Synthesis of Hydroxyapatite/Ag/TiO$_2$ Nanotubes and Evaluation of Their Anticancer Activity on Breast Cancer Cell Line MCF-7

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KEYWORDS
Hydroxyapatite; Nanotube; TiO$_2$; Anticancer; Breast cancer; MCF-7

ABSTRACT: In this research, TiO$_2$ nanotubes were synthesized by anodized oxidation method and were covered with a hydroxyapatite-silver nanoparticles using photodeposition and dip coating for loading silver nanoparticles and coated hydroxyapatite (HA). The morphological texture of TiO$_2$ nanotube and Ag-HA nanoparticles on TiO$_2$ nanotubes surface were studied by field emission scanning electron microscopy (FESEM), energy dispersive X-ray spectroscopy (EDAX analysis) and X-ray diffraction (XRD). The MCF-7 cell lines were treated with concentrations 1, 10 and 100 µg/ml of TiO$_2$ nanotubes and HA/Ag/TiO$_2$ nanotube for 24 and 48h. Finally, the cell viability and IC50% were evaluated using MTT assay. The results show that the HA/Ag/TiO$_2$ has more positive effect on enhancing the cell death compare to TiO$_2$ nanotubes and also exerts a time and concentration-dependent inhibition effect on viability of MCF-7 cells.

INTRODUCTION

In recent years, there has been steady advancement in the biomedical field [1]. In recent times much attention has been focused on Titania and Ti alloys. The nanotubular Titania is of a promising and important prospect in solar cells [2, 3], environmental purification [4], photoysis water [5], gas sensor [6, 7] and bio-application [8, 9] due to its unique highly ordered array structure, good mechanical and chemical stability, excellent corrosion resistance, high specific surface area and biocompatibility. However, in order to improve its biocompatibility, Titania surface should be modified. The particular morphology of nanoscale can affect
surface roughness and increase the strength of adhesion and spreading and proliferation of cells on the nanostructured titanium. High energy level also associated with increased levels of nano raw surface protein intake to the level of the cell interaction. Therefore, the distribution of surface charge and chemistry can play an important role in cell adhesion on the surface. One of the important advantages of Titanium dioxide nanotubes survey related to the adhesion, growth and differentiation of cells other than Titanium nanostructures that these features would ultimately increase cell death [9]. Adhesion, spreading, growth, and differentiation of mesenchymal stem cells are critically dependent on the tube diameter of vertically oriented TiO$_2$ nanotubes [10].

On the other hand, MCF-7 breast cancer cells treated with colloidal silver, significantly reduced the dehydrogenase activity, resulting in decreased NADH/NAD$^+$, which in turn induces cell death due to decreased mitochondrial membrane potential [11].

Effect of hydroxyapatite (HA) particles on the survival of gastric cancer cells of SGC-7901, nano-hydroxyapatite significantly decreased cell viability and induction of apoptosis in these cells so that the DNA fragment and morphological changes occur in cells [12].

In this paper we wish to disclose our initial results on the synthesis of composite TiO$_2$ nanotube covered with HA and silver, their uniform dispersed chemical surface as anticancer drug on MCF-7 cells line and finally their cytotoxicity studies. Anodization method in combination with photodeposition and dip coating method was employed as convenient method for the preparation HA/Ag/TiO$_2$ nanotube. Field Emission Scanning Electron Microscopy and X-Ray Diffraction were used to study the morphology and crystallinity of the HA/Ag/TiO$_2$. FT IR studies have confirm the loading of hydroxyapatite onto the TiO$_2$ nanotube. Finally cell viability was measured on MCF-7 cells using a MTT assay.

**MATERIALS AND METHODS**

**Synthesis of titanium dioxide nanotube arrays**

High-purity titanium foil (99.6%, 0.5 mm thick) was sonicated in acetone, isopropanol for 30 sec. The cleaned titanium foils were anodized at a constant potential of 60 V in an ethylene glycol solution containing 0.3 wt% NH$_4$F and 2vol%H$_2$O for 30 min in a two-electrode configuration with a Platinium cathode [13].

After anodic oxidation, the samples were rinsed with ethanol and water and dried in air. The resulting amorphous Titania nanotube arrays were annealed at 500 °C for 3 h with heating and cooling rates of 2 °C min$^{-1}$ in air to crystallize the tube walls and improve their activity.

The structures of the anoized TiO$_2$ nanotube arrays were characterized by scanning electron microscopy (SEM, XL 30 Philips company). X-Ray diffraction studies were carried out with an X-ray diffractometer (Philips XPert) using Cu k$\alpha$ radiation. The FT IR spectra of the sample were recorded on a Bruker FTIR 27 Tensor spectrometer. The spectra were recorded from 400-4000 cm$^{-1}$.

