Adherence of amino acids functionalized iron oxide nanoparticles on bacterial models E. Coli and B. subtilis

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Abstract. Magnetic iron oxides nanoparticles (NPs) functionalized with lysine (Lys) and arginine (Arg) was obtained by following chemical co-precipitation route in basic medium. The synthesis was performed by mixing ferrous chloride (FeCl₂•4H₂O), ferric chloride (FeCl₃•6H₂O) and the specific amino acid in a molar ratio of 1: 2: 0.5, respectively. High pH sample was washed several times with distilled water to reach a pH similar to distilled water (Ph=7) after the synthesis process, part of the NPs obtained was dried. Of the measurements of XRD and MS was obtained that the samples are magnetic nanoparticles of maghemite of about 9 nm in diameter. Of the FTIR and zeta potential measures was obtained that the amino acids Lys and Arg were correctly functionalized at magnetic nanoparticles, referred to herein as M@Lys and M@Arg. In order to demonstrate the capture and adhesion of the nanoparticles to the bacteria, scanning electron microscopy (SEM) was performed. The obtained visualization of both bacteria shows that they are coated by the magnetic particles. In addition, M@Lys (B. subtilis) were cultured to verify the inhibition of growth measured by colony forming units (CFU), the concentrations of M@Lys were 1.75x10² g/mL and 0.875x10² g/mL. After the confrontation obtained efficiencies of 75.63% and 98.75% respectively for the third dilution. While for the fourth dilution were 90% and 98.57% respectively were obtained for each concentration of nanoparticles. Hinting that a high efficiency of bacterial capture at very low concentrations of NPs, which gives us a tool to capture nanobiotechnology bacteria in liquid cultures with application to capture them in wastewater. Based on our results we concluded that NPs functionalized with the amino acids Lys and Arg adhere to the bacteria efficiently in low concentrations.

1. Introduction

The traditional chemicals disinfectants such as sodium hypochlorite, ozone, antibiotics, among others commonly used for water disinfection are left behind due to the more than 600 by-products derived from these, many of them carcinogenic ([1] [2]). In addition, the use of antibiotic-based disinfectants generates resistance in the microorganism which implies the inversion and synthesis of new disinfectants with more potent or conjugated antibiotics which has been proven to have a side effect and an unfavorable impact on the surrounding biota ([3] [4]). According to the current regulation in Peru of the Estándares Nacionales de Calidad Ambiental para el Agua (D.S.002.2008 ECA AGUA), category 1: Population and recreational. It shows parameters and concentrations of permissible components in the water that reaches the population, including the category of coliforms where we
find thermotolerant coliforms, total coliforms, fecal enterococci, Escherichia Coli, parasitic forms, Giardia duodenalis, salmonella and Vibrio cholerae. In the aforementioned list we observed the presence of Gram-positives (V. cholerae) and Gram-negatives (E. coli, fecal enterococcus) bacteria. In this context, the functionalized magnetic nanoparticles (NPs) are a very interesting alternative because they have shown a great application potential in water treatment processes, especially for the bacteria elimination ([5]). In this study, magnetite NPs (Fe$_3$O$_4$) functionalized with two amino acids (arginine and lysine) denominated as Mgh@Arg and Mgh@Lys respectively were synthesized. In general, this functionalization will be referred as M@AA. The synthesis and functionalization of M@AA will be performed by the chemical route of co-precipitation ([6] [7]). The M@AA application tests were performed on the bacteria: Escherichia Coli (E. Coli) and Bacillus Subtilis (B. Subtilis), which have shown affinity for these nanomaterials functionalized with amino acids ([5]). This is a result of its organic composition of the cell wall. Both bacteria have been used as a models of Gram-negative and Gram-positive bacteria. The results obtained from the synthesis, functionalization and application of these functionalized nanomaterials, with the objective of being applied in the future in the capture of bacteria for the disinfection of water and giving a great scientific contribution in the basic research, lays the bases for further studies in the use and application of nanomaterials for an environmentally friendly industry. Also, the high efficiency of these processes reach scales of high impact not only environmental, but also social, such as for the reuse and cleaning of treated water used for irrigation, enhance the purification of water for human consumption and avoid the loss of this vital resource ([2] [8] [9] [10]).

