

Anti-adhesion and antibacterial activity of silver nanoparticles and graphene oxide-silver nanoparticle composites

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ABSTRACT

The rise of nanotechnology has allowed the development of several inorganic nanoparticles with strong biocidal properties against bacteria, fungi, and viruses. Among them, silver nanoparticles (AgNPs) stand out as one of the most promising antimicrobial nanomaterials. Graphene oxide (GO) is another attractive nanomaterial with antimicrobial properties. Although the antimicrobial effect of AgNPs and GO is known, the development of hybrid materials of GO-AgNPs has considerable interest in various applications since they may exhibit synergistic bactericidal properties that exceed the yields of the individual components. The aims of this work were to evaluate the antimicrobial activity and anti-adhesion properties of AgNPs and GO-AgNPs nanocomposites for potential applications in antimicrobial coatings. The antimicrobial activity was tested by agar diffusion method. It was found that activity varied according to the synthesis procedure of the nanomaterials. *Pseudomonas aeruginosa*, *Bacillus cereus* and *Kokuria rhizophila* were the most susceptible strains. The nanocomposite GO- AgNPs synthesized using the *ex-situ* method exhibited the highest antibacterial activity against all the assayed strains. Similar results were obtained for bacterial adhesion inhibition tests. Thus, GO-AgNPs nanohybrids could be applied as antibacterial coatings to prevent bacterial biofilm development.

Keywords: Silver nanoparticles, graphene oxide, bacteria, biofilms.

1. INTRODUCTION

One of the most effective strategies for the prevention of microbial colonization is the development of functional materials with high antimicrobial properties. In this respect, the antimicrobial efficacy of nanoparticles (NPs), including metal and carbon-based NPs, has been widely studied [1–4].

Among the great variety of antibacterial materials, silver NPs (AgNPs) are marked out as antimicrobial reagents with high capability due to their large surface area and slow release properties [5–8]. They have been used as biocide agents in biomedicine, food, cosmetic and textile applications [9–13] and the responsible mechanism is not yet completely clarified [14,15]. There are studies that point out different cellular targets and actions such as: disturbance of the cell membrane, alteration of cellular DNA and proteins, electron transport, nutrient uptake, protein oxidation, or membrane potential; or the generation of reactive oxygen species (ROS), which lead to cell death. [14, 16, 17]. Despite the proven efficacy of AgNPs, they can lose antibacterial activity due to self-aggregation or precipitation [18]. These problems could be avoided by using graphene or graphene oxide as supporting matrix for the AgNPs [19].

Graphene is a two-dimensional material composed of a hexagonal sp²-hybridized carbon network [20, 21] giving a large superficial area, while graphene oxide (GO) is a chemically modified graphene featuring hydroxyl, carboxyl and epoxy functional groups [22]. Graphene-based materials are attractive

nanomaterials because of their unique chemical, physical, electric, mechanical, thermal and antimicrobial properties which made them useful for several applications such as biomedical, energy, nanoelectronic, biosensors, among others [20-24].

Accordingly, silver nanoparticles assembled on graphene oxide sheets (GO-AgNPs) have been exploited as novel antibacterial systems [25,26] exhibiting antibacterial activity against Gram-negative *Escherichia coli*. Also, in-situ synthesis of GO-AgNPs with good antibacterial activity has been extensively reported [27, 28]. However, the potential for these nanocomposites to prevent biofilm formation has not been explored. Thus, the aim of this work was to evaluate the antimicrobial activity and anti-adhesion properties of AgNPs and GO-AgNPs nanocomposites for potential applications in antimicrobial coating formulations.

2. MATERIALS AND METHODS

2.1 Synthesis and characterization of the nanocompounds

Hummers and Offeman method was used to obtain GO as reported in GARZON *et al.* [29]. Ten milligrams of GO were suspended in 30 ml of deionized water by ultrasonic bath resulting in a colloidal suspension. Hybrid GO-AgNPs were obtained by two routes of synthesis. The first one was *ex-situ*, using reducing agent of silver nitrate solution (Merck®) and gelatin solution (Sigma Aldrich®) as a stabilizing and reducing agent as in ZHANG *et al.* [30]. The second route was *in-situ* synthesis where AgNPs were nucleated and grown on GO sheets. A homogenous solution of GO was mixed with a silver nitrate solution. After that, the temperature was raised to 100°C. At this time, sodium citrate was added dropwise until the solution turned gray, as described in FONSECA DE FARIA *et al.* [31]. In both cases, the particle synthesis was evaluated by UV-visible measurements (200-600nm) which were recorded using a Rayleigh UV1601 UV/VIS-spectrophotometer. Micrographs of nanohybrids were obtained by transmission electron microscope (TEM)-(TECNAIG) operated at 200 kV.

