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Original Article

Chemotherapeutic effects of doxorubicin loaded PEG coated TiO_2 nanocarriers on breast cancer cell lines

Ayca Tas[1], Nese Keklikcioglu Cakmak[2], Erkan Gumus[3], Mustafa Atabey[4], Yavuz Silig[5]

[1]Sivas Cumhuriyet University, Faculty of Health Sciences Department of Nutrition and Dietetics, Sivas,Turkey
[2]Sivas Cumhuriyet University, Faculty of Engineering, Department of Chemical Engineering, Sivas,Turkey
[3]Sivas Cumhuriyet University, Faculty of Department of Histology Embryology, Sivas,Turkey
[4]Sivas Medicana Hospital, General Clinic of Surgery, Sivas, Turkey
[5]Sivas Cumhuriyet University, Faculty of Medicine, Department of Biochemistry, S ivas, Turkey

Abstract

Aim: Breast cancer is the most common frequently diagnosed malignancy among women and leading cause of cancer death in women worldwide. The aim of this study is 1) to increase the biocompatibility of the titanium dioxide (TiO_2) nanoparticles (NPs) by coating it with PolyEthylene Glycol (PEG) and to develop a new nanostructure system and 2) to determine anticancer activity of doxorubicin (DOX) loaded PEG-TiO_2 on MDA-MB-231 cell lines.

Material and Methods: TiO_2 nanoparticles used in this study were synthesized, coated with PEG, and PEG-TiO_2 nanostructure system was loaded by DOX. UV analysis was performed on the prepared solutions. The synthesized drugs were applied to the MDA-MB-231 breast cancer cell line and cytotoxic effect of these drugs were determined by using MTT method. The MDA-MB-231 cells were treated with different concentrations of TiO_2 (5-100 µg/ml) for 24, 48 and 72 hours. Apoptosis and necrosis were determined by fluorescence microscopy using the Hoechst 33258 (HO) /propidium iodide (PI) double staining.

Results: The effects of TiO_2, PEG-TiO_2, DOX, and TiO_2-PEG-DOX on the MDA-MB-231 cell line were compared with the control group and IC50 values were determined for 24, 48 and 72 hours.

Conclusion: In this study, it was shown that the effect of TiO_2-PEG-DOX nanostructured system on MDA-MB-231 cell line was inhibition growth in cancer cells and induction of apoptosis when compared with control group and DOX.

Keywords: Breast Cancer; MDA-MB-231; TiO_2-PEG-DOX: DOX.

INTRODUCTION

Breast cancer is a common type of cancer and is one of the most common causes of death worldwide (1). However, a significant reduction in mortality rates has been observed in breast cancer patients due to advances in diagnosis and treatment methods (2). Surgical, radiotherapy and chemotherapy are the main treatment methods for breast cancer treatment. Since traditional chemotherapeutic agents affect the whole body system by blood, there are significant disadvantages such as systematic side effects, tissue damage, gastrointestinal stress, and the body's multidrug resistance (MDR) (3). P Glycoprotein (P-gp) expression in the membrane of cancer cells is considered to be the main mechanism of multidrug resistance (4). P-gp is a 170 kDa plasma membrane protein and is encoded by the multidrug resistance gene 1 (MDR-1). The P-gP protein acts as an energy-dependent outflow pump, accelerating the excretion of chemotherapeutic drugs from the tumor cells, thereby reducing drug cytotoxicity (5-7). DOX is one of the most widely used chemotherapeutic agents. This therapeutic used is limited due to normal tissue toxicity, especially in ovarian, colon and breast cancers. In addition, MDR to DOX is a separate problem (8-10). Different DOX formulations and modifications, which may escape from membrane carrier proteins such as P-gP, have been a new research topic in nanotechnology (11,12). The development of nanotechnologically drug applications has revolutionized cancer therapy (13). In addition, it has been suggested that P-gP-mediated drug resistance can be overcome by the use of nanoparticle-based drug applications in cancer treatment and thus reverse the MDR (14,15). Recently, TiO_2 nanoparticles have been used as drug delivery systems for different chemotherapeutic agents such as paclitaxel, DOX, daunorubicin, temozolomide and camptothecin

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Corresponding Author: Ayca Tas, Cumhuriyet University, Faculty of Health Sciences Department of Nutrition and Dietetics, Sivas,Turkey
E-mail: aycatas@cumhuriyet.edu.tr

(16-20). Many unique properties of TiO_2 such as good biocompatibility, low toxicity, chemical stability and photocatalytic have made the use of biomedical industry attractive (21-23). Furthermore, biocompatibility of TiO_2 nanoparticles to increase is modified using PEG (24). For example, modification of the surface of the nanoparticles with PEG may prolong the circulation time of the nanoparticles in the blood and increase the likelihood of escaping the mononuclear phagocytic system (25,28). In this study, we generated TiO_2-PEG-DOX complex, whose cytotoxicity and antitumor efficiency were evaluated in human MDA-MB-231 breast cancer cells.