2. Materials and Methods

Synthesis and characterization of the functionalized magnetic nanoparticles

The magnetic nanoparticles were synthesized in the Laboratory of Ceramics and Nanomaterials in the Faculty of Physical Sciences of the UNMSM, following the chemical method of co-precipitation synthesis in basic medium from ferric and ferrous salts. The obtained samples were characterized by X-ray diffraction (XRD), Mössbauer spectroscopy (MS), Z potential ($\zeta$) and infrared spectroscopy (FTIR), which were performed at the Faculty of Physical Sciences of the UNMSM, National University of Trujillo and Texas A&M University. Finally, a stock of functionalized magnetite nanoparticles was obtained at concentrations of Mgh@Arg (15.1 mg/ml) and Mgh@Lys (17.5 mg/ml), of which three concentrations were tested for Mgh@Lys of 1.75 and 0.875 mg/ml in bacterial culture.

Culture for Gram-positive bacteria (B. subtilis)

The culture of Gram-positive bacteria was prepared in a volume of 100ml of growth medium with 15 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl. Bacillus subtilis of the NRRL strain was used as the biological model of positive bacteria. Both cultures were homogenized at 200 rpm in a temperature-controlled homogenizer. Thereafter, E. Coli will be allowed to grow for a period of 16 hours at 37 °C while B. subtilis will be cultured for 32 hours at 30 °C.

Bacterial count

A volume of 10ml will be taken into conical tubes and centrifuged for 10 minutes at 4000 g at 4 °C. The obtained pellet will be isolated and washed three times with sterile saline (0.9% NaCl at pH 7) to remove all medium. After this, it is resuspended in saline solution and a stock of bacteria is separated
and cultured in growth nutrient agar. The quantification was performed by counting of CFU/ml. This procedure was repeated after the confrontation with the nanoparticles.

**Bacterial capture by nanoparticles**

The functionalized magnetic nanoparticles will be homogenized in physiological saline solution for the dispersion and conjugation of the bacteria, taking care of the pH values using HCl and NaOH. The bacterial capture will be performed in the shaker tempered at 200 rpm for 20 minutes.

**Quantification of post-exposure bacteria to functionalized nanoparticles**

In the same way in which the bacteria were quantified to verify their growth and viability, the bacteria were centrifuged, diluted and cultured in order to know the amount of CFU/ml and to verify the bacterial growth.

**Observation of bacteria captured by nanoparticles by Scanning Electron Microscopy**

During the bacterial capture procedure, 1 ml of solution was taken, which will be gradually fixed with 2% Glutaraldehyde (diluted with phosphate buffer). After the gradual exchange of saline solution by the fixative solution, the sample will be left for a period of 12h for the correct fixation of the bacteria so these do not lose their form for the visualization under the electron microscope of the contact of the bacterium with functionalized nanoparticles.

After 12h, the bacteria were washed with phosphate buffer pH 7, and after that the dehydration in the alcohol battery from 20 º, 30 º, 40 º, 50 º, 60 º, 60 º, 70 º, 80 º, 90 º, 100 º and finally by acetone, each of these steps was performed for 10 minutes each. The last two steps were repeated in duplicate.

After dehydrating the sample, it went through a process of desiccation at critical point where the sample already fixed and dehydrated loses the water completely, taking advantage of the critical point of the same 5 °C and the exchange by CO2. This procedure was carried out in the Laboratory of Specialized Equipment (LSE) of the Faculty of Biological Sciences of the UNMSM, in the desiccator at Critical Point EMS.

After the desiccation process was carried out at the critical point, the sample was completely dry. It was adhered to a metal support with self-adhesive conductive carbon tapes. Once the bacteria captured by the nanos on the surface of the support entered a process of coating with plasma gold, this process will be carried out with the LSE Metalizer of FBS-UNMSM, brand SPI supplies.