2.2 Antimicrobial activity

The antimicrobial activity of the AgNPs and GO-AgNP nanocomposites was evaluated using the agar diffusion method (Kirby-Bauer method) [32]. These screening was carried out against Gram-positive and Gram-negative bacteria: *Pseudomonas aeruginosa* PAO 1, *Escherichia coli* (ATCC11229), *Acinetobacter* sp. (KM349193, NCBI-GenBank), *Bacillus cereus* (ATCC 10876), *Staphylococcus* sp. and *Kocuria rhizophila* (ATCC 9341). Inocula were prepared in Muller-Hinton (M-H) broth, from fresh 24 h cultures, all with an $OD_{(600nm)} \approx 0.1$ ($\approx 10^8$ CFU.ml⁻¹). M-H agar plates were inoculated by swab, in three directions, except for *Staphylococcus* sp., which was seeded by spreading 200 µl of the culture with a Drigalsky spatula. Three sterile filter paper disks were placed on each plate. On the filter paper, 10 µl of each nanomaterial was poured. The plates were incubated 20 h at 30 ± 2 °C. The inhibition zones were measured considering: ≤ 6 mm null antibacterial activity and >6 mm positive antibacterial activity. The assay was performed in triplicate.

2.3 Coating deposition

AISI 430 stainless steel (SS) coupons, previously sterilized with UV light, were immersed in AgNPs and nanohybrid solutions for 24 h at 4 °C, in darkness to form a coating. Then, coupons were removed from the solutions and dried in the laminar flow bench. The contact angle of the SS coupons without and with the different coatings was measured by the drop method using an optical microscope with an image analyzer.

2.4 Bacterial adhesion inhibition test

Inhibition of bacterial adhesion on AISI 430 SS coupons was evaluated in multi-well plates. In each well, 1 ml of the *P. aeruginosa* inoculum with an $OD_{(600nm)} \approx 0.1$ ($\approx 10^8$ CFU.ml⁻¹) and coupons with and without coatings were placed. After 24 h at 30 ± 2 °C the coupons were drawn from the culture and rinsed with sterile distilled water. Then, one coupon of each condition was scrapped with sterile scalpel in 1ml of physiologic solution, and serial dilutions were seeded in nutrient agar plates to perform bacterial counts.

2.5 SEM observations

Coupons with and without coatings, before and after the exposure to the *P. aeruginosa* culture were observed in the scanning electron microscopy (SEM) (FEI Quanta 200, ThermoFisher, USA). Prior to SEM observations, samples were fixed in 2.5% v/v glutaraldehyde in phosphate-buffered saline (PBS), dehydrated in ethanol (from 20 to 100% concentration) and metalized with Au.

3. RESULTS AND DISCUSSION

3.1 Synthesis and characterization of the nanocompounds.

Figure 1 shows UV-visible spectra of AgNPs (a) and the GO-AgNPs aggregates synthesized via the *ex-situ* (b) or *in-situ* (c) method. Figure 1a) shows the UV-visible spectrum of the AgNPs synthesized with gelatin, which is comparable with the literature where the spherical AgNPs have a peak of absorbance between the range 420-480 nm depending on the shape [30, 33]. Figure 1b) shows the characteristic peak of AgNPs but with less intensity than the UV spectra in Figure 1a), due to the presence of GO in the sample [30]. In the case of GO-AgNPs *in-situ*, it was possible to observe the characteristic peaks of both nanomaterials, as indicated in Figure 1c). Around 250-300 nm, the typical motion in C=O bonds from n- π transition in the GO was obtained. In contrast to *ex-situ* synthesis, only the AgNPs peak was observed. TEM micrographs in Figures 1 d); 1 e) and 1 f) confirm the spherical and Ag NPs attached on GO sheets were obtained, as shown in figures 1e) and f).

