Yuliana Sanchez¹, Mayra Mora^{1,2}, Manuel Palencia¹

¹ Research Group in Science with Technological Applications (GI-CAT), Department of Chemistry, Universidad del Valle, Cali - Colombia.

² Mindtech Research Group (Mindtech-RG), Mindtech s.a.s., Barranquilla - Colombia

Corresponding author: manuel.palencia@correounivalle.edu.co

Graphical abstract

A first approach to the development of new drugs with leishmanicidal activity via control of surface properties of silver nanoparticles

Abstract

Silver nanoparticles (AgNPs) are widely recognized for their application mainly in the area of medicine, due to their therapeutic potential against infections by Gram-negative and Gram-positive bacteria and even protozoa of the *Leishmania* genus. However, its use is limited by its hemolytic properties when in contact with blood flow. Because the surface properties of AgNPs can be controlled by the nature of the metal surface, the aim of this work was to evaluate the effect of different surface stabilizing agents on the antimicrobial and hemolytic activity of AgNPs. For this, AgNPs were synthesized by chemical reduction with different stabilizing agents, these being poly(vinyl pyrrolidone), sodium citrate, *N*-methyl-D-glucamine and the meglumine antimoniate in molar ratios Ag:stabilizing agent, 1:1, 1:3, 1:6 and 1:10. Nanoparticles were characterized by ultraviolet-visible spectroscopy and dynamic light scattering. In addition, the AgNPs stabilized with

Keywords

Nanostructured systems
Capping agent
Silver nanoparticle
Chemical reduction
Leishmaniasis

meglumine antimoniate were characterized by atomic absorption spectroscopy. Finally, the antimicrobial activity of AgNPs was evaluated against *E. coli* and *S. aureus*, while its hemolytic activity was evaluated by ASTM method F756-13. The results obtained show a dependence on the hemolytic and antimicrobial properties of AgNPs with the nature and concentration of the stabilizing agent.

Una primera aproximación al desarrollo de nuevas drogas con actividad leishmanicida mediante el control de las propiedades superficiales de nanopartículas de plata

Resumen

Las nanopartículas de plata (AgNPs) son ampliamente reconocidas por su aplicación principalmente en el área de la medicina, debido a su potencial terapéutico contra infecciones por bacterias Gram-negativas y Gram-positivas e incluso protozoarios del género *Leishmania*. Sin embargo, su uso se ve limitado por sus propiedades hemolíticas cuando están en contacto con el flujo sanguíneo. Debido a que las propiedades superficiales de las AgNPs pueden controlarse por la naturaleza de la superficie metálica, el objetivo de este trabajo fue evaluar el efecto de diferentes agentes de estabilización superficial sobre la actividad antimicrobiana y hemolítica de las AgNPs. Para ello, se sintetizaron las AgNPs mediante reducción química con diferentes agentes de estabilización, siendo éstos la poli(vinilpirrolidona), citrato de sodio, *N*-metil-D-glucamina y el antimoniato de meglumina en relaciones molares Ag:Agente estabilizante 1:1, 1:3, 1:6 y 1:10. Las nanopartículas se caracterizaron mediante espectroscopia ultravioleta-visible y dispersión dinámica de la luz. En adición, las AgNPs estabilizadas con antimoniato de meglumina se caracterizaron por espectroscopia de absorción atómica. Finalmente, se evaluó la actividad antimicrobiana de las AgNPs frente a *E. coli* y *S. aureus*, mientras que su actividad hemolítica se evaluó mediante el método ASTM F756-13. Los resultados obtenidos evidencian una dependencia de las propiedades hemolíticas y antimicrobianas de las AgNPs con la naturaleza y la concentración del agente estabilizante.

Palabras clave

Sistemas
nanoestructurados
Agente estabilizante
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Corresponding author:

manuel.palencia@correounivalle.edu.co

A first approach to the development of new drugs with leishmanicidal activity via control of surface properties of silver nanoparticles

Yuliana Sanchez¹, Mayra Mora^{1,2}, Manuel Palencia¹

¹ Research Group in Science with Technological Applications (GI-CAT), Department of Chemistry, Universidad del Valle, Cali - Colombia.

² Mindtech Research Group (Mindtech-RG), Mindtech s.a.s., Barranquilla - Colombia

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Abstract

Silver nanoparticles (AgNPs) are widely recognized for their application mainly in the area of medicine, due to their therapeutic potential against infections by Gram-negative and Gram-positive bacteria and even protozoa of the *Leishmania* genus. However, its use is limited by its hemolytic properties when in contact with blood flow. Because the surface properties of AgNPs can be controlled by the nature of the metal surface, the aim of this work was to evaluate the effect of different surface stabilizing agents on the antimicrobial and hemolytic activity of AgNPs. For this, AgNPs were synthesized by chemical reduction with different stabilizing agents, these being poly(vinyl pyrrolidone), sodium citrate, *N*-methyl-D-glucamine and the meglumine antimoniate in molar ratios Ag:stabilizing agent, 1:1, 1:3, 1:6 and 1:10. Nanoparticles were characterized by ultraviolet-visible spectroscopy and dynamic light scattering. In addition, the AgNPs stabilized with meglumine antimoniate were characterized by atomic absorption spectroscopy. Finally, the antimicrobial activity of AgNPs was evaluated against *E. coli* and *S. aureus*, while its hemolytic activity was evaluated by ASTM method F756-13. The results obtained show a dependence on the hemolytic and antimicrobial properties of AgNPs with the nature and concentration of the stabilizing agent.

