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# *In vitro* evaluation of anticancer activity of sodium hyaluronate-titanium dioxide bionanocomposite

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### ABSTRACT

The purpose of the current research is to optimize the synthesis of sodium hyaluronate-titanium dioxide nanocomposite with the highest anticancer activity. To this end, the Taguchi method was followed to design nine experiments with different ratios of sodium hyaluronate biopolymer, titanium dioxide nanoparticles and stirring times. The results of scanning electron microscopy (SEM) confirmed the synthesis of the nanoparticle and nanocomposite. The comparison of anticancer activity level of synthesized nanocomposites using MTT assay showed that the nanocomposite synthesized in the conditions of experiment 9 (8 mg/ml of titanium dioxide nanoparticles, 2 mg/ml of sodium hyaluronate biopolymer and 60 min stirring time) had the maximum anticancer activity against Michigan Cancer Foundation-7 (MCF-7) cell line. According to the results, the Taguchi method can be employed as an effective and useful strategy to save time and cost in order to determine the optimal conditions for the synthesis of sodium hyaluronate-titanium dioxide nanocomposite with the most favorable anticancer activity.

### INTRODUCTION

Given the dramatic medical advances in the treatment strategies over the past decades, researchers have not yet succeeded in treating some diseases such as AIDS [1], autoimmune diseases [2], microbial infections [3], chronic pain [4] and cancer [5,6]. Cancer refers to a set of diseases resulting from uncontrolled cell proliferation. Researchers have already introduced various factors disrupting cell activity, including genetic factors, radioactive substances, chemicals, toxic materials, or overexposure to radiation like sunlight [7]. Cancer is one of the prevalent diseases in the modern world that increasingly affects people. Globally, now, about one out of six deaths are due to cancer. The cancer deaths worldwide are projected to reach over 11 million people by 2030 [8,9].

In recent years, the use of nanotechnology has opened a promising window for cancer treatment. Nanoparticles (NPs) can be produced from a wide range of materials, most commonly, silicates, non-oxide ceramics and metal oxides.

Lately, metal oxides have been considered for pharmaceutical application in humans due to their sustainability in different conditions [10,11]. Among the metal oxides considered in the treatment of cancer is titanium dioxide (also known as Titania). Titania is an inorganic solid white material that is not classified as hazardous according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS). The major problem in biological use of TiO<sub>2</sub> nanoparticles, like other metal nanoparticles, is the high tendency of aggregation, which reduces contact surface area and biological activity. Among the solutions to overcome this problem is the use of TiO<sub>2</sub> nanoparticles in combination with other materials in the form of nanocomposites [12,13]. Biopolymers are among the matrix compounds that are of interest in preventing the aggregation of metal oxide nanoparticles. Sodium hyaluronate biopolymer can be produced by a microbial method that is highly purified and suitable for medical applications [14].

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## AIM

The aim of this study is to use the Taguchi method to determine the optimal conditions for the synthesis of sodium hyaluronate-titanium dioxide nanocomposite with the highest anticancer activity.

## MATERIALS AND METHODS

The sodium hyaluronate biopolymer was synthesized by *Streptococcus zooepidemicus* (IBRC10919) prepared from the Iranian Research Organization for Science and Technology (IROST) in a Brain Heart Infusion Broth (BHI broth) medium. The TiO<sub>2</sub> nanoparticles were synthesized using the sol-gel method described in our previous study [15].

For the synthesis of sodium hyaluronate-titanium dioxide nanocomposite with the most anticancer activity, based on the Taguchi method in the Qulitek-4 software, nine different nanocomposites were synthesized by in situ method (Table 1). Thus, different levels of sodium hyaluronate biopolymer (2, 4 and 8 mg/ml) and various levels of TiO<sub>2</sub> nanoparticles (0.5, 1 and 2 mg/ml) at stirring times of 30, 60 and 90 min were compared to determine the optimal conditions for the synthesis of sodium hyaluronate-titanium dioxide nanocomposite.