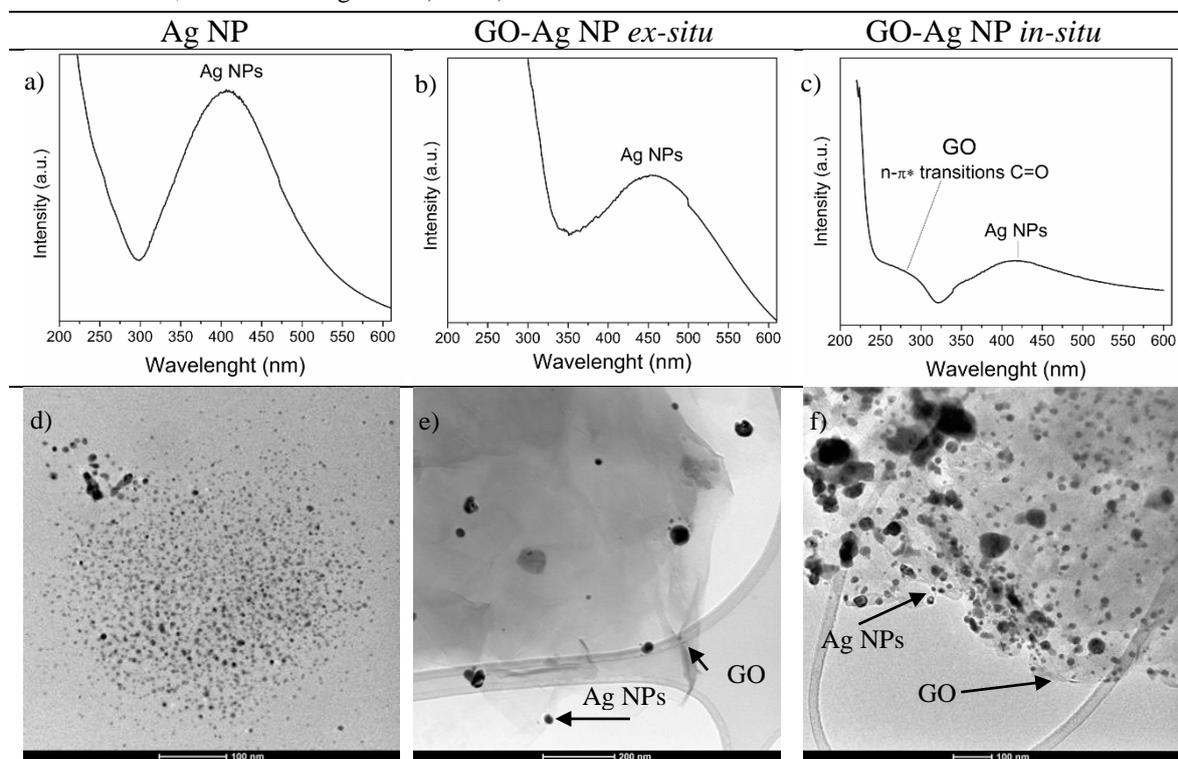


Figure 1: UV-visible spectra and TEM images: a) and d): AgNPs; b) and e): GO-AgNPs *ex-situ*, c) and f): GO-AgNPs *in-situ*.

3.2 Antimicrobial activity of AgNPs and GO-AgNPs nanocomposites

All the nanomaterials showed a certain degree of antibacterial activity as it can be seen in some of the plates used in the diffusion test (Figure 2). The average size of the inhibition zone measured for all the strains is presented in Figure 3. *P. aeruginosa* and *K. rizophila* were the most susceptible strains. The nanocomposite GO-AgNPs synthesized using the *ex-situ* method exhibited the highest antibacterial activity against all the assayed strains. Similar results regarding the enhanced antibacterial activities of GO-AgNPs vs. AgNPs were also reported by ZHANG *et al.* [34] who studied their effect against both Gram-negative *Escherichia coli* and Gram-positive *Bacillus subtilis*.

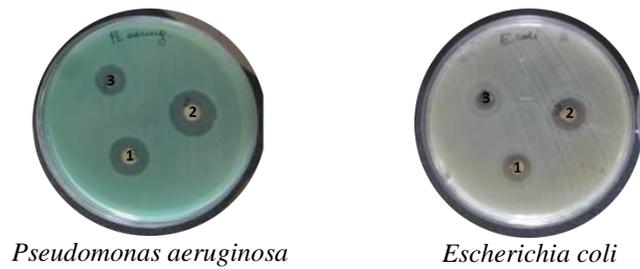


Figure 2: Photograph of one plate seeded with *P. aeruginosa* (left) and one seeded with *E. coli* (right) showing the inhibition zones produced by AgNPs (1), GO-AgNPs *ex-situ* (2) and GO-AgNPs *in-situ* (3).