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1. Introducción

Neglected tropical diseases (NTDs) are a group of infectious diseases that predominate in tropical and subtropical areas of low economic resources such as Africa, Asia and America, due to insufficient sanitary infrastructure, poor housing conditions, malnutrition and the consequent weakness of the immune system [1]. According to the World Health Organization, leishmaniasis is classified as

one of the main NTDs, without control and high priority, registering at least 700 thousand new cases and 20 thousand annual deaths worldwide, affecting not only people living in countries where the disease is endemic, such as Colombia, it also represents a risk for people who transit in those areas [2].

Leishmaniasis is a zoonotic disease caused by an intracellular protozoan of the genus *Leishmania* that belongs to the Trypanosomatidae family and is

transmitted by the bite of infected female sandfly vectors commonly known as drain flies [3]. This disease is classified into three main forms: cutaneous leishmaniasis, mucocutaneous and visceral. About the 2 million new cases that occur per year, 1.5 million correspond to cutaneous leishmaniasis. Particularly in Colombia, the number of cases has increased in recent years, according to Sistema Nacional de Vigilancia en Salud Pública (SIVIGILA), 12,232 and 13,696 cases of leishmaniasis were presented for the years 2009 and 2010, respectively, of which 98 % corresponded to the cutaneous presentation of leishmaniasis [4].

For the treatment of this disease, mainly pentavalent antimonials (meglumine antimoniate or Glucantime® and sodium stibogluconate or Pentostan®) are used, which are first-line medications and are administered parenterally. These medications are not ideal due to their toxicity, the hospitalization requirement for their administration and the serious side effects that include pancreatitis, cardiotoxicity and hepatotoxicity [5]. Likewise, the ineffectiveness of these treatments has been reported due to the resistance that the parasites have generated to the medicine such as the loss of capacity to reduce metals, decrease in the entry of the drug, among others [6]. As second-line medications are the miltefosine known as Impavido® which is administered orally and amphotericin B deoxycholate commercially known as Fungizone® administered parenterally. These medications do not completely eliminate the parasite from the organism, which leads to new occurrences of the disease, in addition, they are expensive medications with numerous side effects [7].

Therefore, due to its level of importance in public health, it is necessary to advance in new strategies that allow formulating drug alternatives for the treatment of this disease, such as nanotechnology, which currently has played a very important role presenting satisfactory results for *in vitro* studies of leishmaniasis [8]. Some cases of nanostructured drug delivery systems are nanoemulsions, solid lipid nanoparticles, polymeric nanoparticles, inorganic nanoparticles (NPs) and liposomes, which improve the characteristics of solubility, increase the therapeutic efficacy of the drug and decrease the side effects of different types of drugs [9].

On the other hand, the *in vitro* leishmanicidal activity of metal nanoparticles has been reported, among which stand out the silver nanoparticles (AgNPs), which are characterized by their antimicrobial properties, their small size, easy preparation, low cost and their multidirectionality of action that precludes the formation of microbial resistance [10]. However, there are properties that limit its application, among which are its hemolytic properties and its high non-specificity to interact with various molecular systems at the cellular level [11, 12].

For its synthesis, stabilizing agents that prevent particle aggregation by mechanisms of electrostatic interaction and/or steric hindrance between the stabilizing agent and AgNPs are commonly employed. An example of the first stabilization mechanism is achieved with sodium citrate molecules which surround the nuclei of Ag generating around it a negative charge density that leads to repulsion between the silver nuclei [13, 14]. While an example of the stabilization mechanism due to steric hindrance is presented by using poly(vinyl pyrrolidone) as a stabilizing agent, which achieves its function because it is a polymer with high molecular weight and has several carbonyl groups that stabilize the Ag nuclei through coordination bonds [15,16].

The effect of the stabilizing agent on antimicrobial and hemolytic properties is not well known. However, different researchers have reported that properties such as size, surface charge, acidity, basicity, susceptibility to ionic strength, specific reactivity with different molecules and permeability through the cell wall of the microorganism can be modulated depending on the type and quantity of stabilizing agent used in order to regulate both properties. For example, Tiwari *et al* (2014) and Berrio *et al* (2017), reported differences in the antimicrobial properties of AgNPs stabilized with sodium citrate and with poly(vinyl pyrrolidone) or PVP, due to the nature of the interaction between the stabilizing agent and the silver nuclei, since when found to media where ionic strength and pH change, the adsorbed citrate can be eliminated by neutralizing the charges losing their effect as a stabilizer, and therefore its antimicrobial and hemolytic properties, an aspect that does not happen with PVP [15, 17].

By the above, the objective of this research was to study the effect of different stabilizing agents

specifically, PVP, sodium citrate, *N*-methyl-D-glucamine and meglumine antimonate on the antimicrobial and hemolytic activity of AgNPs in concentration function. Thus, results are a first indicious for the development of new strategies against of leishmaniasis because bacterial coinfection have been described into different skin wounds associated with leishmaniasis.

