In Vitro Cytotoxicity Study of Pt Nanoparticles Decorated TiO$_2$ Nanotube Array

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Abstract

Titanium dioxide nanotubes were synthesized by anodizing Ti sheets in the ethylene glycol solution and were covered in Pt nanoparticles onto the surface of TiO$_2$NTs using electrodeposition method from using five derivatives of Mannich base Pt complexes which have been used as precursor of platinum. The mean size, shape, elemental composition of the titanium dioxide nanotubes and platinum deposited on the template were evaluated by different techniques such as field emission scanning electron microscope (FE-SEM), transmission electron microscopy (TEM), X-ray diffraction pattern (XRD), and energy dispersive X-ray (EDX) technique. From all these analyses, the TiO$_2$NTs prepared and Pt nanoparticles deposited on it were identified. The diagnoses proved that all the Pt nanoparticles have a size less than 50 nm. The MCF-7 cancer cell lines and WRL68 normal cell lines were treated with concentration 800, 400, 200, 100, 50, 25, 12.5 µg/ml of TiO$_2$NTs and PtTiO$_2$NTs(1) and (2) for 48 hours using MTT assay. IC$_{50}$ and inhibition rate were calculated. The result shows that the PtTiO$_2$NTs have more inhibition effect on cancer cell lines than TiO$_2$NTs array.

Key words: Electrochemical deposition, Platinum nanoparticles, Titanium dioxide nanotubes

Introduction:

Most recent research on titanium dioxide nanotubes interested in the doping or deposition of metal ions like chromium, iron, cobalt, nickel, copper, palladium, platinum, sliver, and zirconium (1) and like boron, carbon, nitrogen and fluoride as non-metal in addition to using the metal oxides such as manganese dioxide had increased its applications in several fields (2). Among the varied nanostructured oxide materials, special attention has been directed toward TiO$_2$ nanotubes as result of developing some feature like low-cost and it has a large surface area compared to the volume (3). The titanium nanotubes are used as catalysts in accumulation boiling, photocatalysis (4), electrochromic device (5), resistant to corrosion (6), H$_2$ gas generation (7), solar cells (8), sensors, memory device (9), catalyst support (10), wastewater. Conjointly consistent with several researchers, the titanium oxide nanotubes could have been employed in drug-eluting stents and for the native unleash of antibiotics, drug delivery in cancer and tumor therapy. It is also widely used in dental implants and bones (11). Using titanium nanotubes in nanomedicine has a promising future in treating many diseases because improving cell adhesion, growth and differentiation (12,13), as well as its use in drug delivery. Later findings proved the strong relation between the cell responses and nanotube dimensions (14). Some of methods used to improve the performance of TiO$_2$NTs are to load nanotubes with some antibiotics such as vancomycin (15) or decorate the surface of TiO$_2$NTs with different nanoparticles such as gold (16) and sliver (17). Platinum medicine is still one of the most important treatments for
cancer, one of the most effective materials in the treatment of human cancers in particular (18).

Platinum nanoparticles have many properties that can be used in practical applications, including the manufacture of electronics and internal electrodes (19), durable proton exchange membrane fuel cells and biology and biochemistry applications (20). In this study five Mannich base Pt(IV) complexes were used as a source of platinum instead of platinum salt (H₂PtCl₆•6H₂O) because the ligand prevents the aggregation of Pt nanoparticles when decorated on the surface of nanotubes. In this research titanium nanotubes were prepared and different sizes of Pt nanoparticles deposited on the surface of titanium nanotubes using different deposition times 3 minutes when using PtL₄, PtL₂, PtL₃ and PtL₅ and 6 minutes when use PtL₄ at the fixed concentration to show the effect of the size and density of platinum nanoparticles and their effect on inhibition of cancer cells compared to nanotubes alone by using MTT method in 620 nm. This research aims to synthesize Pt nanoparticles of different size from Mannich base platinum complexes as a source of platinum after deposition on TiO₂NTs and study the cytotoxic effect of these nanomaterials on breast cancer cell lines.