The different susceptibilities to AgNPs may be due to different cell wall structures and the different antibacterial mechanisms of Ag against different cells [35-37]. For instance, Gram-negative bacteria possess a thin peptidoglycan layer (7-8 nm thickness), whereas Gram-positive bacteria possess a thick peptidoglycan layer (about 20-80 nm thickness) [38], which is more resistant to Ag⁺ diffusion.

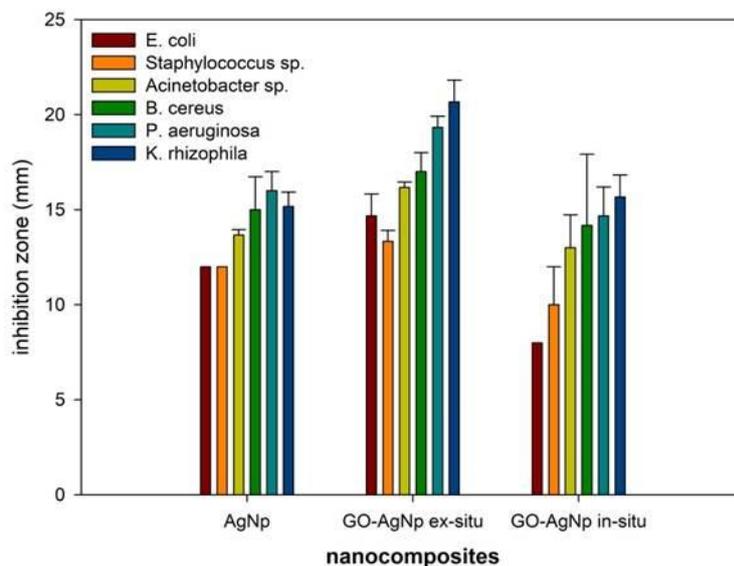


Figure 3: Inhibition zone (mm) of the AgNPs and the nanocomposites GO-AgNPs *ex-situ* and GO-AgNPs *in-situ* against all tested bacterial strains.

3.3 Bacterial adhesion inhibition test

Before the adhesion test, the surface of the coated and uncoated SS coupons was characterized through water contact angle (WCA) measurements (Figure 4) and SEM observations (Figure 5). The WCA of the clean SS coupon and those coated with the nanomaterials did not show significant differences. WCA values smaller than 90° are characteristic of hydrophilic surfaces with good wettability and adhesion and high surface energy [39]. These surfaces are more able to bind bacteria or cells, as compared with extremely hydrophobic or hydrophilic surfaces [40]. It has been reported that surface with WCA between 40 to 70° enhanced cell adhesion and growth [41]. From the above, it can be assumed that the materials tested in this work would allow the development of bacterial biofilms.

