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Antimicrobial Activity against *Escherichia coli* of Cu-Ni Nanoalloy and Combination of Ag Nanoparticles, Obtained by Different Method

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Abstract: *Escherichia coli*, is a pathogenic bacterium that causes serious infections, whose therapeutic treatment is threatened by the emergence of multiple resistance to conventional antibiotics. In recent years, metal nanoparticles (NPs) have been studied for their antimicrobial capacity and their possible applications as an alternative to antibiotics against different pathogens. NPs also vary in synthesis techniques; either by chemical, physical and biological methods. The objective of this work was to study the possible antimicrobial capacity of Cu-Ni nanoalloys obtained by a method called citrate-gel. The antimicrobial capacity of the NPs mentioned above was evaluated in vitro by the agar diffusion method. Most of the NPs evaluated showed antibacterial activity against the strain of *E. coli* studied. When combining chemical and biological NP, synergistic effects are observed with an increase in antibacterial activity in some cases. We can conclude that NPs derived from chemical and biological synthesis could be used as antimicrobials against *E. coli* and when these are combined, antibacterial effects increase. In the future, these applications of nanomaterials could be used as an alternative to the use of antibiotics against infections that have limited treatments.

Key words: Nanoalloys Cu-Ni, antibacterial activity, human pathogens, nanotechnology.

1. Introduction

Nanotechnology is considered as a vital current technology in the 21st century based on its economic and scientific potential [1]. In recent years, it has experienced exponential growth in different disciplines, for example within the field of health [2].

Infectious diseases are a set of diseases or disorders caused by pathogenic microbes (bacteria, viruses, fungi, protozoa, parasites) that directly affect human health [3]. Antibiotics are often used to treat these infections, but classic antimicrobial treatments are not efficient due to the emergence of resistant microorganisms, therefore, research is currently based on the development of new antibacterial strategies [4, 5].

Within the range of pathogenic microorganisms, some bacteria can cause serious infections. *E. coli* is the most common Gram-negative bacterial pathogen, and due to increased selective pressure to use antimicrobial agents [6], some high-risk multidrug resistant strains have evolved, which poses great

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clinical and epidemiological challenges. In this context, the contribution of nanotechnology is very important, as nanoparticle based processing presents a very promising approach [3, 7].

Metal ions, either alone or in complexes, have been used to disinfect fluids, solids and tissues for centuries, however nanomaterials improve the physical, chemical and biological properties of non-nanometric particles [8, 9]. The use of nanoparticles as an alternative to the use of antibiotics with some other advantages such as their low toxicity, broad spectrum biocide, chemical stability, long period of action and thermal resistance [10].

Some metal nanoparticles obtained by chemical synthesis, such as gold (Au) and silver (Ag), are well known for their antibacterial effects and are still in use despite their high cost [1]. In this context, nanomaterials composed of copper (Cu) and nickel (Ni) are a good alternative because they retain the antimicrobial properties and are cheaper than gold and silver [11]. On the other hand, metal nanoparticles of biological synthesis have also received much attention for its antibacterial activity [12].

Recent studies have shown that the properties of the materials depend on the shape of the particles, the surface area, the homogeneity, the degree of crystallinity of the particles, and should influence the antimicrobial properties. These characteristics are closely related to the preparation technique [13-15].

The nanoalloys also vary in synthesis techniques; either by chemical [16], physical [17] and biological based methods [12]; most of the Cu-Ni nanoparticle synthesis methods are very expensive, cheaper alternatives are sought, among them the citrate-gel method has emerged [13, 18].

Wang et al. [19] prepared four kinds of coatings, Cu coating, Ni coating, NiCu coating and CuNi coating, their nanoparticles were prepared by electroplating and then the antibacterial activity was evaluated and the NiCu coating was found to show excellent antibacterial activity compared to the Ni coating.

Therefore, the purposes of this study were the synthesis and characterization of Cu-Ni nanoparticles prepared using precursors synthesized by the citrate-gel method, and the evaluation of the antimicrobial properties of the nanoalloys obtained on a representative bacterium such as *Escherichia coli*.

2. Material and Methods

2.1 Materials

Nickel nitrate ($Ni(NO_3)_2$ $6H_2O$) (Fluka), copper nitrate ($Cu(NO_3)_2$ $2.5H_2O$) (Rieldel–de Haën), ammonium hydroxide (Biopack), and citric acid (Anedra) were used. The gases employed were 99.999% pure N_2, a mixture of H_2 (5%) and N_2 and a mixture of O_2 (10%) and N_2. The gases were purified using adequate traps to retain water and oxygen.

