Detection of antibiofilm formation by silver nanoparticles created by tetracycline antibiotic

Alaa Z. Hameed and Nehia N. Hussein

Division of Biotechnology, Department of Applied Science, University of Technology, Baghdad, Iraq.
*E-mail: 100103@uotechnology.edu.iq

Abstract. Silver conjugated Tetracycline, was created and characterized by ultraviolet visible spectrophotometry (UV-vis), Fourier transform infrared (FT-IR), X-Ray Diffraction Patterns (XRD). Using antibacterial assays, the effects of tetracycline alone and drugs-conjugated with silver nanoparticles were tested against Gram-ve Pseudomonas aeruginosa isolates by well diffusion assay. The UV-vis spectra of silver-drug Nano conjugates showed a characteristic surface Plasmon resonance band in the range of 400–450 nm. FTIR analysis demonstrated the involvement of Hydroxyl groups in both drugs in the stabilization of silver nanoparticles. (XRD) showed that they cubic structure of silver and, antibacterial assays showed that biosynthesis silver nanoparticle conjugation enhanced antibacterial potential of Tetracycline compared with drug alone.

Keywords. Antibiotics, Anti biofilm, Nanoparticles, Antibacterial activity.

1. Introduction

Drug-resistant microbes are a main reason producing microbial re-emergence [1]. P. aeruginosa is main human pathogens causative to urinary tract infections [2]. Nanoparticles has shown abundant possible led to effectiveness of numerous Nano conjugates towered pathogenic microbes [3]. Nanoparticles have been often used as effective coatings to inhibit bacterial adhesion to surfaces as well as bactericidal agents [4]. Among these (AgNPs) have shown growth inhibitory as well as bactericidal effects [5]. The high surface area of AgNPs leads to high antimicrobial activity in compared with the silver metal [6]. This study aimed to create AgNPs by tetracycline antibiotic, and evaluation the anti-biofilm activity and cytotoxicity against red blood cells.

2. Materials and Methods

2.1. Synthesis of AgNPs by Drug

Five mL (0.1 mM) Tetracyclin solution was added to five mL (0.1 mM) silver nitrate aqueous solution, the mixture was magnetically stirred for 10 min. 20 µL of 5 mM freshly prepared NaBH₄ was
added in mixture. Turned color from yellow to Greenish from transparent upon addition of a reducing agent indicating the reduction of silver ions and the formation of Tetra-AgNPs [7].

2.2. Characterization of AgNPs

Tetr.-AgNPs were exposed to whole analysis (UV-vis, FT-IR, SEM, and XRD) (8,9) UV-Vis spectral analysis this was done by using UV-Vis spectrophotometer (PG- T80+ UV/Vis spectrophotometer, England) from 350-700 nm at a resolution of 1 nm. XRD measurements of the silver nanoparticles solution drop-coated on glass were done on Shimadzu XRD-6000 model with 40 kv, 30 mA with Cu kα radiation at 2θ angel. X-Ray powder diffraction is a rapid analytical technique primarily used for phase identification of a crystalline material and can provide information on unit cell dimensions. The crystallite domain size was calculated from the width of the XRD peaks, assuming that they are free from non-uniform strains, using the Scherer formula:

\[ D = \frac{0.94 \lambda}{\beta \cos \theta} \]

FT-IR analysis demonstrated the involvement of Hydroxyl groups in both drugs in the stabilization of silver nanoparticles.

2.3. Test pathogen

The cultures of 10 bacterial isolates of P. aeruginosa were obtained from the Microbiology Laboratory at Al Azizya-Hospital.

2.4. Antibiotics sensitivity test

Eleven different antibiotics were selected. Ciprofloxacin, Tobramycin, Azithromycin, Ceftraxime, Trimethoprim, chloramphenicol, erythromycin, gentamicin, and Clindamycin. Tests for antibacterial activity were performed with standard antibiotic discs. The inhibition zone was measured after incubation period at 37°C [8].

2.5. Antibacterial assay

Antibacterial potential of AgNPs synthesized by Tetracyclin. Was done by agar well diffusion assay towered P. aeruginosa. Nutrient broth was inoculated with isolates of P.aeruginosa bacterium for 3 hours at 370C, and the turbidity was compared with 0.5 McFarland standard. The culture (100µL) was poured on the Muller Hinton Agar(MHA) and spread using a spreader and left for 10 min to settle down, well with 8mm in diameter were made using gel puncture, nanoparticles solution (50 µL) was poured in each well and incubated for 24 hr at 370C. The diameter of zone inhibition was measured in millimetre after incubation period, and was recorded as mean ± SD of the triplicate experiment [9].

