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Fabrication of docetaxel surfaced Fe_3O_4 magnetite nanoparticles and their cytotoxicity on 4 T1 breast cancer cells

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Abstract

Background: In the recent years, there is an increasing attention to the using of Fe_3O_4 magnetite nanoparticles (MNPs) as drug delivery systems. Application of this nanoparticles could profit advantages of nanomedicine to enhance biological activity of pharmaceutical ingredients.

Methods: Fe_3O_4 MNPs were synthesised by a chemical method and characterized by transmission electron microscopy and energy-dispersive spectroscopy techniques. In the next step, docetaxel-coated Fe_3O_4 MNPs were prepared, using precipitation method. The surface chemistry of docetaxel-coated Fe_3O_4 MNPs as well as their thermal decomposition characteristics were examined using fourier transform infrared spectroscopy and thermogravimetric analyzer equipment, respectively. The cytotoxicity assay was conducted on 4 T1 breast cancer carcinoma by MTT assay to evaluate the possible *in vitro* antiproliferative effects of docetaxel-coated Fe_3O_4 MNPs.

Results: During precipitation process, docetaxel molecules were precipitated on the surface of Fe_3O_4 MNPs by the ratio of 3:100 w/w which indicates that each milligram of coated Fe_3O_4 MNPs averagely contained 30 μg pure docetaxel compound. Docetaxel showed antiproliferative effects against mentioned cell line. The highest concentration of docetaxel (80 $\mu\text{g}/\text{ml}$) caused about 80% cell death. However, the results demonstrated that much lower amounts of docetaxel will be needed in combination of Fe_3O_4 MNPs to produce the potent antiproliferative effect compared to docetaxel alone. Dose response cytotoxicity assay of docetaxel-coated Fe_3O_4 MNPs against 4 T1 breast cancer cells showed that lower amount of docetaxel (0.6 $\mu\text{g}/\text{ml}$) can exhibit higher cytotoxic effect against this cancer cell line (90% cell death).

Keywords: Docetaxel, Fe_3O_4 magnetite nanoparticles, Antiproliferative effect, 4 T1 breast cancer cells

Introduction

Today, side effects of anti cancer drugs are still considered as a major problem in chemotherapy of cancer diseases (1). In the recent years, new drug delivery systems have been developed to reduce the side effects of these drugs (2–4). These systems mainly include nanotubes (5), liposomes (6), dendrimers (7) and nanoparticles (8). The potential biomedical applications of Fe_3O_4 magnetite nanoparticles (MNPs) are well discussed in the literature (9). Due to their magnetic properties, these nanomaterials have received particular attention as possible drug carriers (10). The

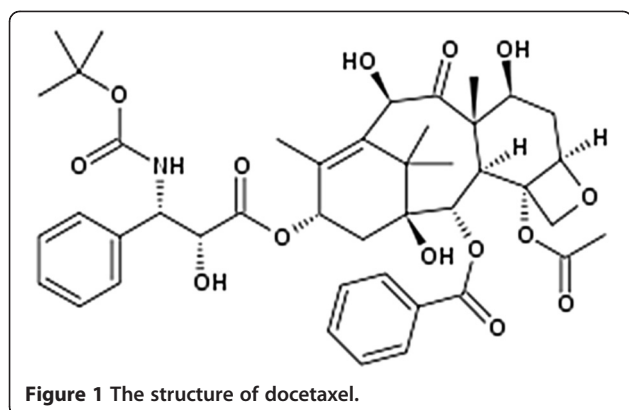
conjugation of chemical or natural anticancer drugs to Fe_3O_4 magnetite nanoparticles (MNPs) can lead to increase the uptake of anticancer agents by targeted tumor and improve their therapeutic index (11). For example, a significant difference in antiproliferation effect of a natural product, umbelliprenin, and its Fe_3O_4 MNPs-conjugated form was observed *in vitro* model (12).