**Material and Methods**

All chemicals were purchased from commercial sources H₂PtCl₆•6H₂O (99.9%), S-(1-benzothiazole-2-ylamino)methyl]-H-benzimidazole-2-yl)4-nitrobenzotheioate (PtL₄), S-(1-(Pyrazine-2-carboxamido)methyl)-1-H-benzimidazole-2-yl)4-nitrobenzotheioate (PtL₅), N-((2-((Morpholinomethyl)thiol)-1H-benzimidazol-yl)methyl) pyrazine-2-carboxamide (L₁), N-(2-(Morpholin-N-methyl)mercaptop-1H-benzimidazole (L₅), S-(1-Morpholinomethyl)-1-benzimidazole-2-yl)4-nitrobenzotheioate (L₅).NH₄F (99.5%), ethylene glycol 99.8% and Ti, Pt foil (99.6,99.99%) and thickness 0.25 mm. Solvents and reagents were used as received. The nanostructures were characterized by SEM, TEM, XRD, EDX, FT-IR, Transmission Electron microscopy (TEM) was recorded on Philips CM (10). EDS. Atomic weight and atomic number of all prepared nanoparticles were carried out by energy dispersive X-ray spectroscopy (EDS) XFlah6-10 Detector –Bruker. X-ray diffraction was measured using Shimadzu ray 6000.

**Preparation of Complexes**

Preparation of Trichloro S-((1-benzothiazole -2-ylamino) methyl]-H-benzimidazole-2-yl)4-nitrobenzotheioate, Ptominum (IV), Chloride.0.5hydrate (PtL₄), Trichloro S-1-(pyrazine-2-carboxamido) methyl]-1-H-benzimidazole-2-yl) 4-nitrobenzotheioate (PtL₅) and PtL₆. Dichlorobis (2-(Morpholin-N-methyl)mercaptop-1H-benzimidazole) (PtL₇). Dichloride. Hydrate (PtL₈). Dichloro Bis S-(1-Morpholinomethyl)-1-benzimidazol-2-yl)4-nitrobenzotheioate (PtL₉). Dichloride. Dihydrate (PtL₁₀). The Pt complexes were prepared according to the literature (21, 22). The Mannich bases reaction occurs in ethanol with platinum salt, 1:1 and 1:2 molar ratio for L₁, L₂, L₃ and L₄, L₅ respectively. The mixture was then refluxed for 3 hours.; the color solid complexes were formed, and then filtered, washed with ethanol and dried in disector.

**Preparation of TiO₂ Nanotubes**

Titanium dioxide nanotubes was prepared according to the literature (8). Titanium foils were cut into the suitable size (1x2 cm²). A direct current power supply (matrix E3612A) was utilized as the voltage source for the anodization. The anodization process was executed in a homemade plexiglass cell with two electrode arrays; titanium foil as the working electrode and Pt mesh utilized as the counter electrode in constant potential at 25°C. The distance between the substrates and the counter-electrode was approximately 1.5 cm. Degreased by sonication in detergent, deionized (DI) water, ethanol and acetone respectively for 10 minutes dried in an oven at 100°C for 15 minutes. For the anodization process, the electrolyte used was 0.5 wt% ammonium fluoride (NH₄F), (99.5%) in anhydrous ethylene glycol (99.8%) of purity at room temperature. The anodized substrate was then soaked in a water bath at 40°C for 20 min to remove the organic electrolyte. The anodization was performed for one hour at 40 V. After the occurrence of the anode, annealing in the oven with a temperature at 550°C was done (8).