2. Experimental section

2.1 Reagents

Silver nitrate (AgNO_3 ; 98 %; Aldrich), poly(vinyl pyrrolidone) (PVP; 40 kDa, Aldrich), sodium citrate (SC; 99 %; Aldrich), *N*-methyl-D glucamine (NMDG; 99 %; Aldrich) and sodium borohydride (NaBH_4 ; 98 %, Aldrich) were used as precursors for the synthesis of AgNPs. Potassium (V) hexahydroxoantimonate ($\text{K}[\text{Sb}(\text{OH})_6]$; Aldrich), acetone (Aldrich) and NMDG were used to obtain the meglumine antimoniate (MA). Additionally, *Escherichia coli* and *Staphylococcus aureus* (UCO 2297, from strain collection of GI-CAT research group of Universidad del Valle, were used as bacterial models. Barium chloride (BaCl_2), sulfuric acid (H_2SO_4 , Merck), Mueller-Hinton agar (Aldrich) and tryptic soy broth (Thermo Fisher Scientific) were used in the antimicrobial tests. For hemolytic tests it was used a saline solution phosphate buffered (DPBS) made from dipotassium phosphate ($\text{K}_2\text{H}_2\text{PO}_4$, Merck), potassium chloride (KCl, Merck), sodium chloride (NaCl ; Merck) and dibasic sodium phosphate (Na_2HPO_4 ; Merck). Potassium hexacyanoferrate trihydrate ($\text{K}_3\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$, Merck), potassium cyanide (KCN; Merck) and sodium bicarbonate (NaHCO_3 ; Merck) were used for the preparation of Drabkin reagent.

2.2. Synthesis and characterization of the meglumine antimoniate (MA)

MA was synthesized by the method described by Demicheli with some modifications [18]. For this, 0.004 moles of NMDG were dissolved with 0.004 moles of $\text{K}[\text{Sb}(\text{OH})_6]$ in 25 mL of water with constant stirring, it was brought to a pH of 7 and heated at 60 °C for three hours. Subsequently, precipitation was induced by adding 75 mL of cold acetone in an ice bath and the solid generated was

filtered and dried at 90 °C. It was characterized by Fourier-transform infrared spectroscopy (FTIR, Shimadzu FTIR-8400) using the potassium bromide (KBr) pill method; by proton nuclear magnetic resonance spectroscopy (^1H -NMR, Bruker 400 MHz Ultrashield); Elemental analysis (AnalyzerFlashen 1112 Series) and atomic absorption spectrometry (Perkin Elmer Analyst 100) and the percentage yield was determined by gravimetry. These results were contrasted with the commercial active ingredient present in the drug Glucantime®, which was obtained by drying at 90 °C.

2.3. Synthesis and characterization of AgNPs

AgNPs were synthesized in triplicate using the chemical reduction method of silver salts described by Berrio *et al.* [15]. To this end, different molar ratios AgNO_3 :PVP were added separately in a beaker, these being 1:1, 1:3, 1:6 and 1:10. The mixtures were stirred for 15 minutes and then added 0.75 mL of the NaBH_4 solution (10 mM), dropwise, with constant stirring and in an ice bath for 1 hour. The dispersions obtained were characterized by visible ultraviolet spectrophotometry (UV-Vis, UV-1700 Pharmaspec Shimadzu), FTIR and dynamic light scattering (DLS, Zetasizer 90). For characterization by FTIR it was necessary to perform a previous centrifugation a 15000 rpm and subsequent drying at 90 °C. This procedure was repeated replacing the PVP with SC, NMDG and MA as stabilizing agents where the reproducibility of the synthesis and its stability were studied as a function of time over a period of 0, 24 and 72 hours. AgNPs stabilized with MA synthesized were characterized further by atomic absorption spectrometry.

2.4. Evaluation of antimicrobial activity

The antimicrobial activity of the synthesized AgNPs was determined from the measurement of their ability to inhibit bacterial growth in both Gram-negative and Gram-positive bacteria by the turbidimetry method [19]. For this, a calibration curve was prepared in the McFarland scale of 1.0 to 10.0 CFU/mL from a suspension of barium sulfate prepared with BaCl_2 (0.048 M) and H_2SO_4 (0.18 M) and then its turbidity was measured by UV-Vis at 625 nm. Subsequently, each of the

synthesized AgNPs, as well as the stabilizing agents, were dispersed in triplicate over 5 mL of trypticase soy broth in a concentration of 30 g/L. Then, 100 μ L of bacterial dispersion was inoculated on the previously sterilized broth and each of the solid (0.05 g) and liquid (1.0 mL) samples were deposited. Finally, the systems were incubated at 37 $^{\circ}$ C for 24 hours and bacterial growth was determined by UV-Vis at the same wavelength. The measurements were evaluated against *Escherichia coli* as a model of Gram-negative bacteria and *Staphylococcus aureus* as a model of Gram-positive bacteria. In addition, amoxicillin was used as a positive control and sorbitol as a negative control.