**Deposition of silver nanoparticles on titanium dioxide nanotube**

In order to prepare Ag nanoparticles deposited on TiO$_2$, a silver nitrate (AgNO$_3$) aqueous solution with a concentration of 0.014 M was prepared. The TiO$_2$ nanotube film was dipped on this solution. These samples were dried at room temperature after dipping for 30 min and irradiated under UV light (at 254 nm) from a G15W/T8 Sylvania tube lamp for 2 h.

The silver metal was photoreduced and fixed onto the TiO$_2$ surface by a photodeposition operation. The samples were washed ultrasonically twice in deionized water to obtain the high purity samples for the next procedure which coated the hydroxyapatite [14].
Deposition of hydroxyapatite on Silver loaded Titanium dioxide

For coating hydroxyapatite particles on silver deposited titanium dioxide nanotube, the modified TiO$_2$ nanotube film was immersed on an aqueous solution of 0.1 M hydroxyapatite for 30 min. Then white solid appeared and the sample was dried on air [15].

Cell culture

MCF-7 cells (NCBI C135, National Cell Bank of Iran), were obtained from Pasteur Institute of Iran and cultured in standard culture medium, which consisted of Dulbecco’s Modified Eagle Medium (DMEM) supdplemented with 2 mM Glutamine, antibiotics (100 U/l penicillin, 100 μg/ml streptomycin) and 10% fetal bovine serum (FBS) and incubated at 37 °C in a humidified 5% CO$_2$ atmosphere.

Stock solutions of the studied TiO$_2$ nanotube and HA/Ag/TiO$_2$ nanocomposit were prepared in dimethyl sulfoxide (DMSO, Sigma Aldrich) at a concentration of 1000 μg/mL, then sonicated for 4 h to achieve a homogenous suspension of nanocomposite and sterilized by filtration through Millipore filter, 0.22 μm, before use, and diluted by cell culture medium to various working concentration. The DMSO solvent was used due to solubility problems.

MCF-7 cells were seeded (5000 cells per well) into 96 -wells flat-bottom microtiter plates and incubated for 4 h prior to the addition of filtered 3 different concentrations of the studied compounds. Final concentrations achieved in treated wells were 0.1, 1 and 100 μg/mL. Each concentration was tested in quadruplicate on each cell line. The final concentrations (≤0.1%) of DMSO, were non-toxic to the cells. Only complete medium was added to the cells in the control wells. The incubation time was 24 and 48 h, during the period the control cells showed exponential growth.

MTT assay

The evaluation of the MCF-7 cells survival and proliferation was done using the tetrAzolium-based colorimetric assay (MTT) [16] and modified later [17]. MTT (3 - [4, 5-dimethylthiazol - 2 - yl] - 2, 5 - diphenyltetrazolium bromide; SIGMA) is a yellow water-soluble tetrazolium dye reduced by live cells into a purple formazan product insoluble in aqueous sloution . Briefly, cells were incubated for 24 and 48 h and then, 20 μl of MTT solution (5 mg/mL) in phosphate buffer saline (1/10 of total volume in a well) was added to wells. The MCF-7 cells were incubated for 4 h in humified atmosphere (5% CO$_2$) in the dark. Afterward, the supernatants were removed and the purple crystals were solubilized in 100 μL of DMSO. The plate was shaken for 15 min by a shaker incubator in order to dissolve the formazan crystals. The absorbance was measured at wavelengths of 570, 630 nm in an ELISA plate (Stat Fax-2100, USA) reader. The results were reported the average of 3 replications and expressed as cytotoxicity and viability perenatage by the following equation:

\[
\% \text{ Cytotoxicity} = \frac{\text{absorbence of toxicant}}{\text{absorbence of negative control}} \times 100
\]

\[
\% \text{ Viability} = 100 - \% \text{ Cytotoxicity}
\]

RESULTS AND DISCUSSION

Figure 1 shows FESEM images of TiO$_2$ nanotube (TNT) fabricated in EG-based electrolyte at 60 V for 30 min after anodization. The TNT with open mouth-tube morphology was obtained after optimized ultrasonic agitation (Figure 1a). The image indicates that the TiO$_2$ NT have an inner pore diameter of approximately 100 nm, average wall diameter of 45 nm and well aligned NTs vertically oriented from the Ti foil substrate. The
presence of well-aligned TNTs would promote direct charge transport. Therefore, the well-oriented TNTs may present the role of make distribution of surface charge on TNTs morphologies and subsequently effect on cell adhesion on the surface. Figure 1b shows the bottom surface morphology of TNT after peeling off from underlying Ti substrate. The image shows that TNTs are closed at bottom surface.