Docetaxel (Figure 1) is an insoluble semi-synthetic analogue of Taxol. Docetaxel leads to a significant decrease in free tubulin, required for microtubule formation, and results in inhibition of mitotic cell division between metaphase and anaphase, preventing further cancer cell progeny (13). In this study, docetaxel-coated Fe_3O_4 MNPs have been fabricated to determine the antiproliferative effect of this anticancer agent in combination with Fe_3O_4 MNPs against 4 T1 breast cancer cell line. The main hypothesis is

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evaluation of *in vitro* cytotoxicity of docetaxel in combination of Fe₃O₄ MNPs on 4 T1 breast cancer cells compared to the cytotoxicity of docetaxel alone.

Materials and methods

Synthesis of Fe₃O₄ magnetic nanoparticles and their characterization

A previously reported method was used for synthesis of Fe₃O₄ MNPs (14). Briefly, deionized water (200 ml) was deoxygenated by bubbling nitrogen gas for 1.5 h, then 50 ml of NH₄OH (1 M) was added and the mixture was stirred with mechanical agitation at 1000 rpm. Then, amounts of 6.76 g FeCl₃·6H₂O (2.5 mmol) and 4.97 g FeCl₂·4H₂O (2.5 mmol) were separately dissolved in 50 ml distilled water to make 0.5 M solutions. Subsequently, amounts of 10 ml ferrous chloride and 20 ml ferric chloride solutions were added to the ammonium hydroxide solution and the reaction mixture was stirred for 2.5 min at 1000 rpm. At this stage, a black precipitate formed which was washed four times with deionized water. Finally, using a magnet, the prepared Fe₃O₄ MNPs were separated from the solution. Freshly prepared Fe₃O₄ MNPs were characterized by transmission electron microscopy (model EM 208 Philips) and energy-dispersive spectroscopy (EDS).

Preparation of docetaxel-coated Fe₃O₄ nanoparticles and their surface characterization

In order to coat docetaxel on the surface of Fe₃O₄ MNPs, a simple precipitation method (Figure 2) was used as follows: A 2 mg/ml dichloromethane solution of docetaxel (Cipla, Mumbai, India) was prepared and reserved in a sealed container. Fe₃O₄ MNPs (100 mg) were dispersed in 5 ml distilled water. Thereafter, amount of 5 ml dichloromethane solution of docetaxel was added drop-by-drop to the suspension of Fe₃O₄ MNPs over a 15 min period under continuously stirring (300 rpm) condition, and using a laboratory magnetic stirrer. The resulting mixture was maintained in a static regime at 45°C for 24 hours. Docetaxel-coated Fe₃O₄

MNPs was separated from the solution by a magnet. Finally, the capped nanoparticles were washed three times with distilled water. A stock suspension of Fe₃O₄ MNPs and docetaxel-coated Fe₃O₄ MNPs were prepared (2 mg/ml) and used for antiproliferative bioassay. The surface chemistry of the docetaxel-coated Fe₃O₄ MNPs was studied using Fourier transform infrared spectroscopy (Nicolet Magna 550). Also thermal decomposition characteristics of docetaxel-coated Fe₃O₄ MNPs were determined using Thermogravimetric Analyzer TGA equipment as well.

Cell culture

The 4 T1 breast cancer cell line was obtained from the National Cell Bank of Iran (NCBI), Pasteur Institute of Iran, Tehran (Iran). The cells were cultured in a RPMI-1640 medium with 10% fetal calf serum, 100 u/ml penicillin and 100 µg/ml of streptomycin in 5% CO₂ at 37°C for one week and then used for cell proliferation assay.

Cell proliferation assay

Thiazolyl blue tetrazolium bromide assay (MTT) was performed to evaluate cell proliferation where 2 × 10⁴ cells were seeded in 96 well plates and incubated with 0, 10, 20, 40 and 80 µg/ml of docetaxel-Fe₃O₄ MNPs, bare Fe₃O₄ MNPs or docetaxel alone for 24 hours. Then, 15 µl MTT (0.5 mg/ml) was added to each well, and the plates were incubated for 2 hrs. Then, the medium was replaced with 200 µl of DMSO and the absorbance was recorded at 570 nm (12). Each experiment was repeated three times and MTT assay was performed in 3 replicates for each experiments. The percentage of inhibition rate (IR) was measured using the following equation:

$$IR (\%) = [1 - OD_{exp}/OD_{con}] \times 100$$

Where OD_{exp} and OD_{con} were optical densities of treated and untreated cells, respectively. Finally, The percentage-inhibition curve was obtained by plotting "the percentage of inhibition" (i.e. [absorbance of test wells/absorbance of control wells]/100) against the concentration of cytotoxic compounds, compared to control (i.e. untreated) cells.