2.5. Evaluation of hemolytic activity

This test was performed following ASTM F756-13, in order to determine the hemolytic index of nanostructured systems on blood cells using the method of cyanomethahemoglobin (CMH) [11]. For this, a hemoglobin solution of 10 mg/mL was prepared in DPBS from a blood sample with a hemoglobin concentration of 139 mg/mL obtained at a local clinical laboratory. Then, 7 mL of this solution was taken and deposited next to the liquid samples (1 mL) and solid samples (50 mg) in test tubes. Then, the systems were incubated for 4 hours at 37 $^{\circ}$ C and centrifuged at 150 gravities for 15 minutes. Subsequently, the supernatant was taken, mixed with 1 mL of Drabkin's solution and quantified by UV-Vis at 540 nm using a calibration curve between 0.5 and 10 mg/mL of hemoglobin in the Drabkin reagent. The procedure was performed in triplicate and acetic acid and mannitol were used as positive and negative control, respectively.

3. Results and discussion

3.1. Synthesis and characterization of meglumine antimoniate

The infrared spectra of the $\text{KSb}[\text{OH}]_6$, NMDG and MA was graphed in Figure 1. Thus, in the spectrum of the precursor $\text{KSb}[\text{OH}]_6$ are observed a band associated with the stretching vibration of the -OH bonds in 3169 cm^{-1} and a band of the Sb-O about 594 cm^{-1} . On the other hand, in the NMDG spectrum there are three characteristic bands associated to the stretching vibration of the -

NH bonds, flexural vibrations by scissoring of the -NH bond and vibrations of the stretching of the -CO bond, around 3362 cm^{-1} , 1629 cm^{-1} and 1100 cm^{-1} , respectively. While in the spectrum of the MA a band is associated with the flexural vibrations by scissoring of the -NH bond in 1629 cm^{-1} and another band around 3459 cm^{-1} is related to the stretching vibrations of the -OH bonds which, compared with the bands around 3000 cm^{-1} of the precursors, suffered a shift to higher wave numbers as a result of the strengthening of the free OH bonds of the NMDG and of the $\text{KSb}[\text{OH}]_6$ that did not participate in the formation of the coordination complex between the hydroxyls in *cis* position of the NMDG that act as electron pair donors and the metal Sb that thanks to its empty *d* orbitals acts as electron acceptor to finally forming a coordination bond as shown in Figure 2 [20]. Additionally, the greatest evidence that the meglumine antimoniate molecule was effectively synthesized is the Sb-O bond band at 617 cm^{-1} .

Figure 1. Infrared spectra of the precursors $\text{KSb}[\text{OH}]_6$ and NMDG, and of the final product MA. (str. is referred to stretching vibration and sci to scissoring vibration).

The synthesized product was characterized further by $^1\text{H-NMR}$ obtaining the spectrum shown in Figure 2 where the following signals are evidenced: a peak around 2.74 ppm associated with the CH_3 protons denoted as "a"; followed by a multiplet in the range of 3.03 to 3.35 ppm for hydrogens b_1 and g_1 , as well as a second more complex multiplet in the range of 3.43 to 4.08 for hydrogens d, e, f, b_2 and g_2 . The superposition of signals in this region is due to the existence of different microenvironments in the structure, since authors such as Demicheli, Kato and Roberts, together with their collaborators, have reported that as a product of this synthesis, several complexes are obtained in general $(\text{NMDG-Sb})_n\text{-NMDG}$ where n varies from 1 to 4, so it is possible to obtain oligomers and a mixture of complexes with different sizes [18, 20, 21]. For this reason, despite the wide use of the MA for more than half a century, its mechanism of action, molecular structure and binding sites have not been completely elucidated. Additionally, in the same spectrum is evidenced a peak at 4.17 ppm belonging to hydrogen c, as well as a peak at 4.27 ppm displaced at low field for the hydrogens labeled "h" of the hydroxyl groups bonded to the Sb; and finally two doublets at 4.42 and 4.39 ppm are observed for the hydrogens assigned as i and j. Finally, in order to corroborate the correct synthesis of MA, the elementary analysis of this molecule was contrasted with the active ingredient of the Glucantime® drug, the results of which are tabulated in Table 1 and allows to observe an insignificant difference between the percentages of C, H, N, S and others, taking into account that the values obtained are relative to the amount of sample analyzed. This is confirmed by calculating the C/H, H/N, C/N and C/other ratios of both molecules where it is observed that the values of the four relationships coincide. The first three relationships indicate the presence of NMDG, while the C/other ratio shows that the proportion of oxygen and antimony together with the amount of C are the same in both cases. Likewise, the absence of sulfur is evidenced where it is confirmed that the medicine does not contain the excipients related to SO_4^{2-} after evaporation, stating that the molecule obtained corresponds to the active substance of the medicine. The percentage of yield of the synthesis of MA was 73.3 %, this percentage may be associated with

losses of analyte in the filtration and washing of the product obtained.

3.2 Synthesis and characterization of AgNPs

AgNPs synthesized with each of the different stabilizing agents (PVP, SC, NMDG and synthesized MA) were initially characterized by FTIR-ATR in order to determine the functional groups present in the nanostructures. The spectra obtained are plotted in Figure 3.