2.2 Synthesis Procedure

2.2.1 Step 1: Preparation of precursor

A solution was prepared containing the Cu (II) and Ni (II) ions with a 1: 1 molar ratio from their corresponding nitrates. An acute solution of citric acid was added to this solution in order to obtain the following molar ratio C/Me: 0.73 and 1.5, where C is defined as the moles of citric acid and Me as the sum of the moles of Cu (II) and Ni (II). In all cases, the pH of the solution was 1. The resulted solutions were heated to 50 ℃ for 30 min and continuously stirred. The solvent was later removed at 60 ℃ under vacuum condition using a Büchi 461 vacuum rotavapor until a viscous gel was obtained. Dehydration of the sample was completed by gradual heating to 100 ℃ and keeping for 15 h in a vacuum oven.

2.2.2 Step 2: Decomposition

The decomposition of precursors was carried out under non-isothermal conditions by heating the precursors from room temperature to 280 ℃ in a N_2 flow of 100 mL/min and were kept at the same temperature for 1.45 h.

2.2.3 Step 3: Calcination

Calcination was carried out under non-isothermal conditions with a current of O_2 (10%) in N_2 and a flow of 100 mL/min and at a heating rate of 1 ℃/min, until reaching the selected temperature (300 and 500 ℃). The system was kept at this temperature for 1 h.

2.2.4 Step 4: Reduction

Reduction was carried out under non-isothermal conditions, the solid obtained was reduced using a heating program of 5 ℃/min from room temperature to 300 ℃ with a current of H_2 (5%) in N_2 and a flow of 100 mL/min. The system was kept at this temperature for 30 min, and then cooled in a N_2 gas flow. Their reduction products were labeled as B1-300, B1-500 and B3-300, respectively (Fig. 1).

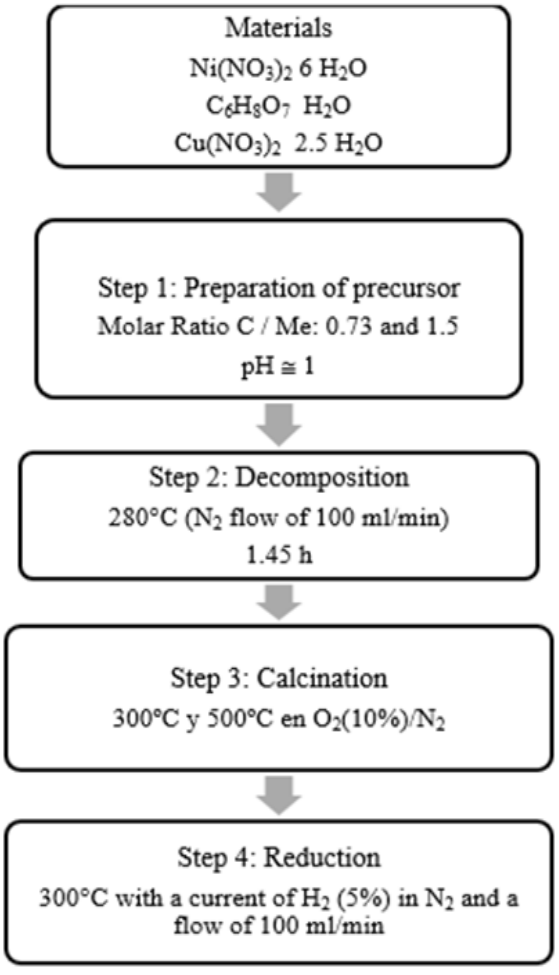

Fig. 1 Synthesis scheme of Cu-Ni nanoalloys using the citrate gel method.

2.3 Characterization of Solids

Different techniques were used for solid characterization, such as, scanning electron microscopy (SEM), energy dispersive spectrometer (EDS), and X-ray diffraction (XRD) techniques were applied to the characterization of the as obtained alloy powders. SEM analyses were performed in a Thermofisher Inspect S50 y Thermofisher Nova NanoSEM 230. EDS analyses were carried out with a 20 keV incident electron beam. High resolution SEM (HR-SEM) studies were performed with a (EDAX Octane Pro). XRD experiments were carried outwith a PANalytical Empyrean, operated at 40 kV and 30 mA, using Cu-Kα incident radiation (λ = 0.15418 nm). Diffractograms of the samples were recorded by scanning the angular interval of 2θ = 10 to 120 ° at a scanning rate of 3 °/min.