MATERIAL and METHODS

Synthesize of PEGylated TiO_2 nanoparticles and Drug loading on TiO_2-PEG

In this study, TiO_2 nanoparticles were produced by sol-gel process (29). Titanium iso-propoxide (TIP) was used as the starting precursor for the production of TiO_2 nanoparticles by the sol-gel method. The surfaces were coated with PEG (molecular weight 1500) to enhance the stability of the TiO_2 NPs. 20 mL of TiO_2 NPs (0.5 mg mL-1) were dropped into PEG solution and stirred for 24 h. The probe sonicator was used at all experimental stages. TiO_2—PEG NPs were separated by centrifugation at 12 500 rpm for 30 min and were dispersed in 20 mL ultrapure water. 1 mL of DOX (1mg mL^{-1}) were drop-wise added into TiO_2-PEG NPs and stirred for another 24 h. The obtained TiO_2-PEG-DOX NPs were collected by centrifugation at 12 500 rpm for 30 min and stored at 4 °C. Furthermore, free DOX in the centrifugal supernatant was also collected to measure the loading efficiency of DOX onto TiO_2-PEG (30).

Characterization

The UV-visible absorption of TiO_2-PEG and TiO_2-PEG-DOX NPs was determined using a UV-visible spectrophotometer (UV-1280, Shimadzu, Japan). The MDA-MB-231 breast cancer cell lines were maintained in DMEM medium, containing 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (10 mg/L). Cells were grown in at 37 °C, 5% CO_2 and 95% air in a humidified incubator. For each cell line, 70-80% confluent cell culture flask was trypsinized and cells were seeded in 96 well plates.

Cytotoxic effect of TiO_2 targeted drug in MDA-MB-231 cells

The cytotoxicity of the TiO_2-PEG-DOX, PEG-TiO_2, TiO_2, and DOX against MDA-MB-231cell lines was performed with the MTT 3- (4.5-dimethylthiazol-2-yl) -2.5-diphenyltetrazolium bromide) assay method (31). Shortly, cells were trypsinized and plated into 96-well plates (Corning, USA) in 0.1 mL of complete culture medium at a density of 1×10^5 cells per well and allowed to attach for 24 h. 1 µL of test substance at concentrations ranging between 5-100 µg/mL were added into each well containing the cells. Test substance was diluted with sterilized water into the desired concentrations from the stock. The plates were incubated at 37°C with an internal atmosphere of 5% CO_2. After 24, 48 and 72 h incubation, with different concentrations of compounds, MTT (5 mg/ml dissolved in PBS) 10 µl/well was added directly to all the wells and incubated for 2 hours at 37°C. The supernatant was carefully removed from each well and 100 mL of DMSO was added to each well to dissolve the formazan crystals. After mixing with a mechanical plate mixer for 15 min, the absorbance of plates were recorded at 570 nm on a microplate reader (Bio-Tek, USA). All drug doses were parallel tested in triplicate and were performed at least 3 times; control samples were run with 1% sterilized water.

Morphological Changes Using Fluorescence Microscope

The quantitative measurement of cell death was performed by Hoechst 33258 (HO) /propidium iodide (PI) staining for apoptosis and necrosis. The blue fluorescent HO nucleic acid stain is a cell permeable dye commonly used to stains brightly the condensed chromatin of apoptotic cells. The red-fluorescent PI is a common dead cell staining dye. Staining procedure was performed with a minor modification of the method described by Syed Abdul Rahman et al (32). Briefly, cells were grown on 6-well plates with cover glass at a density of 1×10^5 cells per well and treated with or without test substances at concentrations that were obtained based upon MTT assay (IC50 values). After 48 hours, the cells were washed with cold sterile PBS and HO and PI were added to each well at final concentrations of 5 µg/ml and 2 µg/ml, respectively. After 1 h of incubation at 37°C, stained cells were washed with PBS and fixed 10% neutral buffered formalin solution for 15 min at room temperature. Then the cells were washed three times with PBS at room temperature for 10 min each. Stained cells were examined and photographed on fluorescence microscope (Olympus BX 51) in the appropriate wave length on the 40X objective. Stained cells were examined and photographed on fluorescence microscope (Olympus BX 51) in the appropriate wave length on the 40X objective. At least 200 total target cells were counted and the numbers of each five cellular states were recorded and analyzed for quantification of apoptosis and necrosis. The experiment was conducted in triplicates.