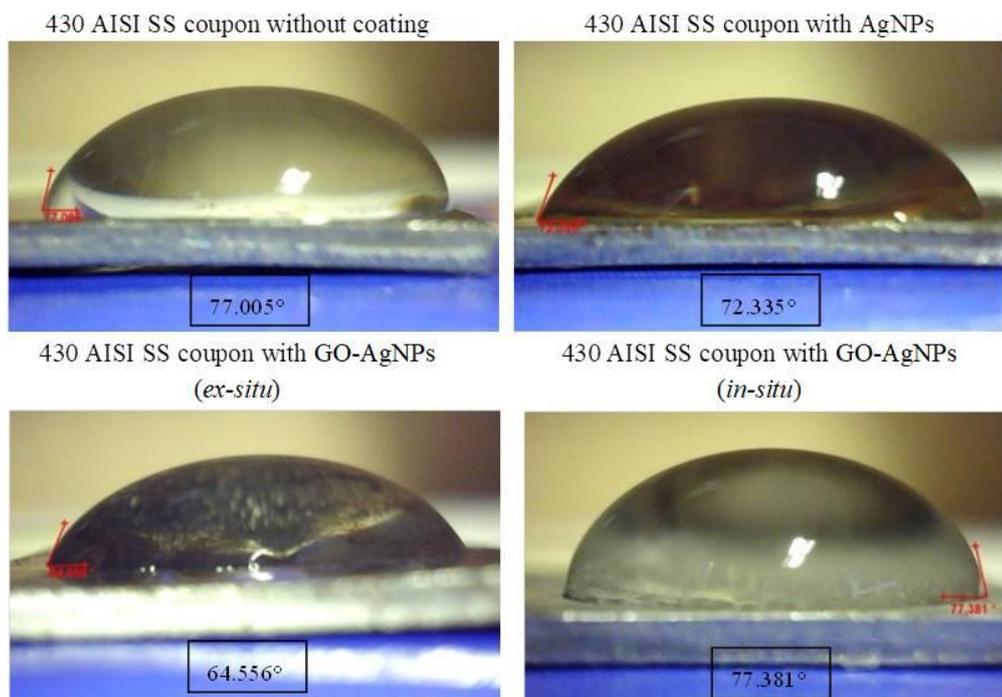


Figure 4: Water drop on the SS surfaces without and with coatings for contact angle measurements. The values of the contact angle obtained are indicated in each photograph.

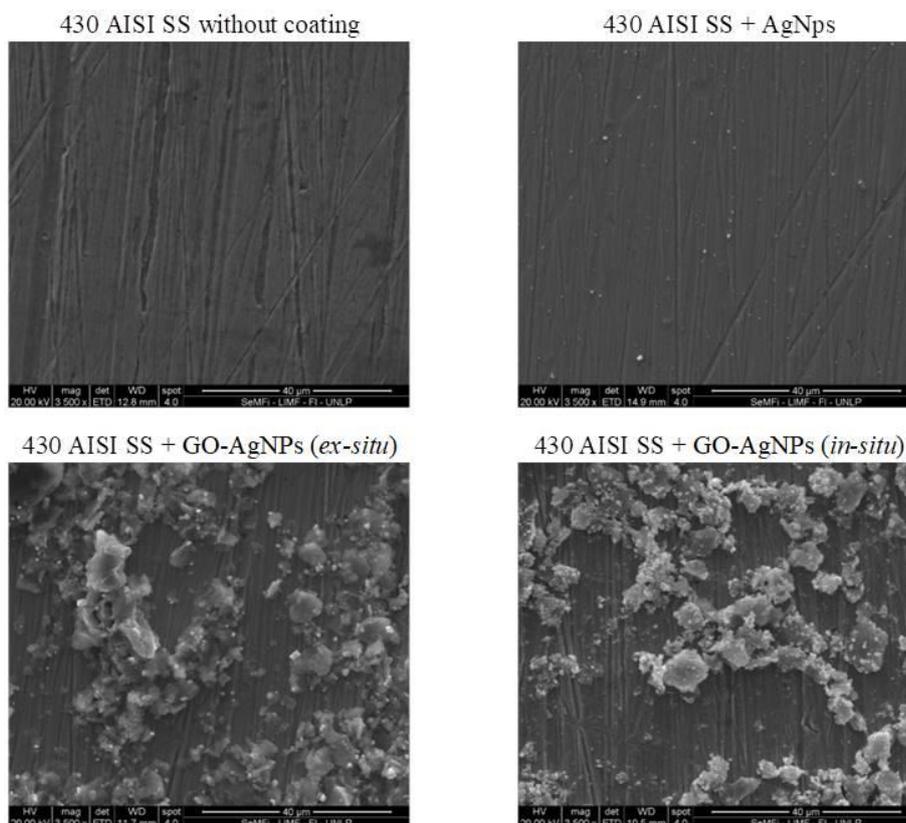


Figure 5: Microphotographs of SEM images of the SS coupons uncoated and coated with the different nanomaterial, before the bacterial adhesion test.

The number of *P. aeruginosa* cells adhered on the coupons were 10^5 - 10^6 CFU.cm⁻² for control coupons, 10^4 - 10^5 CFU.cm⁻² for GO-AgNPs (*in-situ*) coupons and values lower than 10^2 CFU.cm⁻² on AgNPs and GO-AgNPs (*ex-situ*) coupons, indicating that the later materials possessed antifouling activity. These results were corroborated by the SEM observations (Figure 6). GO-AgNPs suspensions were reported to inhibit 100% of *P. aeruginosa* cells adhered to stainless steel surface after 1 h of contact between the supported cells and the nanohybrid material suspended in the culture medium [31]. In our study, the nanomaterials immobilized on the SS surface, provoked a decrease in the number of attached bacteria. However a complete inhibition of biofilm formation or the death of all the bacteria was not observed. Although most of the studies note the importance of dissolved silver ions in the antibacterial effect, several reports claim that leaching silver ions alone cannot account for the silver nanoparticles' cytotoxicity. The size and morphology of the nanoparticles also affect the biocidal performance [42].