In the four spectra of Figure 3 bands in common are observed, such as the band around 3300 cm^{-1} associated with the presence of $-\text{OH}$ groups of the solvent that was not completely evaporated and stretching vibrations of the $-\text{CH}$ bond around 2898 cm^{-1} while in the spectrum of AgNPs stabilized with PVP the greatest evidence of the presence of this polymer is the band corresponding to the stretching vibration of the carbonyl group in which

Figure 2. $^1\text{H-NMR}$ spectrum and possible structure of the MA synthesized molecule.

Table 1. Elemental analysis of active principle: Meglumine antimoniate

Sample	C	H	N (%)	S	Other
Glucantime®	24,9	5,2	4,1	0,0	65,8
MA	18,9	4,3	3,4	0,0	73,4

Figure 3. FTIR spectra of AgNPs stabilized with PVP, SC, NMDG and MA, respectively.

a displacement at lower wave numbers is observed due to oxygen coordination with the atoms of Ag^0 , in addition to the evidence of the $-\text{CN}$ bond around 1172 cm^{-1} and a band associated with the vibration of the $\text{Ag}-\text{O}$ bonds in 636 cm^{-1} [22]. Likewise, the characteristic bands of the SC can be observed in AgNPs stabilized with these molecules, such as, the symmetric and asymmetric stretching vibration of $\text{C}=\text{O}$ at 1616 cm^{-1} and 1387 cm^{-1} and, again, the presence of the band attributed to the $\text{Ag}-\text{O}$ bond in 603 cm^{-1} . In the spectrum of NPs stabilized with NMDG, bands associated with the vibrations of the secondary amine at 1600 cm^{-1} and stretching vibrations of the $-\text{CO}$ bond at 1074 cm^{-1} are observed. And, finally, in the spectrum of AgNPs stabilized with MA, are observed bands corresponding to the stretching of the $-\text{CO}$ bond in 1035 cm^{-1} and the vibration with respect to the Sb-

O bond in 873 cm^{-1} [18]. This characterization is evident in each case, the presence of the stabilizing agent together with the AgNPs even after the centrifugation process that is carried out in order to eliminate the excess of stabilizer present in solution.

On the other hand, the correct synthesis of AgNPs was evidenced by UV-Vis spectroscopy, following the band around 400 nm associated with the phenomenon of surface plasmon resonance (SPR) as a result of the collective oscillation of the electrons found in the surface layer of AgNPs [23]. In addition, the reproducibility of the process of obtaining AgNP was studied taking into account that each synthesis was performed in triplicate. The results are plotted in Figure 4.

From the spectra of Figure 4 it is important to highlight several aspects, the first one, that the synthesis method is reproducible as long as the concentrations of the reagents, the stirring speed and the addition of NaBH_4 are controlled, as was done during the development of the proposed methodology. In addition, the only NPs that presented asymmetry in the Gaussian curve associated with the SPR were the AgNPs stabilized with NMDG due to a greater polydispersity of sizes since the shape of the SPR absorption band is dependent on the interaction between electromagnetic radiation and the colloidal particles in solution, and in turn with the shape, size and distribution of said particles.

This fact was evidenced in Table 2, where the hydrodynamic radio values associated with the size of the AgNPs were reported, presenting a greater standard deviation in the sizes of the AgNPs obtained with NMDG. So, the greater the polydispersity of sizes, this interaction could be affected and the result would be reflected in a change in the shape of the absorption band.

The above was seen qualitatively in Figure 5 where the color differences between the AgNPs obtained with NMDG as a stabilizing agent are evidenced, since the dispersions for the 1:1, 1:3 and 1:6 ratios presented a color gray, however, for the 1:10 ratio they presented a yellow color (as with the AgNPs obtained with the other stabilizing agents), a fact that was attributed to a greater stabilization of the nanoparticles, and implicitly a lower polydispersity of sizes, due to the increase in the concentration of NMDG with respect to the amount of silver. Thus, in the case of the 1:1, 1:3 and 1:6 molar ratios

Figure 4. UV-Vis spectra of AgNPs with PVP, SC, NMDG and MA in molar ratios Ag⁺:stabilizing agent 1:1, 1:3, 1:6 and 1:10 for the reproducibility study.

Table 2. Wavelength values (λ) of the SPR and size distribution of the AgNPs.

Sample	Molar ratio	λ (nm) 0 hours	λ (nm) 24 hours	λ (nm) 72 hours	Radio (nm)
AgNPs:SC	1:1	394,0	393,5	394,0	24,4 ± 6,8 & 91,3 ± 18,5
	1:3	394,0	394,5	395,5	21,0 ± 5,3 & 91,3 ± 29,1
	1:6	396,5	397,5	400,5	32,7 ± 9,7 & 190,0 ± 67,7
	1:10	395,0	396,5	399,5	21,0 ± 5,8 & 106,0 ± 30,8
AgNPs:NMDG	1:1	390,0	390,5	391,0	5,6 ± 1,9 & 68,1 ± 46,4
	1:3	386,5	389,0	389,0	2,7 ± 0,7 & 43,8 ± 29,9
	1:6	385,5	387,5	388,5	2,3 ± 1,5 & 58,8 ± 38,5
	1:10	394,0	396,0	396,0	15,7 ± 3,9 & 106,0 ± 37,4
AgNPs:MA	1:1	403,5	403,5	403,5	11,7 ± 4,1 & 106,0 ± 54,1
	1:3	408,5	409,0	409,0	18,2 ± 5,8 & 122,0 ± 61,8
	1:6	408,5	408,5	408,5	24,0 ± 5,8 & 122,0 ± 36,7
	1:10	408,5	408,0	406,5	6,5 ± 2,1 & 91,3 ± 53,2

Figure 5. Dispersions of AgNPs using different molar relation Ag:NMDG 1:1, 1:3, 1:6 and 1:10.