Statistical analysis

In this study, experiments were carried out in three replications and the results are presented as means with standard error of the mean and standard deviation. Our results were analyzed using one-way analysis of variance (ANOVA) and Tukey multiple comparisons test. A p value less than 0.05 was used for statistical significance. All statistical analyses were determined using by the statistical software program GraphPadPrism7 (GraphPad Software, San Diego, CA, USA).

RESULTS and DISCUSSION

Synthesis and characterization of TiO_2-PEG and TiO_2-PEG-DOX

Titanium dioxide nanoparticles have been widely used in biomedical due to low toxicity, excellent biocompatibility, high photocatalytic and sonocatalytic efficiency. TiO_2-NPs are typically accumulated in biological medium; hence nanoparticle samples used in toxicological studies should

be effectively dispersed in water for in vitro and in vivo applications. In order to ensure the stability of the TiO_2 nanoparticle in the water probe sonicator were performed in this study. But sonication couldn't prevent long term aggregation of nanoparticles and in order to form stable dispersions. In this study, pegylation was also performed to ensure the stability of TiO_2 nanoparticle in water. Figure 1 show the UV–Vis spectra of TiO_2 nanoparticles in water. In the UV region TiO_2 exhibited high absorbance. From the UV/Vis spectra, the TiO_2 nanoparticle can absorb most light with wavelengths less than 400 nm.

TiO_2 nanoparticles were first synthesized, then the TiO_2 NPs were coated with PEG to increase their stability and biocompatibility, and then DOX was added to the TiO_2-PEG NPs to form the TiO_2-PEG-DOX NPs. To determine whether the DOX was successfully loaded into TiO_2-PEG NPs, a UV analysis analysis was performed. As seen in Figure 2, the characteristic peak of the DOX is at 488 nm. The figure is also clearly shows that the peak DOX present at 488 nm was adsorbed onto the TiO_2-PEG surface.

