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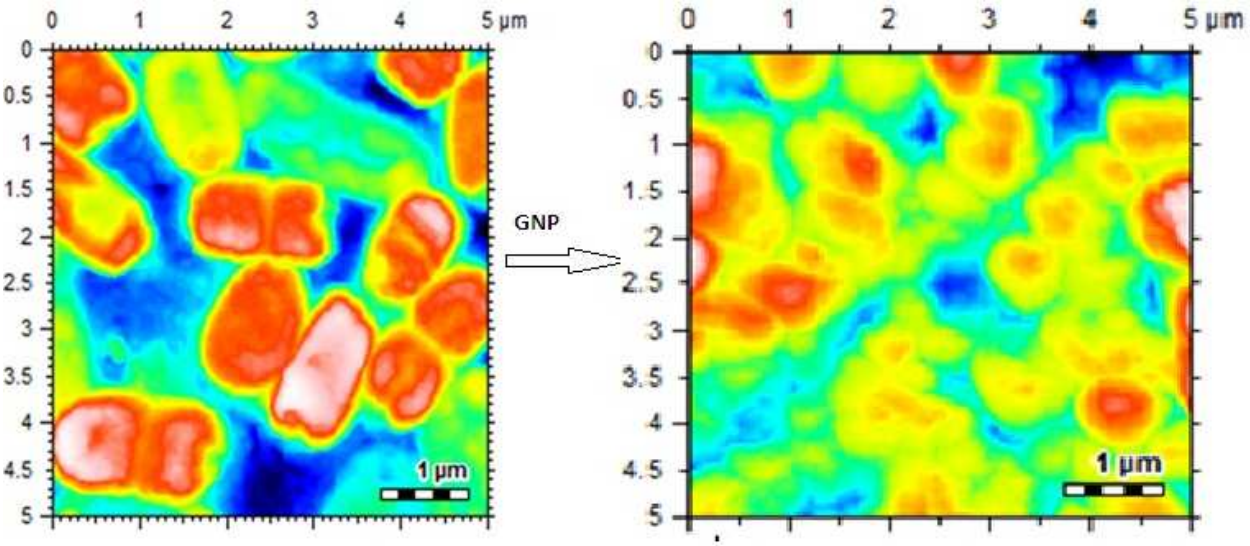
TOC

Monitoring the anti bacterial action of gold and silver nanoparticles via AFM



# Morphological analysis of the antimicrobial action of silver and gold nanoparticles stabilized with ceftriaxone on *Escherichia coli* using atomic force microscopy

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**ABSTRACT**

The antibiotic ceftriaxone was conjugated to Ag and Au nanoparticles. The activity of the conjugates against *Escherichia coli A*TCC 8739 was compared to that of pure ceftriaxone and of unconjugated nanoparticles using Atomic Force Microscopy and more conventional techniques such as the Agar well diffusion method. Conjugation to Ag nanoparticles increased the antibacterial activity of ceftriaxone by about 2 times, and conjugation to Au by about 6 times. Conjugation also appeared to improve the kinetics of the antibiotic. So, for example, membrane damage was barely evident two hours after contacting a cell culture with pure ceftriaxone. In the same time, Ag conjugates severely damaged membranes and Au conjugates completely disrupted the cell morphology.