2.4 Synthesis of Silver Nanoparticles

The silver nanoparticles were used obtained by biological method. For the synthesis of the Ag NPs, one milliliter of an aqueous silver nitrate solution (1 mM) was added to Erlenmeyer flasks containing 100 mL of yeast culture supernatant free of cells. The resulting mixture was shaken at 100 rpm in an orbital shaker for 48 h at 28 ± 4 ℃ in dark. Appropriate controls (uninoculated MH medium plus silver nitrate and yeast culture supernatant without silver nitrate) were run simultaneously. The experiment was carried out in triplicate. The yeasts *Cryptococcus laurentii* (AgNPs-C.l (NPs1)) and *Rhodotorula glutinis* (AgNPs-R.g (NPs2)) they were used as mediators for the biosynthesis [8].

2.5 Antimicrobial Activity

The antimicrobial capacities of the nanoalloys (B1-300; B1-500; B3-300) were evaluated *in vitro* by means of the agar diffusion method. Comparative sensitivity test of samples was carried out against *E. coli* (ATCC 8739). For the assessment antimicrobial, 200 uL of *E. coli* suspension (10^8 CFU/ mL) were

aseptically spread onto plates with selective medium and incubated for 1 h at 37 ± 1 ℃. Subsequently, wells of 7 mm were made aseptically with a punch and were filled with 25 uL of different suspensions of the nanoparticles. For the essay the nanoalloys in powder it was not necessary to perform wells. The AgNPs-C.l (NPs1) and AgNPs-R.g (NPs2) were used with positive control and distilled water was used as a negative control. In addition, combinations of the different nanoparticles mentioned were tested Table 1. To validate the test, we incubate a plaque only with the bacterial suspension and another only with nanoparticles. The plates were incubated for 48 hours at 37 ± 1 ℃. After incubation, the inhibition zones (mm) were measured. The tests were performed in triplicate.

Table 1 Samples evaluated in the antimicrobial activity test.

Number of sample	Sample evaluated	Amount evaluated
1	Negative control	25 µL Distilled water
2	Positive control (AgNP-C.l (NPs1))	25 µL de solution de NPs (6 ppm)
3	Positive control (AgNP- R.g(NPs2))	25 µL de solution de NPs (3 ppm)
4	Powder of B1-300	10 mg of solid
5	Powder of B1-500	10 mg of solid
6	Powder of B3-300	10 mg of solid
7	Solution of B1-300	5 mg/mL
8	Solution of B1-500	5 mg/mL
9	Solution of B3-300	5 mg/mL
10	Combined sample	5 mg B1-300 + 5 mg B3-300
11	Combined sample	5 mg B1-300 + 5 mg B1-500
12	Combined sample	5 mg B3-300 + 5 mg B1-500
13	Combined sample	5 mg B1-300 + 10 µL NPs1
14	Combined sample	5 mg B1-500 + 10 µL NPs1
15	Combined sample	5 mg B3-300 + 10 µL NPs1
16	Combined sample	5 mg B1-500 + 10 µL NPs1

3. Results and Analysis

3.1 Characterization of Solids

Figure 2 shows the diffractograms of the synthesized alloys B1-300, B1-500, B3-300 and the JCPDS 4-836 and 4-850 files corresponding to Cu and Ni, respectively. In Figure 2 from a to c, three peaks are observed, each one of them located at an intermediate position between the corresponding to the peaks of each pure metal. The observed position indicates that, under these working conditions and for all cases, the solid Cu-Ni solution has been formed.

Table 2 shows the values of the lattice parameter "a", the composition and the grain size of the different alloys estimated by means of the Scherrer equation. The results in the table indicate for samples B1-300 and B1-500 that the increase of calcination temperature does not substantially modify the composition of the alloy, where the compositional coefficient x, referred to Cu, changes from 0.50 to 0.51. In sample B3-300, the results indicate that the increase in the C/Me molar ratio modifies the composition of the alloy, where the compositional coefficient x, referred to Cu, changes to 0.56, and that this increase prevents the formation of the solution solid Cu-Ni with a ratio close to 1: 1, obtaining alloys with much greater proportions of Cu.

The calculated particle size was between 10-14 nm, which shows a good similarity with the grain size calculated using TEM techniques in previous works [13].