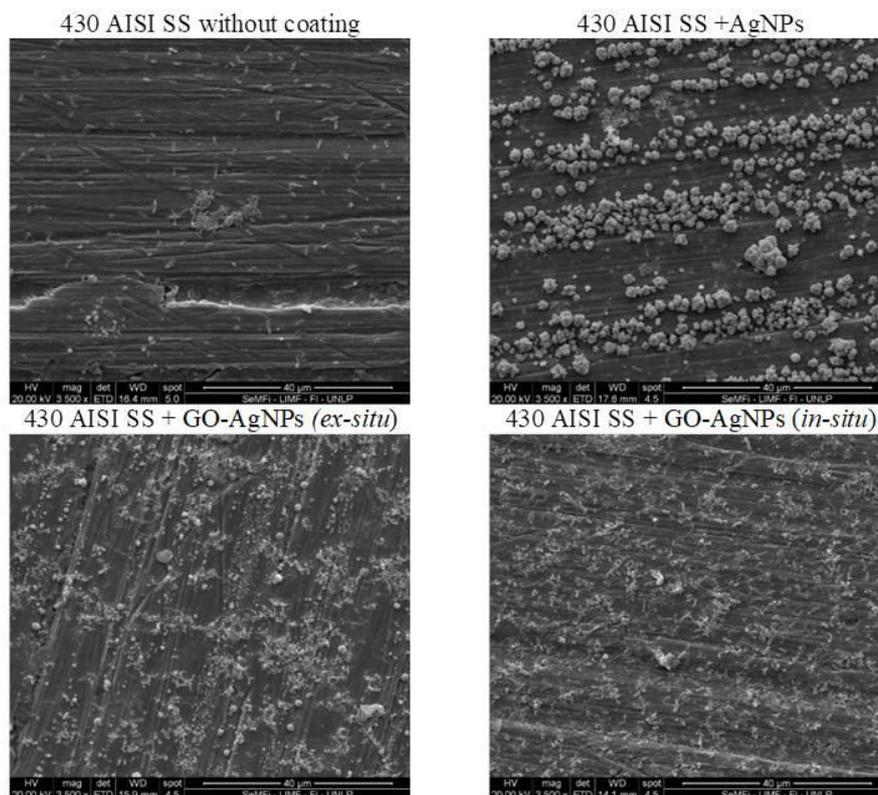


Figure 6: Microphotographs of SEM images of the SS coupons uncoated and coated with the different nanomaterial, after the immersion in *P. aeruginosa* culture. In all the cases, the magnification is 3500x and the scale bar 40 μm.

One advantage of the GO sheets is its role as a supporting and stabilizing agent, and the bacterial activity reduction [31]. The graphene nanosheets are able to wrap around of the bacteria cell maximizing the contact area between the AgNPs material and the bacteria. In addition, graphene material allows the formation of Ag NPs on its surface and minimizes Ag NPs agglomeration, enhancing the biocidal activity of metallic silver [43].

On the other hand, the use of nanomaterials as antimicrobial agents is associated with the bacterial resistance. The excessive use of the conventional disinfectants to control biofilm formation may lead to bacterial strains that are resistant to the chemical substance applied due to the defense mechanism the bacteria have created. GO-AgNPs could be used as an alternative anti-biofilm agent without the adverse effects of microbial resistance frequently attributed to antibiotics, disinfectants and other conventional agents [44].

4. CONCLUSIONS

All the nanomaterials assayed showed antibacterial activity against planktonic bacteria. The nanocompo-

site GO-AgNPs synthesized using the ex-situ method exhibited the highest antibacterial activity against all the assayed strains.

K. rhizophila, *P. aeruginosa* and *B. cereus* were the most susceptible strains.

The nanomaterials were also able to reduce the number of bacteria attached to the SS coupons. AgNps and GO-AgNps (ex situ) produced a reduction of three orders of magnitude in the number of bacteria attached.

The results support the idea that GO-AgNps nanocomposites may be added in antimicrobial paint and coating formulations to diminish the development of biofilms.

5. ACKNOWLEDGEMENTS

The authors are grateful the National University of La Plata Project 11/ I238, CONICET PIP No. 00314 and CICBA 195/17 for the grants received to finance this work. The authors thank Eng. Pablo Seré (from Lab. Anelpire, CIDEPIINT) for the contact angle measurements.

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