In the same laboratory was carried out the visualization of the bacteria captured by the nanoparticles in the Scanning Electron Microscope of the FEI Mark model Inspect S50, in high vacuum mode with the ETD detector to identify the respective ultrastructure.

3. Results and Discussions

**Characterization:**

**X-ray diffraction and Mössbauer spectroscopy**

X-ray diffraction (XRD) measurements were performed on a Bruker diffuser, model D8-Focus, with LYNSEIS detector, using copper Kalfa radiation with an average wavelength of 1.54184 Å. The sweep angle 2θ was 8 to 80 sexagesimal degrees with a pitch of Δθ = 0.01° and time of 2s per data, as shown in figure 1.
Figure 1. X-ray diffractogram of Maghemite with arginine (a) and lysine (b)

The analysis of X-ray diffractograms indicates the presence of maghemite ($\gamma$-Fe$_2$O$_3$) which has indexed the major peaks using PDF N° 39-1346 with widened lines in the samples covered with arginine (M@Arg) and with lysine (M@Lys). On the other hand, by performing a more detailed analysis on the peak (511) (Fig. 2), we see that in the samples there is a distribution of two sizes of crystallites: one of 8.5 nm and 19.2 nm in the Mgh@Arg and another one of 6.4 nm and 16.1 nm for the Mgh@Lys, which was calculated using the Debye-Scherrer equation.

The fitted to the peak (511) located at $2\theta = 57.43^\circ$ of the Mh@Arg used two Gaussians and with a $\chi^2$ of 1.346, with the following parameters to the first line of $Y_{01} = 21.88663$ for the base, $X_{c1} = 57.35115^\circ$ central position, the area $A_1 = 12.17818$ and the line width at half of the maximum height was $w_1 = 1.85978^\circ$ (red line) and for the second peak with $Y_{02} = 21.88663$, $X_{c2} = 57.47709^\circ$, $A_2 = 9.79644$ and $w_2 = 0.82529$ (green line). While the parameters for the Mgh@Lys, the adjustment had a $\chi^2$ of 1.556, whose parameters are for the first line $Y_{01} = 23.64279$, $X_{c1} = 57.30808^\circ$, $A_1 = 15.20828$ and $w_1 = 2.47269^\circ$ (red line) and $Y_{02} = 23.64279$, $X_{c2} = 57.46954^\circ$, $A_2 = 14.13105$ and $w_2 = 0.98138^\circ$ for the second line (green line).

Figure 2. Fitted with 2 Gaussian of the peak (511) of the Maghemite with arginine (a) and lysine (b)

The Mössbauer Spectroscopy (MS) measurements at room temperature were performed with a transducer with sinusoidal signal, a source of Co$^{57}$ and 1024 channels (Fig. 3). The fitted were made
with the software NORMOS and for the sample Mgh@Arg is shown a sextet with widened line widths typical of a magnetic field distribution (B_{hf}) due to the small particle size less than 100Å (10 nm), corresponding to a superparamagnetic relaxation. The distribution has a mean field B_{hf} = 46.7T and an isomer shift (IS) of 0.333 mm/s characteristic of Fe^{3+} of the maghemite, as shown in the upper part of figure 3a and, to the right, its distribution of hyperfine magnetic fields. For the sample Mgh@Lys was adjusted with a distribution of hyperfine magnetic fields with a mean field B_{hf} = 43.16T and with IS of 0.387 mm/s characteristic of Fe^{3+} as seen in the lower left of figure 3a and. to the right, its distribution of B_{hf} fields; the sextet corresponds to a magnetic field of 47.21T with IS of 0.325 mm/s, and a quadrupolar perturbation ε = -0.002 mm/s. When one compares the two Mössbauer spectra, we observe that the Mgh@Lys spectrum is more collapsed than the Mgh@Arg due not only to the small size of the particles, but also to the effect of the covering that affects the magnetic dipole interaction.

Figure 3. ME at RT from the Mgh@Arg in the the upper left, with Mgh@Lys at the bottom and to the right, their respective distributions of hyperfine magnetic fields.