**Preparation of PtNPs/TiO₂NTs**

Platinum nanoparticles were deposited onto the annealed TiO₂ by using an electrochemical (reduction) method at a constant potential in a typical two-electrode system with the prepared TiO₂ nanotube as the working electrode, Pt sheet as the counter electrode. The electrolyte solution was prepared by dissolving the 2mM from five complexes PtL₁, PtL₂, PtL₃, PtL₄ and PtL₅ in 100
ml mixture solvent (dimethyl formamide DMF, ethanol, deionized water (1:1:1)). Electrodeposition time was set at 3 minutes, while the PtL₄ at 6minutes while the electrodeposition voltage was fixed at 7 V and pH=5.5. The prepared Pt modified TiO₂NTs was washed several times with deionized water for 3 minutes to remove the residue of the solutions that are not deposited above the template, and then dried in air.

Cytotoxic Assays

Cytotoxicity effect of TiO₂NTs and PtNPs when deposition on TiO₂NTs on MCF-7 and WRL68 cancer cell line, and normal cell lines were done in a sterile area using the biosafety conditions of the airflow cabinet. MCF-7, WRL68 cell lines used in this study were equipped from Biotechnology Center/Al-Nahrain University. The cells were cultured in (MEM) modified eagles medium with serum ((100 U/ml) of antibiotic, ((100 μg)) of streptomycin/ml in incubator with (5% CO₂ at 37 °C). The survival or death of cells were determined using (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl Tetrazolium bromide ((MTT)) which is diagnosed by using spectrophotometer. Plated in 96-well sterilized microliter-plates at a density of (1x105 cells/well). After twenty-four hours, Cells were treated with different concentrations of prepared compounds starting from the lowest concentration and incubated in (5% CO₂) atmosphere with high humidity. After forty eight hours of compounds exposure, the cells were incubated with (0.5 mg/ml, MTT) distilled water for another four hours at thirty-seven degrees.10% of salt (sodium dodecyl sulphate) then incubated for two hours. Absorption was measured at the wave length 620 nm on a multi-well ELISA plate reader (23).

Results and Discussion

Hitachi S-4160 Field emission scanning electron microscope (FE-SEM) was utilized to diagnose the surface morphology of TiO₂ nanotubes template Fig. 1(A,B,C,D,E,F). Template was scratched with a steel blade so as to observe the nanotubes of the side, as shown in Fig. 1 (A1,B1,C1,D1,E1,F1). The process of anodizing led to the arrangement of nanotubes vertically. Generally, the nanotubes had lengths in the range 3 - 5 μm, and average diameters 83 nm, range from (51.8-95.7) .There were no differences when compared the observed morphology of the annealed crystalline nanotubes and transmission electron microscopy Fig. 2. TiO₂NTs may serve as the active sites or platform to deposit nanocrystals and able to promote unidirectional charge transport due to the one dimensional feature of the nanotubes. The aggregated Pt nanoparticles formed for (PtL₄) were larger than the other particles upon electrodeposition at 6minutes as depicted as in Fig. 1 (B, B1). While other which observed in Fig. 1 (C,C1,D,D1,F,F1,E,E1), the Pt nanoparticles were dispersed uniformly on the tube mouth of the TiO₂NTs at 7V, 2 mM and 3minutes, some Pt nanoparticles were found to have embedded into the TiO₂NTs. However, Pt nanoparticles prepared at 7 V, 2 mM for 6 minutes, became larger than Pt synthesized at 3minutes (24). The EDX unmistakably demonstrates that Pt, Ti and O are the major elements of composition which assures the existence of Pt decorated on TiO₂NTs substrate as appear in Fig. 3.
Field emission scanning electron microscope (FE-SEM) was supported by transmission electron microscope (TEM) technique and similar results have been shown. TEM images of the TiO$_2$NTs and Pt/TiO$_2$NTs are summarized in Fig. 2. Both scans, show similar results in size and shape of nanotubes in average diameter (83nm) and nanoparticles (less than 50 nm) and deposition of platinum nanoparticles on the internal and external walls of TiO$_2$NTs, Fig. 2 a and b.

