-nitroso-MSA-catechin-AgNPs were demonstrated towards different bacterial strains. All tested nanoparticles demonstrated antibacterial effects, as assayed by the determination of MIC and MBC values. In most of the cases, NO-releasing nanoparticles enhanced the antibacterial effect of catechin-AgNPs. These results highlight the promising uses of NO-releasing AgNPs against resistant bacteria in several biomedical applications.

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