Figure 6. UV-Vis and DLS spectra of AgNPs synthesized with PVP in molar ratios Ag:PVP 1: 1 (A), 1: 3 (B), 1: 6 (C) and 1:10 (D) as a function of time: 0 hours (red line), 24 hours (blue line) and 72 hours (black line).

it was possible that the amount of NMDG was not sufficient to obtain a narrow size distribution and the dispersion tended to undergo agglomeration processes between the nuclei of silver, hence they will have a gray color, since, the closer the AgNPs are, the conduction electrons on their surfaces tend to relocate and are shared between them, decreasing the frequency necessary for the resonance process and implicitly modifying the optics properties of the dispersion of AgNPs [24, 25]. Whereas, when the 1:10 ratio between Ag:NMDG was used, it was possible to achieve a sufficient stabilizing agent concentration to obtain a narrow size distribution, better stabilization and avoid agglomeration premature nanoparticles.

The study of the stability of AgNPs as a function of time was carried out by monitoring the SPR behavior of NPs within 0, 24 and 72 hours. In addition, their size distributions were determined by the phenomenon of dynamic light scattering (DLS) using the Stock-Einstein law where the particle size is related to the diffusion coefficient in the dispersing medium. These results are presented in Figure 6 for the case of NPs stabilized with PVP, using the molar ratios Ag:PVP 1: 1, 1: 3, 1: 6 and 1:10, and the results of AgNPs stabilized with SC, NMDG and MA are tabulated in Table 2.

The UV-Vis spectra of Figure 6 show a slight decrease in the intensity of the maximum absorption of SPR at 72 hours related to the destabilization processes and the slow growth of AgNPs. And on the other hand, the DLS of the NPs allowed observing values of hydrodynamic radii around 255 nm for the 1:1 and 1:10 Ag:PVP ratios, and around 220 nm for the 1:3 and 1:6 ratios. This difference in sizes is associated with the concentration of stabilizing agent in the system and the conformation of the polymer chains in aqueous solution, being that, as the amount of stabilizing agent increased, a greater stabilization of the AgNPs and a greater smaller size of these, hence the size of the AgNPs in the 1:3 and 1:6 molar ratios compared with the 1:1 molar ratio. However, PVP being a low hydrophilic polymer tends to acquire a closed chain conformation in aqueous solution and prefers to interact with itself rather than with the solvent, it being possible that by sufficiently increasing the concentration of PVP in the system as in the 1:10 ratio, interactions between the polymer chains were promoted and the interaction with the Ag nuclei in aqueous solu-

Table 3. Sb content in the synthesized MA and in the AgNPs:MA.

Sample	Sb (ppm)
MA synthesized	923,75 ± 0,15
Ag:MA 1:1	30,00 ± 0,25
Ag:MA 1:3	103,59 ± 0,07
Ag:MA 1:6	171,00 ± 0,07
Ag:MA 1:10	290,25 ± 0,15

tion was decreased, thereby reducing the stabilization capacity and increasing their size [26]. Table 2 summarizes the displacement of the SPR as a function of concentration and time, as well as the values of the size distributions of the AgNPs. From this table, the bimodal distribution in the sizes of AgNPs can be highlighted when SC, NMDG and MA were used as a stabilizing agent. This was attributed to the fact that at the beginning of the reaction before adding NaBH₄, stabilizing agent was in solution with Ag⁺ ions from AgNO₃. Therefore, at that moment the Ag⁺ ions managed to form two types of molecular species with the stabilizing agents, one associated with electrostatic type interactions and another associated with coordination bonds. The first type of species promotes the formation of AgNPs with low average size and narrow size distribution, while the second tends to promote AgNPs with high average size and wide size distribution [27, 28]. Thus, and in contrast to the distribution of sizes when using SC, NMDG and MA, in the case of the PVP a unimodal size distribution was obtained, since it does not have a purely electrostatic nature and achieves the stabilization of the AgNPs through the formation of only coordination bonds.

The maximum absorption bands associated with the phenomenon of SPR, characteristic of AgNPs, are also observed, where it is evidenced that it does not vary significantly in time because the stabilizing agent continues to fulfill its function in the silver nuclei. However, small displacements are seen in the maximum absorption wavelength due to dispersions. To explain this, the synthesis of AgNPs can be described by two stages, a first stage of nucleation where seeds are produced and a second stage of growth or aggregation in the transfer of atoms of Ag to the surface of the nucleus [29]. According to Mie's theory, if the number of particles changes during the growth stage, then the surface plasmon band changes to longer wavelengths due to the increase in particle

size generated in the growth process [26]. Thus, as the size, the particles begin to suffer a destabilization due to the agglomeration and precipitation of the groups of particles caused by the large surface area and the forces of Van der Waals, thus forming unstable aggregates that then reflect a lower number of stabilized NPs and a decrease in absorbance intensity.