Figure 1. FESEM images of TiO$_2$ nanotubes fabricated in EG containing 0.3 wt% NH$_4$F and 2 Vol% H$_2$O via anodization: (a) top surface view, (b) bottom surface view of Ti-substrate after separation of TiO$_2$ nanotubes.

The energy dispersive X-ray (EDX) spectrometry clearly shows the presence of Ti, Ag, Ca, and P elements on the surface of catalyst. Figure 2A indicates the presence of Ag nanoparticle on TiO$_2$ nanotubes. In Figure 2B, the presence of hydroxyapatite (the ratio Ca/P is 1.64) is clear [18, 19].

Figure 2. Energy dispersive X-ray spectroscopy (EDX) spectra of (a) Ag/TNT (b) HA/Ag/TNT.

Figure 3a shows the X-ray diffraction patterns of TNT in the range of 5-70 °C. The appearance of sample diffraction peaks at 2θ = 25.4°, 38.3°, 48.1°, 54° and 62.7° are corresponding to the anatase phase. In Figure 3b, the three dominant peaks such as (211) (2θ = 31.8°), (112) (2θ = 32.2°), and (300) (2θ = 32.9°) indicate the presence of hydroxyapatite particle with the structure of hexagonal crystal which are verified by comparing data obtained with the PDF (Powder Diffraction File) pattern 09-0432. For the composite HA/Ag/TiO$_2$, no crystalline phase of silver formation was observed. This may due to the uniform distribution of silver nanoparticles in the
titanium dioxide surface, or related to the peak of silver at $2\theta=38.4^\circ$ was overlapped by the diffraction peak of $TiO_2$ at $38.3^\circ$ owing to the less content of silver [20].

Interaction and binding of hydroxyapatite particles to TNT was confirmed by taking FTIR spectra (Figure 4). The FTIR spectrum of HA/Ag/TiO$_2$ NT showed characteristic peaks at $1042 \text{ cm}^{-1}$ and $1096 \text{ cm}^{-1}$ for P-O stretching and the orthophosphate (PO$_4^{3-}$) stretching mode was also observed at $602 \text{ cm}^{-1}$ and $570 \text{ cm}^{-1}$. The phosphate bands are identified by peaks at $~962 \text{ cm}^{-1}$. The other peaks related to TiO$_2$ are observed in the sample. The broad band centered at 500-600 cm$^{-1}$ is likely due to the vibration of the Ti–O bonds in the TiO$_2$ lattice. The peaks at 1620-1630 cm$^{-1}$ and the broad
peaks appearing at 3100–3600 cm\(^{-1}\) are assigned to vibrations of OH groups.

![Figure 4. FTIR spectrum of HA/Ag/TiO\(_2\)](image)

**Survival assay**

Viable cells were evaluated by MTT assay, where the viability of cells was determined by the reduction of yellow MTT into purple formazan product by mitochondrial dehydrogenase present in metabolically active cells. Cultured MCF-7 were treated with TNT and HA/Ag/TiO\(_2\) separately (1, 10, 100 µg/ml) for 24 h and 48 h. Table 1 shows IC\(_{50}\) value for MCF-7 cell line treated with TNT and HA/Ag/TiO\(_2\). According to IC\(_{50}\) test, the concentration of HA/Ag/TiO\(_2\) that is required for 50% inhibition of MCF-7 cell proliferation was reduced comparing to concentration of TNT.

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNT</td>
<td>110</td>
</tr>
<tr>
<td>HA/Ag/TiO(_2)</td>
<td>98</td>
</tr>
</tbody>
</table>

Figure 5 shows the cell viability after incubation with different concentrations of TNTs for 24 h and 48 h. TNTs did not induce any change in the proliferation with a concentration up to 100 µg/ml, suggesting the absence of toxicity of the TNT. Subsequently the proliferation of MCF-7 cells reduced significantly at a dose of 100 µg/ml, the cell proliferation was reduced by 59% and 57% respectively in 24 h and 48 h exposure times. It is also noted that incubation time on different
concentrations of TNTs did not induce any significant change in the inhibition of cell proliferation.

![Figure 5](image1)

**Figure 5.** Cytotoxicity of TNT with different concentrations after 24 h and 48 h incubation

Figure 6 shows the cell viability after 24 h and 48 h incubation with different concentrations of HA/Ag/TiO$_2$. Similar to TNTs, it did not induce any change in the proliferation with a concentration up to 100 µg/ml. Subsequently the proliferation of MCF-7 cells reduced significantly at a dose of 100 µg/ml, the cell proliferation was reduced by 51% and 50% respectively in 24 h and 48 h exposure times. Incubation time on HA/Ag/TiO$_2$ shows the significant role in the control of cell proliferation.