Figure 3 corresponds to solid B3-300. Figure 3a and 3b show different magnifications of SEM (emissive mode) micrographs. Figure 3a shows the surface

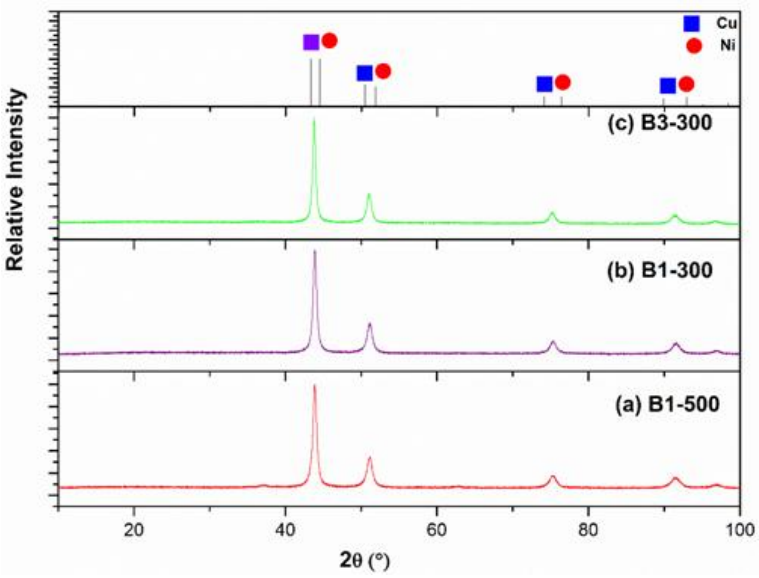

Fig. 2 X-ray diffractograms of the Cu–Ni alloys obtained through the synthesis method: (a) B1-500, (b) B1-300 y (c) B3-300.

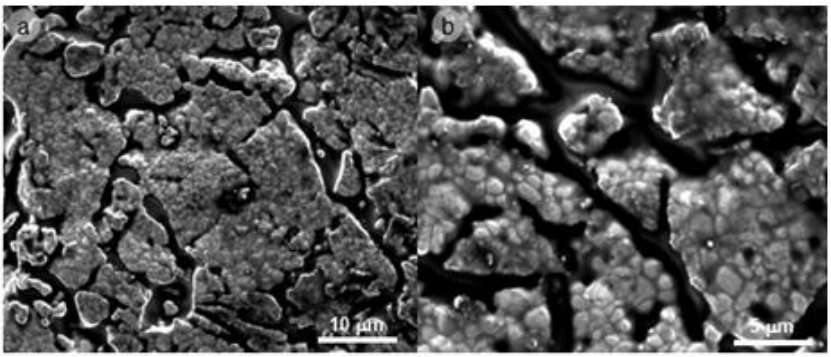

Fig. 3 Sample B3-300. a y b) different magnifications of SEM (emissive mode) micrographs.

Table 2 Lattice parameter "a", composition and grain size of the alloy obtained in the reduction stage.

Sample evaluated	a	Composition Cu_xNi_{1-X}	Grain Size (nm)
B1-300	3.569	$Cu_{0.50}Ni_{0.50}$	13
B1-500	3.570	$Cu_{0.51}Ni_{0.49}$	14
B3-300	3.574	$Cu_{0.56}Ni_{0.44}$	10

morphology of the alloy. Note that it is composed of circular grains with a size of 0.5 µm. The surface shows flat zones and channels. Figure 3b shows a detailed image of the terrace. The compositional mapping is not shown here, but indicates a Ni (54% m/m) and Cu (46% m/m) measured both from the K line signal.

3.2 Antimicrobial Activity

For the validity of this test, a positive control (showing growth) and a negative control (showing no growth) were observed. Figure 4 shows the values of the inhibition halos of all the samples described in Table 1 against *E. coli* and Figure 5 shows the zone of inhibition of some representative samples described therein. An inhibition halo greater than 7 mm indicates that the tested compound is active against the bacteria under investigation [20]. The three types of NPs evaluated in solid state have antibacterial activity against *E. coli*, even the halo of inhibition observed in the three nanoalloys studied is greater than in the case of positive controls. When evaluating suspensions,