Figure 2. (a) TEM images of TiO$_2$NTs; and (b) Pt/TiO$_2$NTs

Figure 1. FE-SEM images: A, A1 TiO$_2$NTs surface and cross section; B, B1, C, C1, D, D1, E, E1, F, F1 Pt/TiO$_2$NTs surface and cross section B, B1 at 7 Vol., 2mM 6 minutes C, C1, D, D1, E, E1, F, F1 at 7V, 2mM at 3 minutes

Figure 3. EDX (A) TiO$_2$NTs template, (B)Pt$_{1,2,3,4,5}$TiO$_2$NTs
XRD analysis was used to confirm the crystal phases of TiO2 nanotubes and the Pt-nanoparticles. The results are shown in Fig. 4, when the sample was heated at 550°C, only anatase phase was detected (25). The XRD patterns of TiO2 nanotubes and Pt/TiO2NTs prepared at 2mM. Plain TiO2NTs were polycrystalline in nature with the existence of hexagonal structure and anatase phase, the XRD pattern exhibited the presence of titanium(JCPDS No. 44-1294), anatase (JCPDS No. 21-1272), diffraction peaks of TiO2 2θ=25.44, 38.20, 48.29, 54.22, 55.30, 62.82, 70.48 and 75.58o can be attributed to the (101), (004), (200), (105), (211), (204), (220) and (215) lattice planes of anatase TiO2, respectively (25). Crystallite size value of TiO2 was calculated 59.6 nm. A comparison of XRD patterns/Pt samples was shown in Fig.4, only anatase phase of TiO2 was observed for all samples , because of the high intensity of the TiO2 peaks and overlapped with Pt nanoparticles peak (due to the large TiO2 crystallite size) compared with XRD of the sample(26).

The crystallite size of the TiO2 nanotubes can be calculated by applying Debye–Scherrer’s equation as below (27):

$$D = \frac{0.94 \lambda}{\beta \cos \theta}$$

where

$D$= Represents the mean size of crystalline

$\lambda$= Represents the wavelength of X-ray

$\beta$= Represents the line broadening in radians

$\theta$= Represents the Bragg angle

Interpretation of Cytotoxic Assay Results

Cells toxicity was evaluated by (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl Tetrazolium bromide ((MTT)) method. Cultured MCF-7 were treated with TiO2NTs and Pt/TiO2NTs at concentration (800, 400, 200, 100, 50, 25 and 12.5μg/ml) for 48 hours. Table 1 shows the statistical results, and the value of IC50 for MCF-7 cancer cell lines and WRL68 normal cell lines. According to IC50 test, the concentration of Pt/TiO2NT that was required for 50% inhibition of MCF-7 and WRL68 cell inhibition was calculated. All data were expressed as means±standard deviations (SD). The statistical analysis was performed using Independent Samples Test (2-tailed (t-test )) at confidence levels of 95%.

The results in Table 1 when deposited the Pt nanoparticles have different grain size on the surface of titanium nanotubes to modify it, and when we compare the values of IC50 for the three compounds Pt/TiO2NTs (1), Pt/TiO2NTs (2), and TiO2NTs, the following are concluded:

1-The nanomaterial Pt/TiO2NTs (1) has platinum of particle size between 22-32 nm which has an inhibitory effect more than platinum of a particle size between 30-45 nm on MCF-7 cell line.

2-When comparing values IC50 of the three nanomaterials Pt/TiO2NTs (1), Pt/TiO2NTs (2) and TiO2NTs, it has been observed that the modification of the titanium-nanotubes surface by different nanoparticles size of platinum, which has a particle size of less than 50 nm, has toxicity against MCF-7 higher than titanium nanotubes alone Pt/TiO2NTs(1)>Pt/TiO2NTs (2)> TiO2NTs.

3-When comparing values IC50 for the two cell lines MCF-7 and WRL68 of the three nanomaterials Pt/TiO2NTs (1), Pt/TiO2NTs (2) and TiO2NT, it was observed that the toxicity of these nanomaterials towards cancer cells were much higher than that of normal cell lines Fig.5.