**Keywords:** *Escherichia coli,* Ceftriaxone, AgNPs, AuNPs, Atomic Force Microscopy

## Introduction

Development of microbial resistant strains against antibiotics and metal nanoparticles are coming up with great imputes, in this regard antibacterial properties of metallic nanoparticles are most promising due to their large surface to volume ratio.[1] Metallic nanoparticles of bare zinc, titanium, copper[2] gold, magnesium[3] and silver are found effective against viruses, eukaryotic microorganism and bacteria. Silver nanoparticles exhibited fabulous antimicrobial potential,[1] and can be used in medicine for dental materials, burn treatment, water treatment, coating stain-less steel materials. The nanoparticles of noble metals are non toxic to human cells and have high thermal stability thus adding value to their anti-microbial potential.[4, 5] There is mounting evidence that conjugation of drugs to nanoparticles enhances their potency. Our own group had shown that conjugation of silymarin to Au nanoparticles results in complete protection from $CCl_4$-induced acute and chronic liver injury[6] and conjugation of a synthetic biocide, 2-4, Dihydroxybenzenecarbodithioic acid to Au nanoparticles results in an enhanced biocidal activity.[7] Ag nanoparticles of some antibiotics like amoxicillin, penicillin and vancomycin showed enhancing antibacterial effect against *S. aureus*.[8]Geoprincy et al. also showed enhanching antibacterial potential of Ag nanoparticles with antibiotics like chloramphenicol, amoxicillin, rifamycin and erythromycin against four major pathogens namely *Bacillus cereus*, *Bacillus subtilis*, *Klebsiellapneumoniea* and *Vibrio cholera.*[9] Conjugation of antibiotics like chloramphenicol, kanamycin, ampicillin and erythromycin to Ag nanoparticles had also shown increased antimicrobial activities against gram-positive and gram-negative bacteria.[10] These are important results, since they may lead to a reduction of the amount of drugs necessary to treat illnesses, thereby reducing side effects and development of drug-resistant bacteria strands. In this work, we show that conjugation to Au and Ag nanoparticles leads to an enhanced and more rapid antibacterial activity of ceftriaxone. Membrane damage was barely evident two hours after contacting a cell culture with pure ceftriaxone. However, in the same amount of time, Ag conjugates severely damaged membranes and Au conjugates completely disrupted the cell morphology. Atomic force microscopy (AFM) was employed for most of the characterization experiments. AFM is a highly suitable tool for the study of bacteria and its great advantage is that samples do not require fixation, conductive coating, or to be imaged in vacuum.[11] Applications of AFM imaging studies include imaging of bacterial nanostructures, genetic

variation and the study of antibacterial effects,[12, 13] making this technique extremely advantageous for the imaging of small highly delicate structures on bacteria.[14]

**Results and discussion**

The morphological analysis and kinetics study of the antimicrobial action of Ag and Au nanoparticles stabilized with ceftriaxone on *Escherichia coli* using AFM was investigated for the first time. The ceftriaxone belongs to 3rd generation antibiotic cephalosporins, which possesses potent activity (both *in vitro* and *in vivo*) against a broad range of bacteria.[15] In this study we used a sensitive strain of *E. coli* ATCC 8739, which is a human pathogen that can cause gastroenteritis, urinary tract infections, and neonatalmeningitis.[16] Synthesis of Ag and Au NPs stabilized with ceftriaxone was carried out in one pot by mixing ionic solutions of Ag and Au with ceftriaxone in the presence of triethylamine as reducing agent and ceftriaxone as stabilizing agent. Formation of Ag and Au NPs accompanied color changes in solution and monitored by UV-Visible Spectroscopy. The UV-Visible spectra of the Ag and Au conjugates (Figure 1) exhibited peaks at 408 and 538 nm, respectively, which can be reconciled with the characteristic plasmonic absorption of Ag and Au NPs.[17, 18] Ceftriaxone conjugation was also confirmed by FT-IR spectroscopy (Figure 2). Important absorption bands appeared in ceftriaxone FT-IR spectrum were 3426 $cm^{-1}$and 3264 $cm^{-1}$which could be assigned to stretching vibrations of N–H and O–H groups respectively, band at 2935$cm^{-1}$ is assigned to stretching vibrations of C–H group, stretching vibration of carbonyl groups (C=O) appeared at 1742 $cm^{-1}$and 1649 $cm^{-1}$ while band at 1537 $cm^{-1}$ is associated with the torsional vibrations of aromatic ring. The bands at 1383 $cm^{-1}$ and 1033 $cm^{-1}$ could be assigned to stretching vibrations of C–N and C–O respectively. Conjugation of ceftriaxone with Ag and Au metals result in merging of bands reduction in absorbance intensities of C=O (1742 and 1650 $cm^{-1}$), N–H (3426 $cm^{-1}$) and O–H (3264 $cm^{-1}$)

stretching.[19] The AFM was used for size determination of Ag and Au nanoparticles which were found to be around 10-50 and 20-40 nm respectively (Figure 3).