3.3 Characterization of AgNPs:MA by atomic absorption spectrometry

Table 3 shows the concentrations of antimony (Sb) in the synthesized MA, as well as in the AgNPs obtained when they were stabilized with said molecule. For this, a calibration curve between 5 and 30 ppm of Sb was used where the absorbance value (Abs) was related to the amount of antimony present in solution ([Sb]), obtaining as an equation of the line, Abs = 0,0079 * [Sb] - 0.0014, with an R² of 0.9991.

The results in Table 3 complement the characterization of item 3.1 for synthesized MA since the presence of Sb in said molecule is confirmed thanks to coordination with the NMDG. However, the percentage of Sb in the sample was 25.6 % per sample mass, which when compared with the theoretical value of Sb in the sample (33.3 %), gives a relative error of 23.1 %, since as mentioned above, it is possible that NMDG forms different oligomers with Sb, not necessarily in an equimolar relationship, which explains the default error of Sb. In addition, the presence of Sb in the dispersion of AgNPs stabilized with MA and an increase in their concentration as the proportion of stabilizing agent increases.

3.4 Evaluation of the antimicrobial activity of AgNPs

The ability to inhibit the growth of *E. coli* and *S. aureus* by each of the synthesized NPs is plotted in Figure 7 where it can be seen that the percentages of inhibition for AgNPs stabilized with PVP against *E.coli* are related to the low mobility of AgNPs in the bacterial cell membrane since a polymer was used that possibly generated a thick bilayer around it as it achieved its stabilization by steric hindrance. However, the 1:3 and 1:6 Ag:PVP ratios obtained high percentage inhibition values since they were smaller NPs allowing easier the

Figure 7. Percentage of inhibition of bacterial growth of AgNPs against A) *E. coli* and B) *S. aureus*.

migration through the cell membranes, otherwise with the ratios 1:1 and 1:10 where larger NPs were obtained. AgNPs with smaller sizes contain a larger surface area that allows them to easily come into contact with the bacteria membrane and with the environmental environment because they contain a greater amount of Ag^+ concentration per unit volume.

In the case of SC-stabilized AgNPs, an increase in antibacterial activity was evident for both *E. coli* and *S. aureus* as a function of the concentration of SC used due to the chelation capacity of this molecule. The chelating capacity of citrate towards metal ions such as Mg^{2+} and Ca^{2+} has been reported, metals that are essential for the absorption of nutrients by microorganisms for their

metabolism [31]. Chelation of these metals could cause an alteration in the permeability in the wall cell and subsequently, the cell death of the microorganism.

Likewise, an effect of the concentration of NMDG with the antimicrobial activity was evident, since the amine of the NMDG at pH 7 is protonated which gives it electrostatic attraction with the phosphate groups found in the cell membrane of the bacteria, more exactly the phosphates of the lipopolysaccharides of *E. coli* and the phosphates of the teichoic acids of *S. aureus* [32]. Also, this mechanism is used to explain the potential antimicrobial activity of AgNPs:MA where percentages above 60 % were obtained taking into account that in the NPs there was not only Ag, but also Sb, which was coordinated with the hydroxyl of the NMDG. The effect of Sb on microorganisms is attributed to the reduction of Sb (V) to Sb (III) which generates a change in cell redox potential causing an imbalance of the bacterial homeostasis [33]. In addition to evidencing an electrostatic interaction, Ag^+ ions can generate an inhibition of DNA replication, disturb the electrical potential and the interaction of AgNPs with the thiol groups of enzymes, which leads to the degeneration of the microorganism's metabolism [34]. These results show a clear dependence on size, concentration and nature of the stabilizing agent with the antimicrobial activity of AgNPs against *E. coli* and *S. aureus*, which is in accordance with literature [35, 36]. Likewise, it was appreciated that the percentages of bacterial growth inhibition were higher in the case of *S. aureus* compared to *E. coli*. This fact was mainly attributed to the difference in the composition of the cell membrane of these microorganisms. Thus, *S. aureus* is a Gram-positive bacterium that has a thick outer layer of peptidoglycans inside its cell membrane, which are functionalized polysaccharides, with amino acids such as alanine and glutamic acid, which due to its structural characteristic makes it possible to interact with the stabilizing agents (coating) of the synthesized AgNPs. For example, the PVP contains an amido group capable of forming hydrogen bonds with the functional groups of peptidoglycans such as $-\text{COO}^-$, $-\text{NH}_3$ and $-\text{OH}$, as well as the presence of London dispersion forces between the carbon chains of the PVP with the polysaccharides of the peptidoglycans. In the case of SC, carboxylate groups play the role of

hydrogen bond acceptors, promoting interaction with said groups. Finally, the presence of –OH groups in the NMDG and MA allow to contemplate the presence of hydrogen bonds, as well as the electrostatic interactions between the amino groups and the phosphates of the teicoic acids, increasing the effect on the inhibition percentages [37].