**Culture of Gram-positive bacteria**

Centrifuge and obtain the bacterial pellet, which was resuspended in 10 ml of saline solution. *Bacillus subtilis* from the NRRL strain was washed three times with saline. Cultures obtained from the dilutions obtained averaged 9.5 x 10^6 CFU/ml.
Clash of bacteria with functionalized nanoparticles

When cultivating the third dilution under sterile conditions, *Bacillus subtilis* of the NRRL strain obtained a post challenge growth of 2.3 x 10⁶ CFU ml for the concentration of M@Lys 1.75 mg/ml; 1.2x10⁵ CFU/ml for 0.875 mg/ml, resulting 75.63 in efficiency and 98.75% efficiency in bacterial capture and inhibition of growth as seen in Fig. 4.

![Efficiency Graph](image)

Figure 4. The efficiency of *B. subtilis* growth inhibition at two concentrations of M@Lys is observed in the bar graphs, at concentrations of 1.75 and 0.875 mg/ml. For the third dilution before cultivation.

Cultivation of the fourth dilution under sterile conditions to *Bacillus subtilis* of the NRRL strain resulted in post-challenge growth of 9.5 x 10⁵ CFU/ml for the concentration of M@Lys 1.75 mg/ml; 1.4 x 10⁵ CFU/ml for 0.875 mg/ml, resulting 90% in efficiency and 98.57% efficiency in the bacterial capture and growth inhibition as seen in Fig. 5.

![Efficiency Graph](image)

Figure 5. The growth inhibition efficiency of *B. subtilis* is shown in the bar graph for two concentrations of NP@Lys at concentrations of 1.75 and 0.875 mg/ml. For the fourth dilution before cultivation.
Evidence of bacterial capture by Scanning Electron Microscopy

After the fixation, dehydration, drying and metallization of the samples, micrographs of E. coli were obtained, where it was successfully observed—as shown in Fig. 6 (a, b, c and d) where the bacterial population is visualized.—Many of them were accumulated by centrifugation, surrounded by the M@Arg nanoparticles.

Figure 6. Four microphotographs are observed a) 6000X at 10 μm scale, b) 6000X at 10 μm in which 4 bacteria (E. coli) with an average size of 1.8 μm are observed, c) 12000X at 5 μm of scale, d) 24000X at 5 μm scale where a bacteria of 2.240 μm is observed. All bacteria totally captured by M@Arg nanoparticles. The microphotographs were obtained at voltages between 20-30 kV, 2.62e-5 Torr pressure and with the ETD detector of the FEI-Inspect S50 Microscope.

On the other hand, B. subtilis was visualized in the same way by the Scanning microscopy. However, its visualization was faster due to its size as we can see in Fig. 7 and 8. The individuals of B. subtilis captured by the M@Lys nanoparticles can be seen.
Figure 7. The *B. subtilis* bacillus is observed at the center of the 5 μm scale image with an approximate size of 3,072 μm, captured by the M@Lys nanoparticles. The photomicrograph was obtained at 15000 magnification, 12.5kV, 1.37e-5 Torr pressure and with the ETD detector of the FEI-Inspect S50 microscope.

Figure 8. There are four microphotographs: a) 6000X at 10 μm of scale, three measurements of *B. subtilis* with an average size of 4.15 μm are observed, b) 12000X at 5 μm where *B. subtilis* is observed with a size of 3,706 μm, c) 24000X at 5 μm scale, d) 12000X at 5 μm scale where a 3,615 μm bacterium is observed, all the bacilli captured by the M @ Lys nanoparticles. The microphotographs were obtained at voltages between 20-30 kV, 4.15e-5 Torr of pressure and with the ETD detector of the FEI-Inspect S50 Microscope.
4. Conclusions

The M@Lys NPs showed more efficiency in the fourth dilution than the third dilution, this could be for bacterial saturation in the solution, also the affinity between M@Lys and gram positive bacteria. The results indicate that at very low concentrations of M@Lys and M@Arg, these can perform an efficient bacterial capture, which opens doors to the reduction of bacterial proliferation in liquid media—with low budget and easy laboratory manipulation—, in the particular case of the application to the bacterial capture in sewage.

5. References

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