Results

Preparation and characterization of Fe₃O₄ MNPs

As mentioned earlier, Fe₃O₄ MNPs were chemically synthesized and their shape as well as size properties were confirmed by transmission electron microscopy. Upper illustration in Figure 3 demonstrates a representative TEM image of synthesized Fe₃O₄ MNPs. TEM image shows that the particles range in size below than 20 nm and possess an average size of 10 nm. The lower

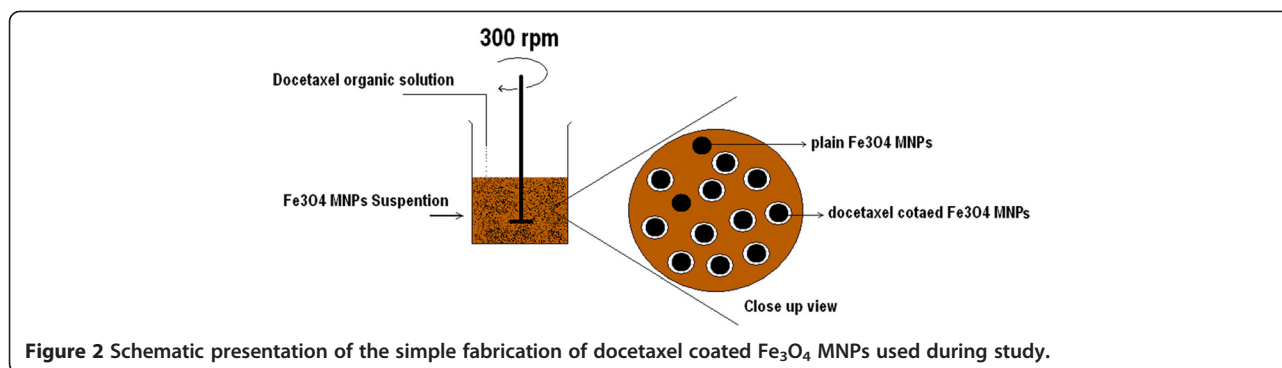
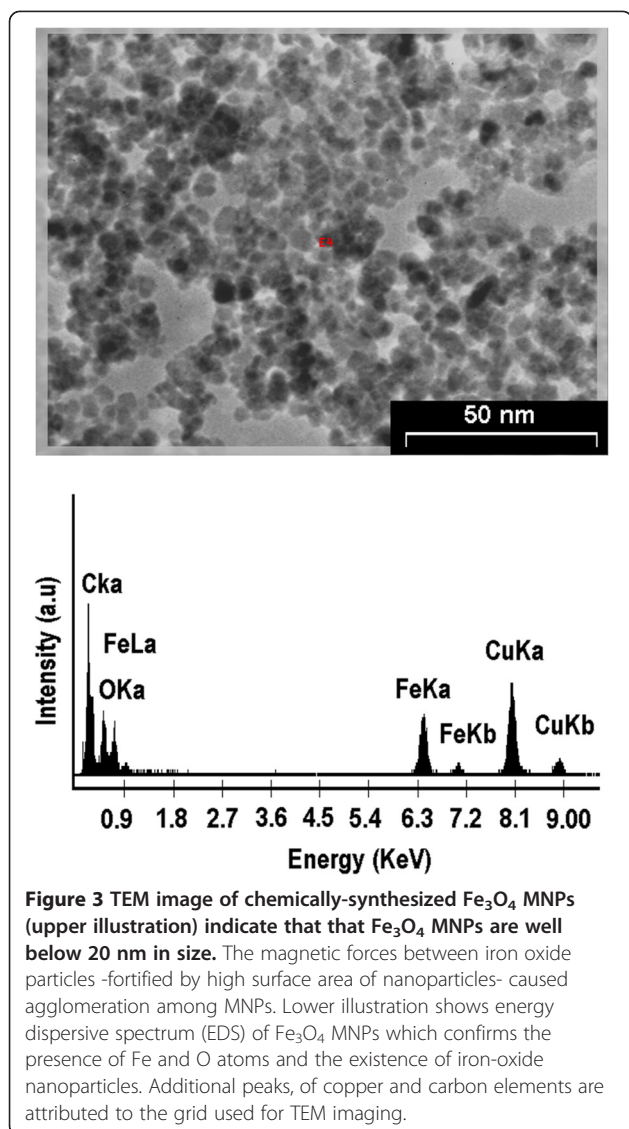