Figure 4. X-ray diffraction pattern of synthesized TiO2NTs and other Pt nanoparticles decorated on it
cancer cells incorporated the nanostructure into the cells; form aggregates in the cells and inhibit migration and proliferation of cancer cells (31).

Conclusions:
Electrodeposition was applied to synthesize Pt|TiO2NTs. The regular crystalline with single-phase formation (anatase). The experiential methods Powder XRD, FE-SEM, TEM, EDX analytical techniques confirmed the presence of TiO2 NTs in anatase phase and Pt nanoparticles decorated on it. In vitro cytotoxicity test has been carried out using the MTT assay method in wave length 620 nm. The study proved that the toxicity of the titanium nanotubes toward cancer cell lines (MCF-7) increased by deposition platinum nanoparticles on it. Also from IC50 Value proved that these prepared nanomaterials have very low toxicity toward normal cell lines.

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Authors' declaration:
- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad.

Table 1. Statistical data and IC50 Values of Pt|TiO2NTs(1), Pt|TiO2NTs(2) and TiO2NTs on cancer (MCF-7) cell lines and normal (WRL68) cell lines in time of exposure 48 hrs

| Conc. μg/ml | Pt|TiO2NT(1) MCF-7 | Pt|TiO2NT(1) WRL68 | Pt|TiO2NT(2) MCF-7 | Pt|TiO2NT(2) WRL68 | TiO2NT MCF-7 | TiO2NT WRL68 |
|------------|----------------|-----------------|-----------------|-----------------|----------------|----------------|
| 800        | 72.40±0.172975 | 29.22±0.691198  | 59.56±0.502162  | 37.22±0.217785  | 49.20±0.136163 | 34.40±0.057769 |
| 400        | 66.56±0.167097 | 8.62±0.445073   | 52.15±0.469597  | 9.50±0.430155   | 43.38±0.0122317 | 12.89±0.867106 |
| 200        | 51.97±0.132842 | 5.63±0.235530   | 48.80±0.912652  | 5.40±0.460815   | 30.97±0.884123 | 8.84±0.511783  |
| 100        | 37.97±0.136704 | 4.40±0.330807   | 39.70±0.302108  | 5.30±0.484152   | 20.94±0.051394 | 7.30±0.237557  |
| 50         | 20.85±0.597523 | 4.32±0.000577   | 22.30±0.389295  | 3.34±0.401623   | 15.80±0.705143 | 5.98±0.256689  |
| 25         | 15.34±0.154717 | 3.13±0.262543   | 11.26±0.267275  | 3.13±0.421099   | 10.50±0.45254  | 4.30±0.436534  |
| 12.5       | 8.22±0.240563  | 3.08±0.896177   | 5.13±0.008737   | 3.08±0.125819   | 7.03±0.0417440 | 3.00±0.325140  |
| IC50       | 191            | 431             | 156             | 450             | 212            | 406            |

Figure 5: The percentage inhibition rate in MCF-7 cell line after treatment with TiO2NTs, Pt|TiO2NTs (1) and Pt|TiO2NTs(2) , 48 hrs compared to normal WRL68 cell line

The viability of the cell depends on the environment or the dominant medium in order to achieve the best response, including cell adhesion or migration and proliferation. Biological effectiveness depends largely on several factors, the most important of which are chemical and physical properties, including surface area, particle size shape and purity of the phase in addition to the concentration of nanoparticles (28, 29).

Therefore a number of reasons have been suggested to inhibit the growth of cancer cell lines, including the Pt-high surface density of nanoparticles which was found to be incompatible with MCF-7 cell adhesion and proliferation (28,29). Therefore, it is important and desirable to find an optimal surface density of Pt nanoparticles to be decorated on TiO2NTs including the nanoparticle and nanotube diameters that effectively kill bacteria, cancer cells and remains favorable to the normal cells.

The reason may be releasing platinum nanoparticles from Pt|TiO2NTs and the breakdown of DNA (30), or maybe attributed to inhibition of
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