Ag and Au NPs conjugates were synthesize in order to enhance the antibacterial activity and kinetics against *E .coli* which was ignored in the past. Anacona et al. reported ceftriaxone transition metal complexes and its enhanced antibacterial activities,[20] Junejo et al. reported silver nanoparticles coated with ceftriaxone and studied its catalytic activity[21] while Savi et al. explored its antibacterial potential.[22] The antibacterial activity of the conjugates was evaluated with conventional biological methods and with AFM. The minimum inhibitory concentration (MIC) was calculated through zone of inhibitions[23] by using ceftriaxone and its Ag and Au conjugates. The MIC of pure ceftriaxone and its Ag and Au conjugates were found to be 2.1 ± 0.71 µg/mL, 4.1 ± 0.32 µg/mL (which correspond to ~ 1.06 mg ceftriaxone) and 4.3 ± 0.57 µg/mL (which correspond to ~ 0.34 mg ceftriaxone) respectively. While MIC of unconjugated Ag and Au NPs were found to be 48 ± 1.5 µg/mL and 73 ± 1.9 µg/mL respectively (Figure 4). AFM investigation validated the results of the zone of inhibition analysis. The MIC of pure ceftriaxone was around 2 times lesser as compared to its Ag and Au conjugates while unconjugated Ag and Au NPs exhibited very poor MIC values when compared with the pure ceftriaxone and its Ag and Au conjugates. Since ceftriaxone represented a small weight fraction of the conjugates (26 wt% for AgNPs and 8 wt% for AuNPs), which indicate that conjugates had a 2-6 times higher activity than pure ceftriaxone.

Untreated *E. coli* cultures exhibited cells with regular shapes and smooth membranes with mean length of 1.5 µm, mean width 0.95 µm and mean height of 0.3 µm, as shown in Figure 5. Cultures were then treated with ceftriaxone at doses of 1 mg and 5 mg respectively, and cell morphology was analyzed as a function of incubation time. The 1 mg sample exhibited rough surfaces after 2 hour (Figure 6 a). Morphology degradation increased with time, and after 8 hours cells were melted and completely degraded. The 5 mg sample exhibited a faster degradation, as shown in Figures 8 a and 9 a. Ceftriaxone-Ag conjugates were found to degrade cells faster than pure ceftriaxone. Ag conjugates in a 1 mg concentration (which corresponded to a ~0.25 mg ceftriaxone concentration) induced cell rupture already after 1 hour (Figures 7 a). A 5 mg Ag conjugates concentration led to melting and complete degradation in as little as 2 hours (Figures 8 b). Ceftriaxone-Au conjugates also degraded cells faster than pure ceftriaxone, as shown in

Figures 6 c, 7 b and 8 c. Significantly, unconjugated Au and Ag NPs at 5 mg, induced only marginal morphological changes even after long incubation time (i.e. >8 hours) as shown in Figure 9 b and c.

The nanoparticles can easily penetrate bacterial cell membrane by adhering to proteins in the cell membrane owing to high affinity of Ag and Au conjugates towards sulfur. Inside the cell the nanoconjugates can interact with protein, phosphorus and DNA and hence can disturb several vital functions and ultimately lead to the death of the cell. [5, 24-27]

The anti-bacterial action of nano-conjugates has been already investigated through high resolution imaging techniques. Morones et al [26] studied the antibacterial potential of silver NPs with transmission electron microscopy revealing ultra-structural features including the ultra-cellular organelles of *E.coli* but it only produced a snapshot in time when all *E.coli* were dead. In the present study atomic force microscopy produced three dimensional view of living *E.coli* by providing: detailed topographic description of shape and surface; phase imaging that allows to measure boundary stiffness, width, height and length.

**4. Conclusion**

In conclusion, Ag and Au nanoparticles stabilized with ceftriaxone were synthesized and characterized by UV-Visible, FTIR andA FM 10-50 and 20-40 nm respectively. Theresults indicated that the nanoparticles prepared were very stable. The antibacterial activity of the nanoparticles against *E. coli* ATCC 8739 was studied. The morphologies of the bacteria were examined using AFM after treatment with the free antibiotic and the nanoparticles stabilized with antibiotic. Quantitative measurements of surface integrity and analysis of increased surface roughness and changes in cell shape indicate that these nanoparticles killed *E. coli*, with higher efficiency compared with free ceftriaxone as indicated by images of morphological changes.

The present study though preliminary, provides useful insight to the improvement of novel antimicrobial nanoparticles. To expose the mechanism of the synergistic effect of chemically synthesized nanoparticles, more sophisticated experimental evidences will be needed. We believe that it will not be long before we will be able to explore morphological alterations in living bacteria while they are taking place.