illustration in Figure 3 depicts the energy dispersive spectrum of Fe_3O_4 MNPs revealed the presence of Fe and O elements peaks. These EDS peaks confirm the existence of iron-oxide nanoparticles. It should be

mentioned that additional peaks, belonging to copper and carbon element are attributed to the grid used for TEM imaging.



Preparation and surface characterization of docetaxel coated Fe_3O_4 MNPs

After drop-by-drop addition of the organic solution of docetaxel to Fe_3O_4 MNPs slurry, docetaxel molecules precipitated on the surface of the Fe_3O_4 nanoparticles by the ratio of about 3/100 w/w (it means that 1000 μg of coated Fe_3O_4 MNPs nanoparticles contained almost 30 μg docetaxel). This ratio was simply determined gravimetrically by weighing the coated Fe_3O_4 MNPs before and after it was washed with chloroform as solvent, for three times as well as the results obtained from thermogravimetric assay. Figure 4 demonstrates weight loss of docetaxel-coated Fe_3O_4 MNPs as a function of temperature which was determined using this method. This weight loss (%3) in the TGA curve indicated carbon oxidation which proved the presence of organic moieties

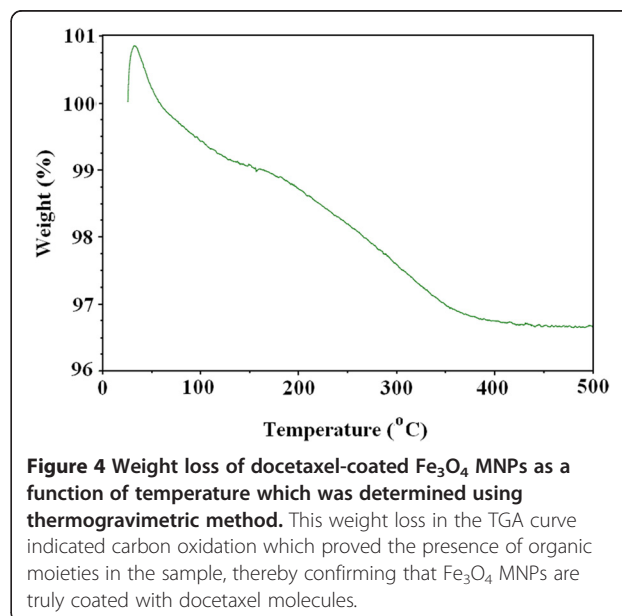


Table 1 The FTIR spectroscopy data of Fe₃O₄ MNPs, docetaxel and docetaxel coated Fe₃O₄ MNPs

Sample	Main peaks wave numbers (cm ⁻¹)
Fe ₃ O ₄ MNPs	584
docetaxel-coated Fe ₃ O ₄ MNPs	<u>2922</u> , <u>2927</u> , <u>1736</u> , <u>1246</u> , <u>1161</u> , <u>1065</u>
docetaxel alone	3487, 3377, <u>2937</u> , <u>1735</u> , <u>1712</u> , <u>1246</u> , <u>1169</u> , <u>1072</u>

Underlined data shows common corresponding peaks in FTIR spectra of docetaxel-coated Fe₃O₄ MNPs and docetaxel.