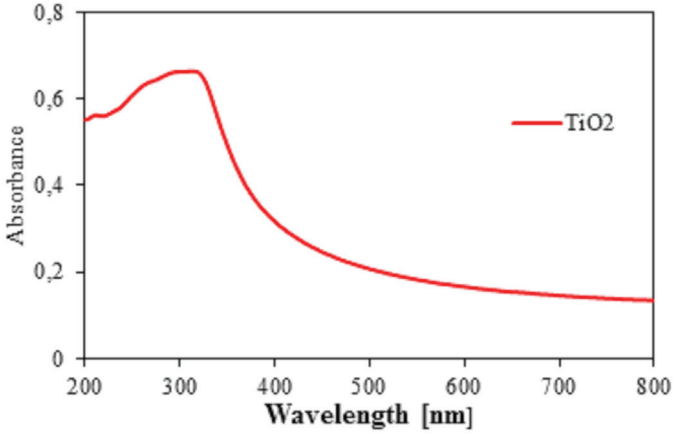


Figure 1. UV-Vis absorbance spectrum of sol-jel TiO_2 nanoparticles in water

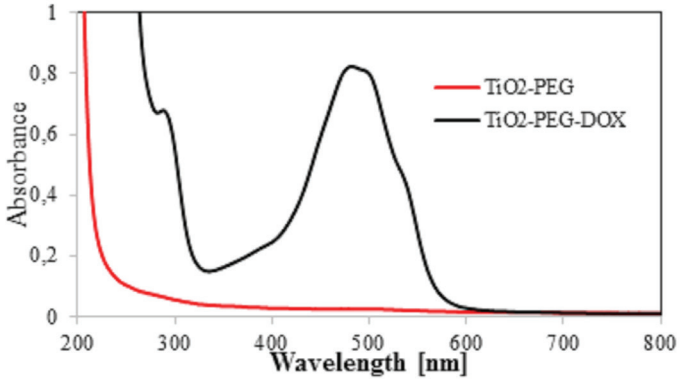
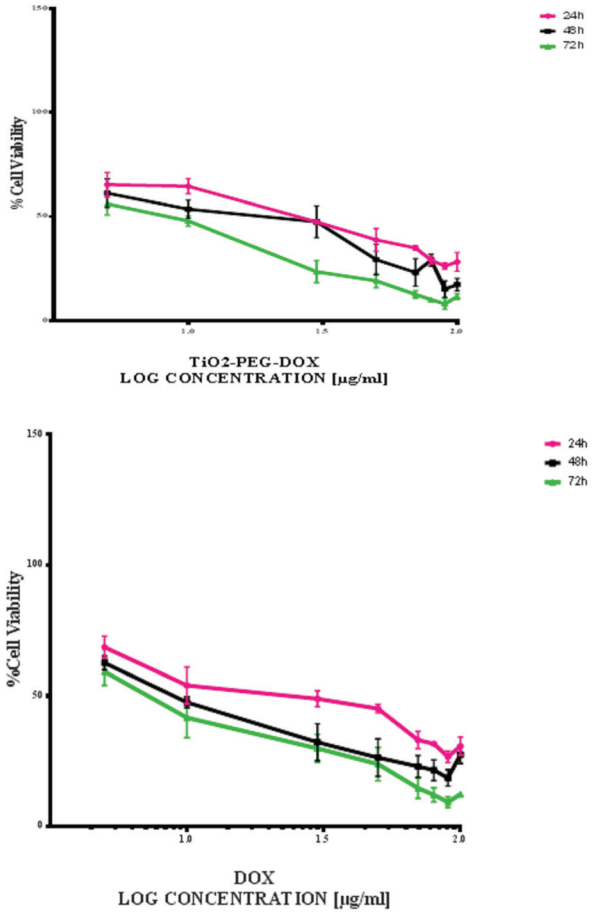


Figure 2. UV-Visible absorption of TiO_2-PEG and TiO_2-PEG-DOX NPs

Cytotoxicity activities of TiO_2, TiO_2-PEG, TiO_2-PEG-DOX and DOX drugs on MDA-MB-231 cells

In recent years, novel approaches have been developed to reverse MDR by using nanoparticles to increase the therapeutic effect and reduce toxicity. Previous studies have shown that TiO_2-DOX nanoparticles exhibit cytotoxic effects in MDR breast cancer cells in vitro (33). By coating the surface of nanoparticles with PEG, they can prevent the rapid clearance of these particles from the renal and reticuloendothelial system (RES) and can greatly increase the half-life of the blood. Therefore, it increases the accumulation of nanoparticles in tumor tissue (13,15,34). Accordingly, based on previous studies, we have successfully synthesized the new TiO_2-PEG-DOX system modified by PEG coating on TiO_2 nanoparticles. In our study, MDA-MB-231 cells were exposed to certain doses of these drugs to determine whether the TiO_2-PEG-DOX, PEG-TiO_2, TiO_2 and DOX drugs had cytotoxic effects (Figure 3). Figure 3 shows changes in cell inhibition for 24h, 48h, and 72 h against increasing concentrations of MDA-MB-231 cell lines. The x-axis shows cell types and varying time points, while the y-axis shows inhibition rates of cancer cells relative to controls. MDA-MB-231 breast cancer cells compared to the control group were found to significantly reduce the survival rate of cancer cells after 24 h, 48 h and 72 h of incubation with TiO_2-PEG-DOX. TiO_2-PEG-DOX, PEG-TiO_2, TiO_2 and DOX drugs were found to be the most active after 72 hours of incubation on MDA-MB-231 cells. Among these drugs, the most active was found as TiO_2-PEG-DOX for 24, 48 and 72 hours, and the IC_{50} values were 5.61 µg/ml, 4.37 µg/ml and 2.61 µg/ml, respectively (Table 1).