**EXPERIMENTAL**

## Materials

Tetrachloroauric acid trihydrate ($HAuCl_4.3H_2O$) and silver nitrate was purchased from Merck, tri ethylamine from Scharlau and ceftriaxone were supplied by Pharmagen limited Lahore Pakistan. *Escherichia coli A*TCC 8739, provided by Industrial analytical centre (ICCBS) University of Karachi, Pakistan, was used to evaluate the antibacterial activity *in vitro*. Deionized water was used throughout experiment for the preparation of nanoparticles and further analysis.

## Synthesis of Ag nanoparticles (AgNPs)

A 1 mM solution of $AgNO_3$ and a 1 mM solution of ceftriaxone were prepared in deionized water. Two volumes of the stock solutions were mixed using a 8:1 Ag:ceftriaxone mole ratio. After stirring for 30 minutes, 0.2 mL of triethylamine was added to the solution. The solution turned from colorless to dark brown immediately; UV-visible spectra were taken after 2 hours stirring. The suspensions were then freeze-dried, the nanoparticles collected and washed repeatedly with water to remove unreacted precursors and reaction by-products.

## Synthesis of Au nanoparticles (AuNPs)

A 1 mM solution of $HAuCl_4.3H_2O$ and a 1 mM solution of ceftriaxone were prepared in deionized water. Two volumes of the stock solutions were mixed using a 15:1 Au:ceftriaxone mole ratio. After stirring for 30 minutes, 0.2 mL of triethylamine was added to the solution. The solution turned from colorless to dark brown immediately; UV-visible spectra were taken after 2 hours stirring. The suspensions were then freeze-dried, the nanoparticles collected and washed repeatedly with water to remove unreacted precursors and reaction by-products.

## Characterization.

UV-Visible spectra were collected with a Thermo Scientific Evolution 300 Spectrophotometer. FT-IR spectra were collected with a Bruker Victor 22 spectrophotometer using KBr pellets.

## Quantification of the weight of ceftriaxone in the conjugates.

Suspensions of Ag and Au NPs respectively, were centrifuged. The precipitated nanoparticles were removed and the supernatant was centrifuged for a second time. This process was repeated three times. The supernatant was then freeze-dried, and the residues weighed. The ceftriaxone

was determined by this method to be 26 wt% for AgNPs conjugates and of 8 wt% for Au NPs conjugates.

**Stability of nanoparticle suspensions**

Temperature, salinity and pH of the suspensions were measured to determine whether the conjugates would be stable under physiological conditions. UV-Visible spectroscopy was employed to characterize the stability of the suspensions, since coagulation is generally accompanied by a shift of the surface plasmon towards longer wavelengths.[28]The suspensions were found to be stable for temperatures up to 100 $^0$C (Figure S.1), for NaCl concentrations up to 2 M (Figure S.2) and for a pH between 6 and 12 (Figure S.3).

**Antibacterial activity and morphological changes of *E. coli* under AFM**

*E. coli* ATCC 8739 were grown on Tryptic soya agar (Oxoid UK) at 37 ºC for 24 hours in static condition and marked as stock *E. coli* culture. 10 µL drops of polylysine was added on freshly cleaved mica slide and left to dry, in the mean while freshly incubated culture of *E. coli* on tryptic soya agar (Oxoid UK) inoculated in distilled water to make $10^6$ cfu of *E. coli* and 5-10 µL droplets of this solution were transferred onto a freshly cleaved mica surface and left to dry. After the sample was dried, it was characterized by atomic force microscopy to check its size and morphology. The two different doses of ceftriaxone like 1 mg and 5 mg were added into vials of distilled water containing $10^6$ cfu of *E. coli* bacteria and incubated it for 2 hours at 37 ºC after incubation 5-10 µL drops of each dose transferred on freshly cleaved mica coated with polylysine separately and left it for dry and was characterized by atomic force microscopy. The same procedure was applied for 4, 6 and 8 hours to check the degradation of *E. coli* cells. Bare silver and gold nanoparticles were also treated in similar way to record the effect of bare silver and gold nanoparticles on *E. coli* bacteria which were taken as negative control. Ag nanoparticles of ceftriaxone doses of 1 mg and 5 mg were treated with $10^6$ cfu of *E. coli* for 1 & 2 hours and was characterized by atomic force microscopy to check the cell changes and noted the effects of these AgNPs. On the other hand Au nanoparticles of ceftriaxone were treated with $10^6$ cfu of *E. coli,* the doses were similar as for AgNPs and incubated at 37 ºC. 5-10 µL of this suspension was transferred on freshly cleaved mica coated with polylysine and left it for dry and then was characterized by atomic force microscopy. On this way we recorded, control treated with