in sample, thereby confirming that Fe₃O₄ MNPs are truly coated with docetaxel molecules. The associated FTIR spectroscopic data of docetaxel-coated Fe₃O₄ MNPs, docetaxel-free Fe₃O₄ MNPs and docetaxel alone, are outlined in Table 1; which further confirmed the presence of docetaxel on the surface of synthesized Fe₃O₄ MNPs. In terms of chemical bonding, a main interaction is expected to occur between Fe₃O₄ MNPs and docetaxel: electrostatic bounds may form between carbonyl oxygen and hydroxyl groups of docetaxel with Fe chelating agent in Fe₃O₄ crystal (Table 1). This interaction lead to disappear of hydroxyl bands centered at 3487 and 3377 cm⁻¹ in FTIR spectrum of docetaxel-coated Fe₃O₄ MNPs. It is possible that these interactions

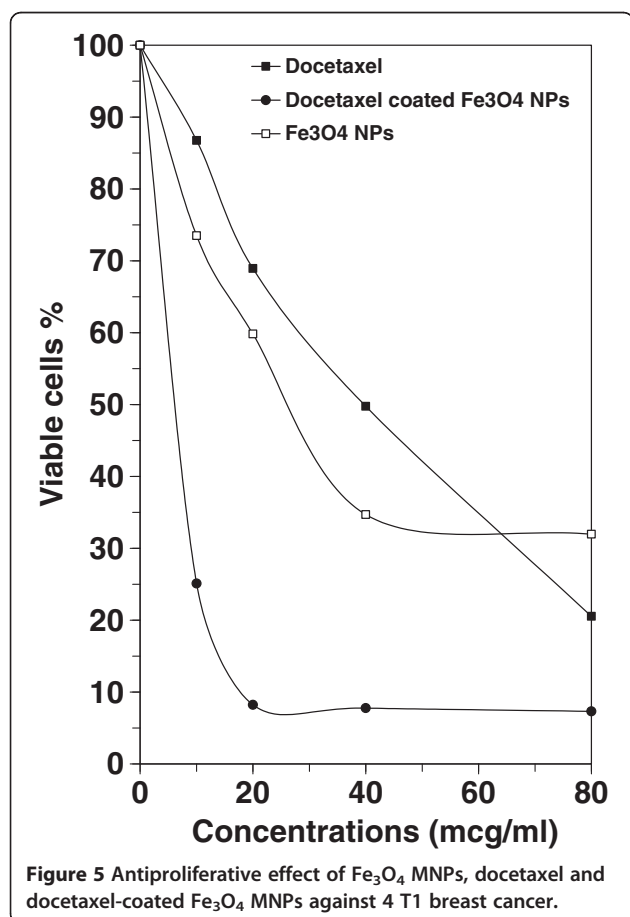
make docetaxel -coated Fe₃O₄ MNPs a suitable carrier with bipolar properties which penetrates into both hydrophilic and hydrophobic (i.e. cell membrane) fluids at the same time.

Antiproliferative effects

Antiproliferative effects of docetaxel-coated Fe₃O₄ MNPs, docetaxel alone, and Fe₃O₄ MNPs alone were *in vitro* evaluated at different concentrations on 4 T1 breast cancer cells. Cytotoxicity analysis of samples suggested a direct dose-response relationship, in which cell proliferation decreased in higher concentrations (Figure 5). Samples were found to have different cytotoxicities on 4 T1 breast cancer cells, with docetaxel-coated nanoparticles being the most potent. The antiproliferative effect of test compounds increased in the following order: docetaxel < Fe₃O₄ MNPs < docetaxel-coated Fe₃O₄ MNPs. Moreover, the necessary concentrations to cause 50% cell death (IC₅₀) were more than 30 and 40 (µg/ml) in cells treated with Fe₃O₄ MNPs alone and docetaxel, respectively. Docetaxel-coated Fe₃O₄ MNPs could cause the same effect at 10 µg/ml concentration. In other words, the combination of docetaxel and Fe₃O₄ MNPs exhibited a much more potent antiproliferative activity than docetaxel and Fe₃O₄ MNPs alone. As clear in Figure 5, docetaxel at concentrations of 20 and 30 (µg/ml), moderately reduced the proportion of viable cells to 70% and 50%, respectively. The cytotoxicity curve for Fe₃O₄ MNPs alone followed almost similar pattern, though sharper. The percentage of viable cell number sharply decreased from 87% at the concentration of 10 µg/ml to 35% at 40 µg/ml concentration. In contrast, however, only the low concentration of 10 µg/ml docetaxel-coated Fe₃O₄ MNPs (equivalent to the concentration of approximately 0.3 µg/ml pure docetaxel), drastically reduced the proportion of viable cells by more than 70%. Also a considerable cytotoxicity (about 90%) was observed for 20 µg/ml docetaxel-coated Fe₃O₄ MNPs against 4 T1 breast cancer cells. In higher concentrations (>20 µg/ml), this proportion declined steadily, until it reached the remarkably-low amount of about 7% at 80 µg/ml concentration.