![Figure 6](image2)

**Figure 6.** Cytotoxicity of HA/Ag/TiO$_2$ with different concentrations after 24 h and 48 h incubation

Figure 7 shows the cell viability after 24 h incubation with TNT and HA/Ag/TiO$_2$. In all the concentration the inhibition of cell proliferation by HA/Ag/TiO$_2$ is more than pure TNT itself. This strongly indicates that modified TiO$_2$ act as a more potent anticancer drug than pure TiO$_2$. 

![Figure 7](image3)
Figure 7. Cytotoxicity of TNT, HA/Ag/TiO$_2$ with different concentrations after 24 h incubation

Figure 8 shows the cell viability after 48 h incubation with TNT and HA/Ag/TiO$_2$. In all the concentration with increase incubation duration to 48 h, the inhibition of cell proliferation by HA/Ag/TiO$_2$ shows significant change than pure TiO$_2$ (TNT) itself. Incubation time would be the factor to influence on cell proliferation by HA/Ag/TiO$_2$.

Figure 8. Cytotoxicity of TNT, HA/Ag/TiO$_2$ with different concentrations after 48 h incubation

CONCLUSIONS

This paper, presents a simple and convenient method to prepare multifunction HA/Ag/TiO$_2$ order array nanotube as potent to be anticancer drug. The presence of hydroxyapatite and silver particle conjugated to tubular structure of TiO$_2$ increase the inhibition of MCF-7 breast cancer cell line than TiO$_2$ nanotube itself and also decrease the IC$50$ value. The effect of concentration and incubation time of TiO$_2$ and HA/Ag/TiO$_2$ on prolirative of MCF-7 cell line was investigated. Thus, the present study will serve as a first step towards investigation the effect of multifunctional composite based TiO$_2$ as potent anticancer drug on different categories of cells line.
ACKNOWLEDGMENTS

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REFERENCES

and interleukin-6 (IL-6). J Immunol Methods. 15;145(1-2),199-203.

Appendix 1: EDAX Analysis of HA/Ag/TNT

| E# | Line | Int | Error | K | X | W| % | ZAF | Formula | Ox| P| Tg | Class | LConf | HConf | Cost |  |
|----|------|-----|-------|---|---|---|---|-----|---------|---|---|----|--|-------|-------|-------|-----|---|
| O  | Kα  | 686.2 | 360.7500 | 0.1578 | 0.0946 | 10.67 | 51.38 | 0.2074 | 0.00 | 94.67 | A | 40.52 | 41.42 | 0.00 |  | |
| F  | Kα  | 70.3  | 360.7500 | 0.0168 | 0.0085 | 5.20  | 7.55  | 0.1635 | 0.00 | 8.62  | A | 5.01  | 5.18  | 0.00 |  | |
| Na | Kα  | 98.4  | 41.4401  | 0.0121 | 0.0065 | 1.68  | 1.83  | 0.5891 | 0.00 | 4.59  | A | 1.63  | 1.73  | 0.00 |  | |
| P  | Kα  | 210.8 | 17.6416  | 0.0083 | 0.0045 | 1.80  | 1.60  | 0.5469 | 0.00 | 5.58  | A | 1.76  | 1.84  | 0.00 |  | |
| Cl | Kα  | 99.9  | 17.9839  | 0.0000 | 0.0000 | 0.81  | 0.81  | 0.8006 | 0.00 | 2.11  | B | 0.59  | 0.64  | 0.00 |  | |
| Ca | Kα  | 182.7 | 17.9839  | 0.0016 | 0.0024 | 1.28  | 1.45  | 0.9942 | 0.00 | 6.18  | A | 2.21  | 2.31  | 0.00 |  | |
| Ti | Kα  | 2181.6 | 17.9839  | 0.6508 | 0.3344 | 57.80 | 30.95 | 0.8844 | 0.00 | 41.21 | A | 37.56 | 38.05 | 0.00 |  | |
| Ag | Lσ  | 95.5  | 17.9839  | 0.0017 | 0.0017 | 1.44  | 1.44  | 0.8125 | 0.00 | 3.07  | B | 1.38  | 1.50  | 0.00 |  | |
| Au | Lσ  | 7.4   | 0.4251   | 0.0628 | 0.0628 | 8.34  | 1.09  | 0.5999 | 0.00 | 2.60  | B | 7.42  | 9.25  | 0.00 |  | |