ceftriaxone, negative control with bare Ag and Au nanoparticles and with ceftriaxone stabilized Ag and Au nanoparticles, images of *E.coli* in similar condition using atomic force microscope (AFM, Agilent Technologies 5500, USA) in the ACAFM mode. We used high frequency Si cantilever having length of 125 µm, force constant 42 N/m and resonance frequency 330 KHz. All samples were prepared and analyzed in a same condition.

**Minimum inhibitory concentration by Agar well diffusion method**

To calculate minimum inhibitory concentration (MIC), Agar well diffusion method[29] was employed. Determination of MIC for Ceftriaxone was measured with or without Ag and Au nanoparticles. In brief, Nutrient agar was used as medium to grow lawn of *E. coli* at the concentration of $10^6$ cells/mL ATCC 8739, duplicate dilution was used to calculate MIC. 60 mm well was made by using borer. 100 µg/mL stock solution of ceftriaxone was used to avoid nonspecific merged zone of inhibition. In each well we added different amount of various concentrations ranging from 80-1 µg/mL were used. The plate was incubated at room temperature for 2 hours to allow the diffusion process to take place before it was incubated for 24-48 hours at 37 ºC. The zones of inhibition were measured by using millimeter scale.

**Acknowledgment**

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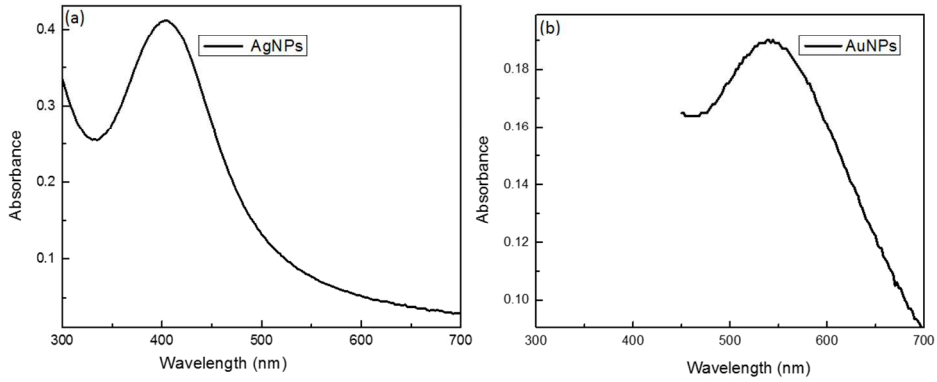
**List of Figures**




UV-Visible Spectra for AgNPs (a), and AuNPs (b) Stabilized with ceftriaxone.
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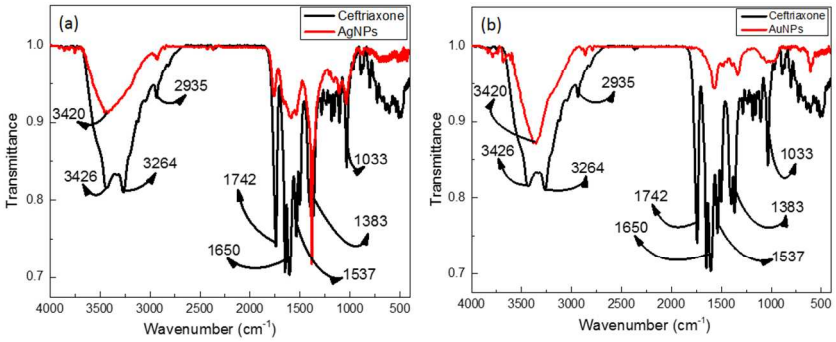