On the other hand, *E. coli* (Gram-negative) does not have peptidoglycans in its external part, but rather a membrane of phospholipids and lipopolysaccharides, where lipids give the membrane a hydrophobic character because its chemical structure is fundamentally hydrocarbon (aliphatic and aromatic) with a large number of C–H and C–C bonds that decrease the dipole moment of the structure. In order for an interaction with this part of the membrane to be achieved, it is necessary that the AgNPs be smaller in order to enter the lipid bilayer and in addition, they must have a hydrophobic surface, which was not present in the majority of the AgNPs synthesized at except for AgNPs stabilized with PVP [38].

3.5 Evaluation hemolytic activity

Figure 8 shows the results of the evaluation of hemolytic activity for each of the nanostructured systems developed, as well as that of the stabilizing agents used and the negative control (mannitol) in order to guarantee the efficiency of the method used.

The positive control (acetic acid) presented a hemolysis percentage of 60.33%, and was not reported in the figure because it was not used to explain the results, but to verify the optimal conditions of the experiment.

According to the American Society for Testing and Materials (ASTM) [39] and the results of Figure 8, it is possible to infer that AgNPs stabilized with MA in all their molar relationships, as well as AgNPs stabilized with NMDG in ratios 1:1 and 1:10 and those stabilized with SC in molar ratios 1:6 and 1:10 presented hemolysis percentages lower than 2 %, considered as non-hemolytic materials. It is important to note that the lowest hemolysis values in general were obtained for the ratios Ag:Stabilizing agent 1:10 for all AgNPs, because at higher concentrations of stabilizing agent, the nanoparticle is trapped by the agent molecules, limiting its release to the medium and

implying little contact with the red blood cell membrane [43].

The other nanostructured systems were classified as moderately hemolytic materials (between 2 and 5%) because the stabilizing agents used for the synthesis of AgNPs do not have hemolytic activity and show an effect on AgNPs. The differences in hemolysis percentages are associated with the physicochemical properties of AgNPs and these in turn of their size, as reported in the literature, where is described the existence of an inverse relationship between size and hemolytic activity of these materials [38]. These results are congruent for AgNPs stabilized with SC in ratio 1:3, where the highest percentage of hemolysis and the smallest sizes were obtained compared to the other ratios used. This is because the dispersions of smaller radius NPs have a greater surface area and therefore a greater contact with the membranes of the red blood cells allowing their superficial adsorption [40, 41]. As all nanostructured systems were in the range of 100 nm to 200 nm radius, this size property promoted a decrease in the contact of AgNPs with red blood cells due to a lower concentration of AgNPs per unit volume.

Another of the stabilizing agents used in the synthesis was the PVP that is considered hemocompatible, inactive and does not present toxicity against the organisms [41]. The hemolytic activity of the PVP stabilized AgNPs is associated with the release of Ag, which interact with the groups thiol of transmembrane proteins found in red blood cells causing their destruction and subsequent release of hemoglobin into the medium [40]. However, a decrease in hemolysis was evidenced by increasing the content of PVP in AgNPs associated with the immobilization of NPs in the polymeric networks of PVP inhibiting contact with red blood cells.

Likewise, the hemolysis percentages obtained for AgNPs with MA and NMDG may be the result of the electrostatic interaction between the positively charged amines of the molecules with the negative charge of the sialic acid found in the red cell membrane glycoproteins [42]. The pH of the medium is 7 which is above the pKa of sialic acid (3.9), which results in deprotonation of the carboxylic group in the molecule. However, around the red blood cell a diffuse double layer is formed with the ions of the medium in order to neutralize the charge, this effect prevents AgNPs

Figure 8. Percentage of hemolysis of AgNPs with their respective stabilizing agents.

from coming into contact with the membrane due to an electrostatic repulsion between ions and stabilizing agents [42]. The reason why ratios 1:3 and 1:6 AgNPs:NMDG not classified as non-hemolytic materials was related to the smaller size they presented compared to the ratios 1:1 and 1:10 AgNPs:NMDG.

4. Conclusions

Based on the results obtained, it is possible to conclude that the wet chemical reduction method is a simple and reproducible method for the synthesis of AgNPs that, in addition, allows the modification of AgNPs surfaces through the use of stabilizing agents of different nature as are the PVP, NMDG, SC and MA. Additionally, it was possible to modulate the surface properties of AgNPs by varying the molar relationships between the silver nuclei and the stabilizing agent. For example, greater antimicrobial activity was obtained for AgNPs stabilized with MA and NMDG as a result of the strong electrostatic interaction between the protonated amine and the phosphate groups of the bacteria membrane. Likewise, a decrease in the hemolytic activity of AgNPs was observed without affecting the antimicrobial activity as a function of the amount of stabilizing agent used. In consequence, using a high molar ratio of Ag:stabilizing agent for all cases, they showed a hemolysis value equal to or below the negative control.

Finally, it was possible to synthesize AgNPs with a new surface stabilizing agent, such as meglumine

antimoniate, with low hemolytic activity and with high antimicrobial activity, being a potential alternative against to the leishmaniasis by the combination of AgNPs and drugs commercially available for this objective. However, new experiments must be performed in order to evaluate this application.

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Conflict of interests

The authors declare that there are no conflicts of interest.

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