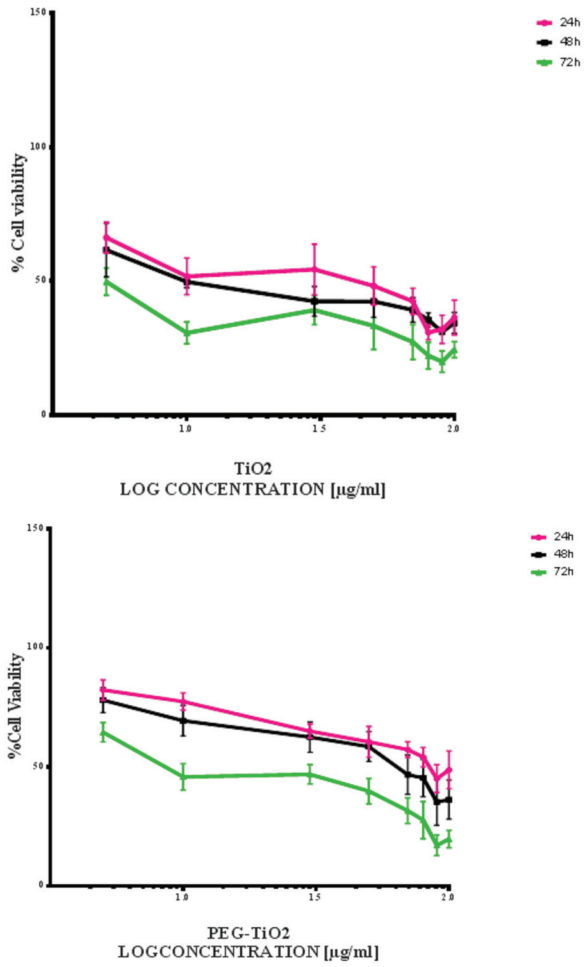


Figure 3. Anti-cancer Activities of TiO_2-PEG-DOX, PEG-TiO_2, TiO_2 and DOX drugs on the MDA-MB-231 cell line

Table 1. Comparison of IC_{50} values between TiO_2-PEG-DOX, PEG-TiO_2, TiO_2, and DOX on MDA-MB-231 after 24 h, 48 h and 72 h of incubation

	IC_{50} (µg/ml±SD*)		
Drugs	24h	48h	72h
TiO_2-PEG- DOX	5.61±0.34	4.37±0.11	2.61±0.05
TiO_2-PEG	65.29±013	49.40±0.21	31.00±0.25
TiO_2	21.78±0.19	14.55±0.22	11.35±0.26
DOX	6.36±0.39	4.93±0.33	3.81±0.38

*Mean standard deviation values of IC_{50} obtained from three independent experimental repetitions after 24 h, 48 h and 72 h incubation for MDA-MB-231cell lines

Du et al. (30), similar to our study, investigated the use of nanoparticles such as TiO_2 for the drug delivery system. TiO_2 nanoparticles were coated with PEG to enhance their biocompatibility and functionalization potential, and then they were activated by DOX. Firefly luciferase-labeled MDA-MB-231-GFP-fLuc breast cancer cell line was used to generate the breast cancer animal model in experiments. In MDA-MB-231-GFP-fLuc cytotoxicity studies, they found that DOX was more cytotoxic than TiO_2-PEG-DOX nanoparticles after 24 and 48 h incubations. In our study, we found that TiO_2-PEG-DOX activity was higher than DOX after 24 and 48 hours incubation. However, they noted that after 72 hours, the TiO_2-PEG-DOX nanoparticles showed more cytotoxic effects in the cells than DOX. The data obtained after 72 hours of incubation were similar to our study. In another study, TiO_2-DOX nanoparticle was determined that exhibited slightly higher anticancer activity compared to DOX on MCF-7 breast cancer cell line. The DOX for the MCF-7/ADM cell line showing MDR did not show any cytotoxic effect. However, TiO_2-DOX nanoparticle has been found to have a cytotoxic effect of 40% (33). As a result, in this study concluded that the use of TiO_2 nanoparticles coated with PEG as nano-carriers in drug delivery is more efficient than DOX. Thus, the drug delivery system makes a great promise, especially the nanoparticles designed to overcome the MDR.

Detection of Apoptosis and Necrosis in MDA-MB-231 Cell Lines Using Fluorescence Microscope

In this present study, morphological alterations of apoptotic cell death were detected by fluorescence microscope double staining of Hoechst 33258 (HO) and propidium iodide (PI). The synthesized drugs showed apoptotic activity in breast cancer cells. The control or untreated cells showed regular intact nuclei with a less bright blue fluorescence after HO staining and the absence of red fluorescence after PI staining. Cells treated with test substances have typical features of apoptosis such as cell and nuclear shrinkage, chromatin condensation, DNA fragmentation, formation of apoptotic bodies. Substance-treated cells were exhibited a brighter blue fluorescence than the control and untreated cells (Figure 4 A). On the other hand, necrotic cells are reorganized, swollen, and unable to maintain membrane integrity. However, less percentage of substance-treated cells were detected in MDA-MB-231 cells after PI staining (Figure 4 A). The results showed that the apoptosis of MDA-MB-231 treated cells was slightly decreased (Figure 4 B). In previous studies, nanoparticles caused cell death by activating the intrinsic apoptotic pathway (35). Overall, the percentages of apoptotic cells in the MDA-MB-231 treated with TiO_2-PEG-DOX and TiO_2 were significantly higher than those in the control or untreated cells (20.1 ± 1.54 and 13.41 ± 2.14 vs. 7.2 ± 1.35 and 3.62 ± 2.01, respectively; $p < 0.05$). There were no significant differences between TiO_2-PEG (7.20 ± 1.35) and both control or untreated cells ($p > 0.05$). Moreover, the TiO_2-PEG-DOX nanoparticles showed more apoptotic effects in the cells than DOX (17.71± 2.13). However, there were no significant differences between TiO_2-PEG-DOX and DOX treated cells ($p>0.05$) (Figure 4). TiO_2, nanomaterials, has special chemical characteristics. TiO_2 is known to interact with water molecules inside cells and creates free radicals (36). Free radicals can cause cell death via DNA and cell membrane damage (37). In previous studies have shown that TiO_2 nanoparticles have the potential to be applied in the near future in clinical trials (38).