2.6. Biofilm formation, Tube method

This method was carried out by growing one colony at 24 hours in TSB medium for 24 hours in test tubes with a volume of 5 ml per tube with adding 1 ml of each concentration of silver nanoparticles concentrations (20, 60, 100) mg / ml, to each tube in When the positive control tube was containing the medium inoculated with bacteria and the negative control tube contained the medium that was not inoculated with bacteria, and after the incubation period for 24 hours, the medium was discarded and the tubes were washed with a phosphate buffer solution of pH 7.3, and the tubes were left to dry, then stained with 0.1% of dye crystal violet for 5 minutes, then the dye was removed from the tubes and
washed with tap water to get rid of the remaining dye and the tubes were left in an upside down position to dry, and the results were recorded as follows:

- The result is given (-) if there is no biofilm production (negative control).
- A score of (+) is given if the biofilm composition is weak.
- The result was given (+ +) if the membrane formation was moderate.
- The result is given (+ + +) if the membrane formation is biotic-dense [10].

2.7. Cytotoxicity assay

The agar-well diffusion method was adopted and the concentrations 20, 60, 100 mg / ml. well with a diameter of 8 mm were made on the surface of the blood agar medium and the prepared concentrations were poured in wells while the physiological solution was added as control, after incubation period (18-24 hours, and 37 ° C) and the effectiveness of the AGNPS was determined by measuring the haemolysis area around each well [11].

3. Results and Discussion

3.1. Characterization of biosynthesized AgNPs

This results in (Figure 1) show the change in color of cell filtrate of Tetracycline solution from pale yellow color to Greenish color after addition the Ag+ ions at 28 h.

Figure 1. Synthesis of AGPS Tetracycline Coated Silver Nanoparticles (A) before treatment. (B) After treatment of silver nitrate.

Figure 2 shows the UV-vis absorption spectra of silver- nano particles, which showed absorbance peak at 390nm.
FTIR of spectrum of tetracycline antibiotic showed the absorption peaks for N-H stretching at 3344 cm\(^{-1}\) and aromatic C-H stretching at 3095.75 cm\(^{-1}\). The vibrational peaks at 2989.66 cm\(^{-1}\) and 1352.10 cm\(^{-1}\) were assigned to CH\(_3\) stretching and at 1662.64 cm\(^{-1}\) refer to C=C stretching, respectively. Aromatic C-H bending was appeared at 1456.26 cm\(^{-1}\). The aromatic in plane and out plane deformation peaks were appeared at 1286.52 cm\(^{-1}\) (Figure 3A) [12]. The results of the spectral absorption of silver nanoparticles showed the disappearance of some bonds at certain ranges that were present in (Figure 3B), and the emergence of some bonds in other ranges and this is evidence of the formation of new compounds upon the synthesis of nanoparticles, and this is very clear at the range 3387.00 cm\(^{-1}\) evidence for the presence of O-H and N-H bonds. The disappearance of the C = C and CH\(_3\) bonds is observed upon the formation of the nanoparticles, after they were present in the drug alone.

**Figure 2.** Uv-Vis spectrum of Silver nanoparticles for (A) Tetracycline antibiotic only, (B) Silver nanoparticles by tetracycline antibiotic.
Figure 3. FTIR analysis of Silver Nanoparticles synthesized for tetracycline antibiotic (A), and (B) for to the silver nanoparticles

XRD pattern shows three strong peaks in the total spectrum of 2θ values in 20–60, as obvious from the peaks at 2θ standards of, 35.48, 29.23, 28.03, respectively for silver. The particle size was 57.55, calculated by using Scherer equation where (Figure 4).

Figure 4. XRD of silver nanoparticles

3.2. Antibiotics Susceptibility test

The result in (Table 1), shows of the antibiotics sensitivity test against the bacterial isolates, the highest recorded effect of the antibiotic Cip in the growth of isolate no. 8 with a diameter of the inhibition region reached (30 mm), followed by isolate no. 6 with a diameter of the inhibition region reached (22 mm), and the diameter of the inhibition region reached (20 mm) for each isolates (4,7,9), followed by the isolation no.3 with a diameter of the inhibition region reached (19 mm), and the isolate no. 5 with a diameter of the inhibition region reached (18 mm), while the antibiotic showed no significant effect in the growth of each of the isolates (1,2,10). As for the other isolates, the antibiotics varied in their effect. (Tob, C, AK, CN) antibiotics showed a different effect in the growth of bacterial isolates, whereas the antibiotics (Az, TMP, CTX E, DA, Tet). There was no significant effect.
Table 1. Antibiotics activity test against bacterial isolates.

| Isolates no. | Inhibition zone diameter in mm |
|--------------|--------------------------------|
|              | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10    |
| Cip          | 6     | 6     | 19    | 20    | 18    | 22    | 20    | 30    | 20    | 6     |
| Tob          | 6     | 6     | 9     | 6     | 10    | 10    | 10    | 13    | 11    | 6     |
| Az           | 6     | 6     | 6     | 6     | 6     | 6     | 6     | 6     | 6     | 6     |
| C            | 6     | 6     | 10    | 15    | 6     | 6     | 6     | 14    | 20    | 6     |
| TMP          | 6     | 6     | 6     | 6     | 6     | 6     | 6     | 9     | 6     | 6     |
| CTX          | 6     | 6     | 6     | 6     | 6     | 6     | 6     | 6     | 6     | 6     |
| E            | 6     | 6     | 6     | 6     | 6     | 6     | 6     | 6     | 6     | 6     |
| DA           | 6     | 6     | 6     | 6     | 6     | 6     | 6     | 6     | 6     | 6     |
| Tet          | 6     | 6     | 6     | 6     | 6     | 6     | 6     | 6     | 6     | 6     |
| AK           | 6     | 6     | 10    | 6     | 6     | 6     | 6     | 13    | 12    | 6     |
| CN           | 6     | 6     | 10    | 6     | 10    | 12    | 10    | 20    | 10    | 6     |