Discussion

Literature review shows that there are numerous reports on cytotoxicity of docetaxel in combination of different carrier (15,16). However, the efficiency of this compound in conjugation with Fe₃O₄ MNPs has not been yet investigated. Here, the antiproliferative effect of docetaxel enhanced in tumor cells when it was coated on the surface of Fe₃O₄ MNPs has been reported. As mentioned earlier, Fe₃O₄ MNPs, docetaxel and docetaxel-coated Fe₃O₄ MNPs, all showed antiproliferative effects on 4 T1 breast cancer cells, increasing in the order: docetaxel < Fe₃O₄ MNPs < docetaxel-coated Fe₃O₄ MNPs. It is noticeable that based on current study, docetaxel has considerable



antiproliferative effect on 4 T1 breast cancer cells in concentrations of not below 10 µg/ml. However, obtained results demonstrated that when coated on Fe₃O₄ MNPs surface, docetaxel have antiproliferative effect, even at the low concentration of 0.3 µg/ml. This literally means that much lower amounts of docetaxel will be needed to produce the same antiproliferative effect as docetaxel alone. As described in above, during precipitation process, docetaxel molecules were precipitated on the surface of Fe₃O₄ MNPs by the ratio of 3/100 w/w which indicates that each milligram of coated Fe₃O₄ MNPs averagely contained 30 µg pure docetaxel compound. Therefore, it is deducible from Figure 5, that the concentration of 80 µg/ml docetaxel caused 79% cell death. However, when combined with Fe₃O₄ MNPs, the average concentration of 0.6 µg/ml docetaxel (which is equivalent to the concentration of about 20 µg/ml docetaxel- Fe₃O₄ MNPs) caused more than 90% cell death. Thus, it is concluded that by coating docetaxel on the surface of Fe₃O₄ MNPs, the required amount of docetaxel for showing a potent antiproliferative effects against 4 T1 breast cancer cells could be considerably lowered (more than 100 fold). As mentioned before, many pharmaceutical compounds have low solubility in biological fluids, which reduces their efficiency in *in vivo* models. Therefore, it is hypothesized that, Fe₃O₄ MNPs may be considered as efficient carriers for these insoluble compounds.

Conclusion

In this paper, the antiproliferative effect of docetaxel (IC₅₀ =40 µg/ml) has been reported and the results revealed that the combination of Fe₃O₄ MNPs and docetaxel is significantly more cytotoxic (IC₅₀ =10 µg/ml) than docetaxel alone. In conclusion, this result is of particular value since it demonstrates that by using metallic nanoparticles in combination therapy, lower amounts of these materials might be required, resulting in reduction of the potential hazards of docetaxel usage. Further investigations are needed to determine the antiproliferative effect of docetaxel-coated Fe₃O₄ MNPs in other cell lines. Moreover, studies on cytotoxic effects of docetaxel-coated Fe₃O₄ MNPs in animal models will be of great value.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

MHY: Collaboration in proliferation assays. ZNN: Collaboration in fabrication of nanoparticles. MRK: Commented on cytotoxic assays. MA: Commented on surface chemistry analysis of the nanoparticles. ARS: Design of the project. All authors read and approved the final manuscript.

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