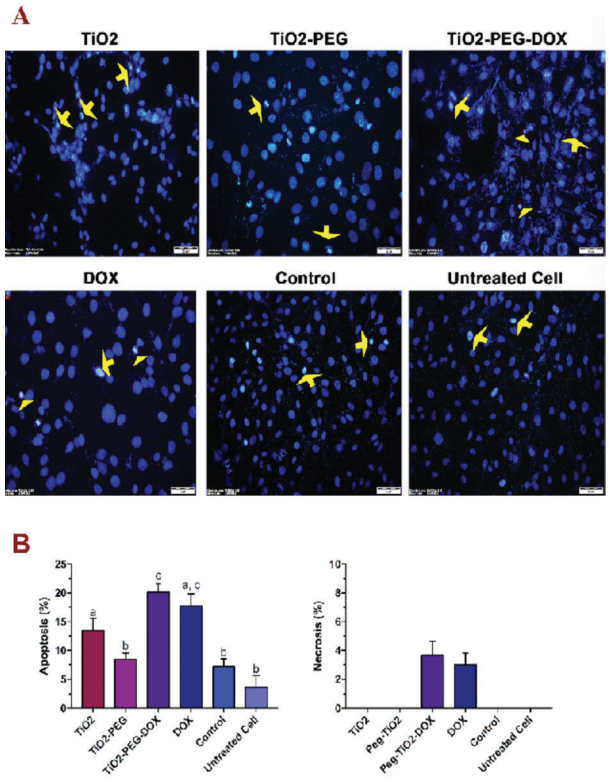


Figure 4. Representative HO/PI staining of MDA-MB-231cells (A). Yellow arrows and arrows head indicate apoptotic and necrotic cells, respectively. Percentage of apoptotic and necrotic cells (B) according to test substance types. Data are presented mean ± SD from 200 cells for each group. aP < 0.05 vs. TiO_2-PEG, TiO_2-PEG-DOX, Control, and Untreated Cells. bP < 0.05 vs. TiO_2, TiO_2-PEG-DOX, and DOX. cP < 0.05 vs. TiO_2, TiO_2-PEG, Control, and Untreated Cells

CONCLUSION

In this work, DOX was loaded onto TiO_2-PEG NPs to form TiO_2-PEG-DOX nanocomposite. The nature of the interaction between DOX and TiO_2 nanoparticles was determined by UV-visible spectra. The results unambiguously confirmed that DOX is adsorbed onto TiO_2-PEG NPs via electrostatic interaction. To ensure the stability of the synthesized nanomaterials, probe sonicator were used at each stage. In addition, the stability of the pegylation process has been maintained for a long time. This study showed that TiO_2-PEG-DOX NPs based drug had a higher effect on MDA-MB-231 breast cancer cells than DOX. In vivo studies are needed to make for TiO_2-PEG-DOX nanoparticle-based drug a future cancer drug candidate. In addition, this nanocarrier based drug can be transformed into a targeted drug by marking the expression of a protein with an increased protein in breast cancer cells and it has the potential to be used as a new therapeutic agent for cancer treatment in the future.

Competing interests: The authors declare that they have no competing interest.
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Ayca Tas ORCID: 0000-0002-7132-1325
Nese Keklikcioglu Cakmak ORCID:0000-0002-8634-9232
Erkan Gumus ORCID: 0000-0001-6432-7457
Mustafa Atabey ORCID: 0000-0002-9226-4358
Yavuz Silig ORCID: 0000-0002-0562-7457

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