IR spectra of Ceftriaxone and its AgNPs (a), Ceftriaxone and its AuNPs (b).
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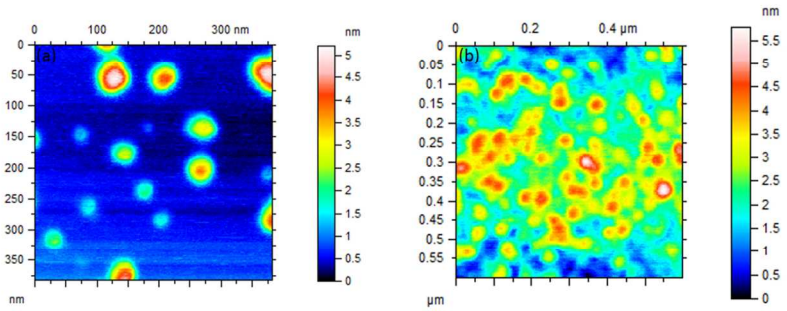


AFM images of AgNPs (a), and AuNPs (b)
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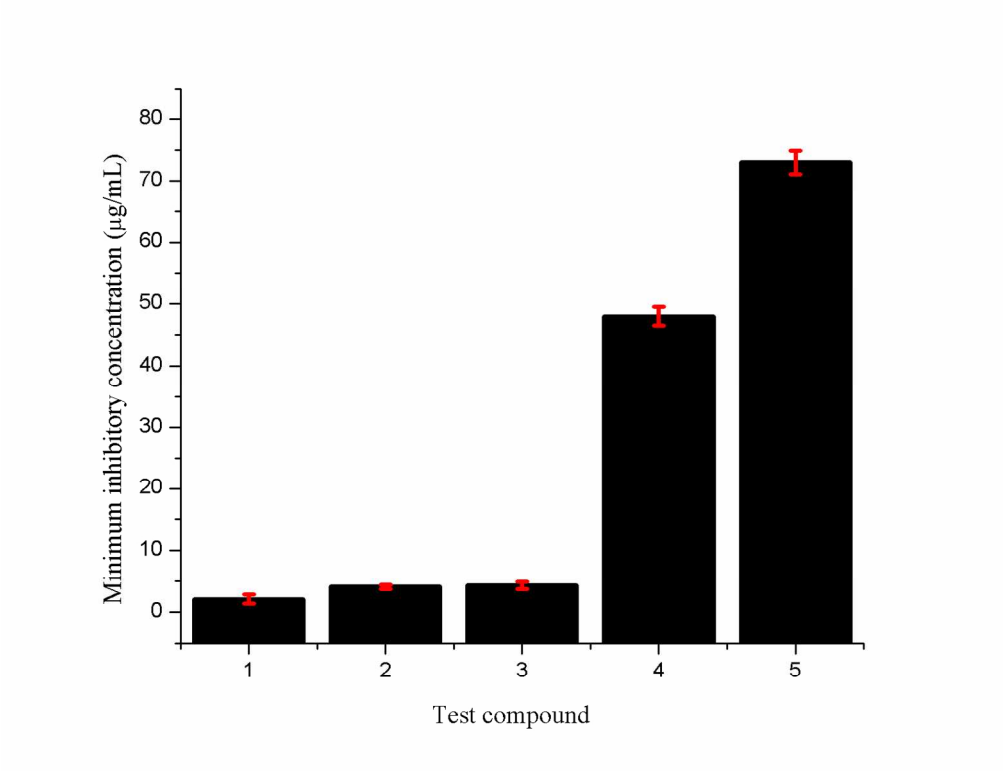


MIC of Ceftriaxone (1), Ag conjugated ceftriaxone (2), Au conjugated ceftriaxone (3), Bare Ag NPs (4), Bare Au NPs (5).
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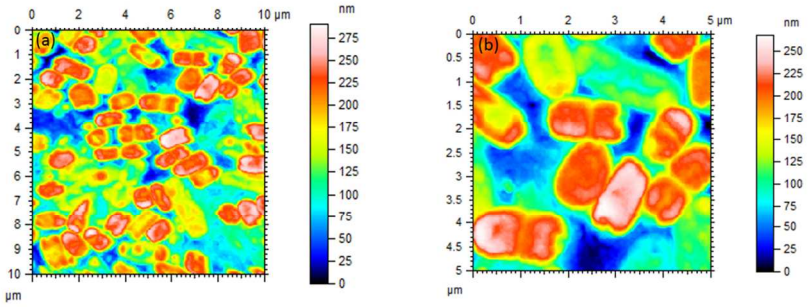