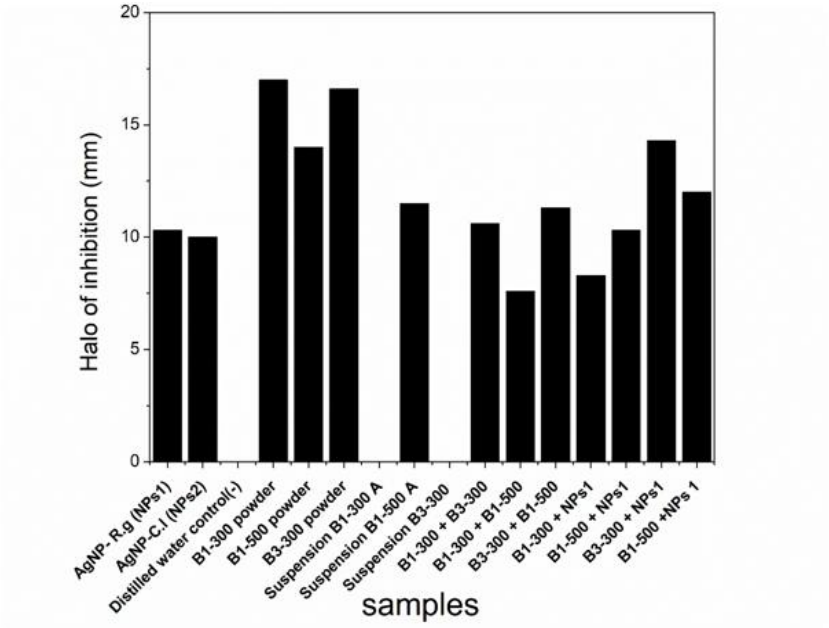

Fig. 4 Average inhibition observed *in vitro* of all samples described in the Table 1 against *E. coli*.

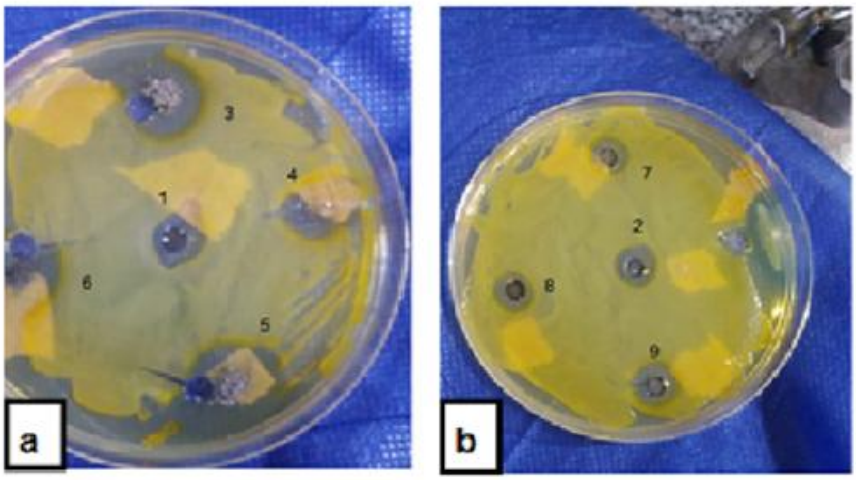

Fig. 5 A, b shows the zone of inhibition covered by some representative samples described in the Table 1 against *E. coli*.

only B1-500 has activity. It is likely that since the metals are not soluble in water, when the dilutions are made the effect is lost. When combining NPs, synergistic effects are observed with an increase in antibacterial activity in some cases.

4. Discussion

Nanostructuring of Cu and Ni alloys opens new possibilities for the design of antimicrobial materials. Nanocrystalline metals due to extremely small grain sizes enhance physico-chemical, mechanical and biological properties compared with the corresponding materials with microcrystalline grain size [21]. Several authors confirm that the method of synthesis is crucial to obtain desirable properties in the alloy; for example, variants are observed in the final particle size, the degree of aggregation and the uniformity of the solid surface [22].

In this work, we focus on analyzing antimicrobial capacity since they are potentially attractive materials in a wide variety of medical applications, such as dental procedures [23]. This study shows that silver

NPs and Cu-Ni NPs have excellent antibacterial activity against *E. coli* that nanoparticles with a greater surface/volume ratio provide more efficient means for antibacterial activity. It is postulated as a mechanism of action, the penetration of nanoparticles in the pores of nanometric scale present in the cell membranes of the bacteria, which after entering degrade components of the cytoplasm eventually causing death [24]. Hou, J., et al confirmed that metallic nanoparticles mainly affect nucleic acid metabolism in the nucleus through alterations in nucleic acid binding [25].

5. Conclusions

The preparation of Cu_xNi_{1-x}nanoalloys by citrate-gel method was verified by structure and morphology analysis

The antibacterial activities of 5 NPs and its combinations against *E. coli* were studied. The 3 types of NPs evaluated in solid state have antibacterial activity against *E. coli*, which can be ascribed to the contact-killing strategy has activity.

The best antibacterial property was obtained only when evaluating suspensions of B1-500. When combining suspensions of differents NPs, synergistic effects are observed with an increase in antibacterial activity in some cases.

Future studies on the biocidal influence of this nanomaterial on other Gram-positive and Gram-negative bacteria are necessary to fully evaluate its possible use as a new bactericidal material.

These results helped us to understand more about antimicrobial properties of Cu-Ni nanoalloys and have important scientific significance. However to draw conclusions about toxicity, the interaction and integration of NPs should be paid more attention in the future research.

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