3.3. Antibacterial Activity of the AgNPs

The results showed that the biosynthesized AgNPs by antibiotic has a strong inhibitory effect on the growth of pathogenic bacteria. The effect of biosynthesized silver nanoparticles was the highest inhibition on the grown thin all isolates of *P. aeruginosa* at concentration of 100%, the highest of inhibition zone reached to of 24.83 mm in isolate no. 10, followed by 24.33 mm in isolate no 1 (Table 2). The present investigation supports the use of biosynthesized AgNPs by Tet. Elicited a strong antibacterial activity. Thus, the biological approach could be an economical alternative to conventional chemical and physical assays of AgNP synthesis and would be suitable for the development of a biological process for commercial large-scale creation. AgNPs have wide application in different fields, such as antibacterial. Therefore, the improvement of their synthesis for nanoparticle production is the main objective of nanotechnology [13]. But the biosynthesized silver nanoparticles by Tet. Have a strong inhibitory effect because silver nanoparticles have a very effective against bacterial and fungal infections. These properties are due to silver nanoparticles having a large surface area of the volume ratio which increase their association with bacterial cell well causing changes in the membrane and thus cell death [14]. That can also release silver ions that interfere with thiol groups in biomass enzymes, which inhibit them. Silver ions also inhibit respiratory enzymes and during the inhibition process reactive oxygen species are generated. (ROS) attack the cell itself and thus die [15]. Silver nanoparticles have the ability to interfere with the sulfur and phosphorus bases of DNA and thus lead to the breakdown of DNA and cell death due to a disturbance in the DNA replication of bacteria and microbes [5]. This agreement with that AgNPs has wide application in different fields, such as antibacterial. Therefore, the improvement of their synthesis for nanoparticle production is the main objective of nanotechnology [16].

Table 2. Antibacterial activity of biosynthesis of AgNPs created by Tetracycline antibiotic.

| Isolates no. | Inhibition zone of different Conc. of AgNPs |
|--------------|--------------------------------------------|
|              | 0.2     | 0.6     | 1        |
| 1            | 18.16 ±0.61 | 19.8 ±0.6 | 24.33 ± 0.4 |
| 2            | 14.5 ±0.5    | 16±1.0   | 0.7± 18.8 |
| 3            | 15 ±0.81     | 15.33 ±0.23 | 18.33±0.47 |
| 4            | 12.6±0.94    | 12.9 ± 0.69 | 16.5 ±0.40 |
| 5            | 14±1.04      | 15.2±0.40 | 16 ± 0.28 |
| 6            | 11.83±0.62   | 14.5±0.40 | 14.16±0.23 |
| 7            | 13.16±1.02   | 14.83±0.23 | 15.0 ±0.7 |
| 8            | 14.5±0.40    | 16.33±0.47 | 17.16±0.23 |
| 9            | 15.5±0.40    | 15.33±0.47 | 20.83±0.62 |
3.4. Determination the anti-biofilm activity for AgNPs

We explored the possibility of using the Ag⁺-tetracycline combinatorial therapy to treat biofilms grown in vitro. *P. aeruginosa* biofilms grown overnight were treated with Ag⁺ and tetracycline combination in different concentration, control positive, and control negative. In the present study, biofilm forming potential of *P. aeruginosa* strains (Figure 5) was assessed by test tube method. Biofilms are microbial networks of living beings follower to one another and an objective surface. Biofilm arrangement shields microscopic organisms from hydrodynamic stream conditions, for instance in the against phagocytosis, urinary tract and host resistance instruments, just as anti-infection agents. Over half of every bacterial disease detailed includes biofilm arrangement [17]. The results appear a difference in the amount of pigment in which the tubes were formed. The positive control tube produced a dense biological membrane (+++). The negative control tube did not have a dynamic membrane (-), the 80% (+), the tubes treated with nanoparticles with a concentration of 100%, formed a weak biological membrane (+).
3.5. Determination the toxicity of AgNPs on blood human

The effect of silver nanotubes in human blood on blood agar was studied at concentrations (20, 60, 100) mg / ml, through the results show there is no inhibition area (Figure 6). Which means that the silver nanoparticles are not able to cause blood cell degradation? The absorption of red blood cells and platelets of silver nanoparticles depends on the size and charge of those particles [18]. It is known that silver nanoparticles are toxic to erythrocytes at high concentrations only. Therefore, care should be taken when using silver nanoparticles at high concentrations [19].

4. Conclusion

Tetr-AgNPs showed bactericidal effects against all tested isolates, and anti-biofilm activity. The exact mechanism of action of these nanoparticles is not precisely understood and it is the subject of future studies along with testing their potential in vivo.

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6. References

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