AFM images of E. coli before treatment (a), (b).
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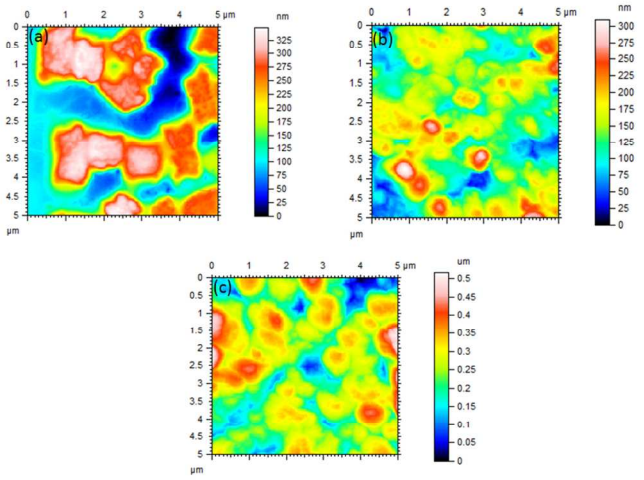


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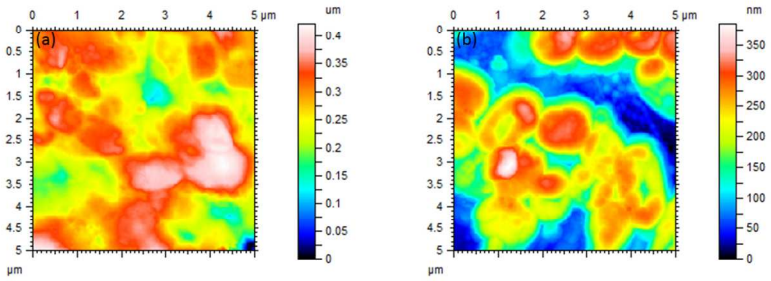


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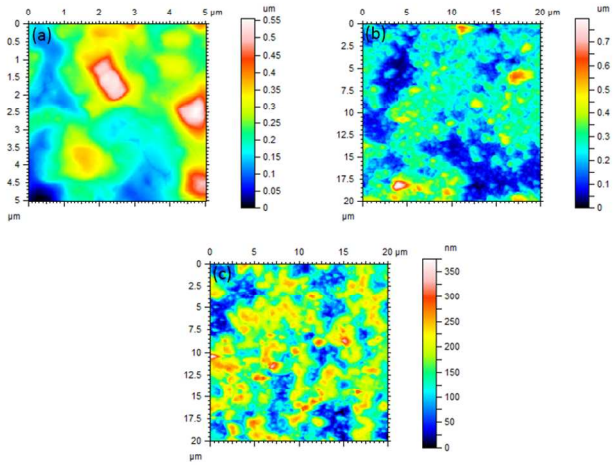


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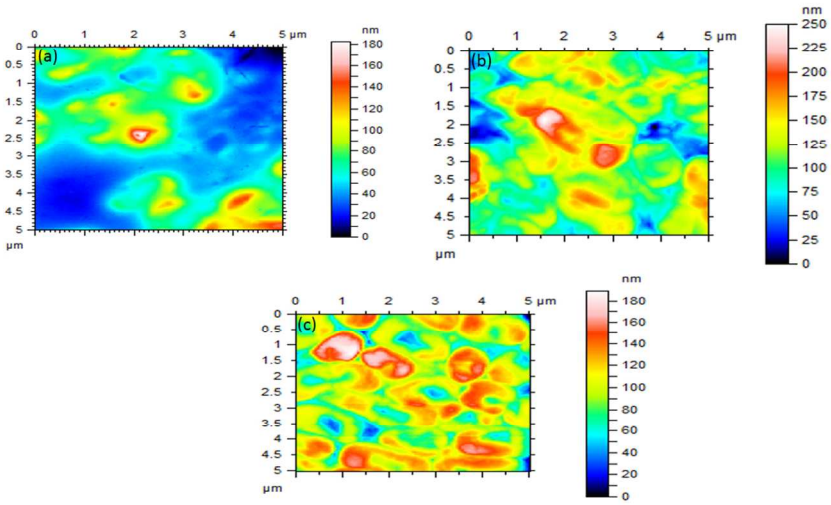


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