**Table 1.** Taguchi design of experiments and results of anticancer activity of sodium hyaluronate-titanium dioxide bionanocomposites

Experiment	TiO <sub>2</sub> (mg/ml)			Sodium hyaluronate (mg/ml)			Stirring time (min)			Cell growth inhibition (%)
	2	4	8	0.5	1	2	30	60	90	
1		2			0.5		30			40.52
2		2			1		60			49.34
3		2			2		90			36.19
4		4			0.5		60			59.94
5		4			1		90			65.25
6		4			2		30			62.92
7		8			0.5		90			50.23
8		8			1		30			64.87
9		8			2		60			68.89

The MTT assay was applied to assess the anticancer activity of the synthesized nanocomposites on MCF-7 cell line prepared at the Pasteur Institute of Iran. For this purpose, the cells in the Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin were incubated in CO<sub>2</sub> and humidity at 37°C. The cells were separated from the flask by trypsin-EDTA and then 180 µl of the prepared cell suspension containing 5 × 10<sup>4</sup> cells was poured into each well of a 96-well plate and cultured in the incubator for 24 h. Next, 20 µl of the suspension containing the nanocomposites was added to each well; the untreated wells were considered as controls. After 72 h of cell treatment with nanocomposite compounds, 20 µl of MTT at a concentration of 5 mg/ml was prepared in Phosphate Buffered Saline (PBS) and added to each well. The surface of the plate was then covered and incubated for 4 h at 37°C with 5% CO<sub>2</sub>. After this time, the supernatant solution of each well was replaced by 100 µl

of dimethyl sulfoxide (DMSO) and the plate was placed on a shaker for 10 min. Ultimately, the optical density was read by ELISA reader at a wavelength of 570 nm [16,17]. All trials of this study included three tests carried out in triplicate and the inhibitory level of cancer cell growth was determined using the following equation:

$$\text{Cell growth inhibition (\%)} = \frac{\text{ODc} - \text{ODt}}{\text{ODc}} \times 100\%$$

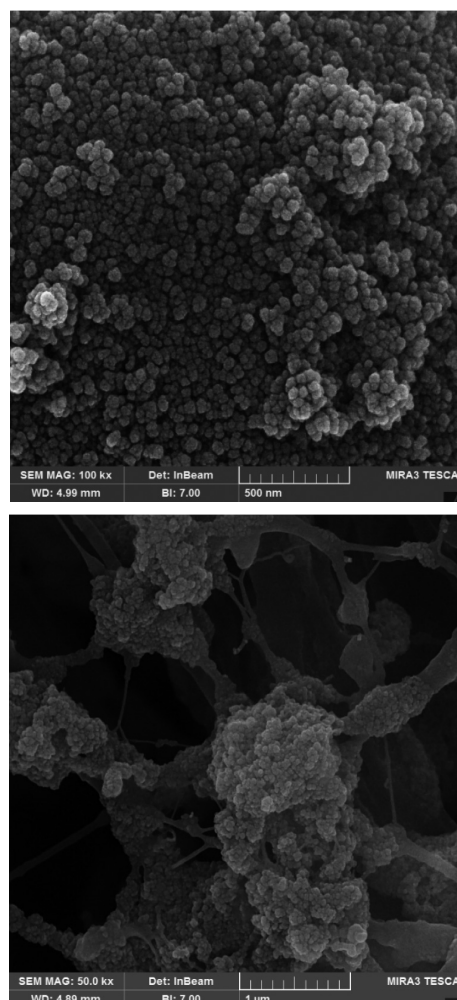
ODt: optical density of treated cells,

ODc: optical density of control cells.

The TESCAN scanning electron microscopy made in the Czech Republic was used to determine the distribution, shape and dimensions of nanoparticles and nanocomposites. Data analysis was performed using Qualitek-4 software.

## RESULTS

The SEM images prepared from TiO<sub>2</sub> nanoparticles and sodium hyaluronate-titanium dioxide nanocomposite are presented in Figure 1. The SEM images reveal that the nanoparticles were almost spherical with an average size of 23 nm, which were partially agglomerated. The image of sodium hyaluronate-titanium dioxide nanocomposite shows the spread of nanoparticles on the surface of the biopolymer, which confirms the formation of the nanocomposite.



**Figure 1.** SEM images of TiO<sub>2</sub> nanoparticles (up) and sodium hyaluronate-TiO<sub>2</sub> nanocomposite (down)

The cytotoxicity level of nine produced nanocomposites is presented in Table 1. The results indicate that the nanocomposite synthesized with 8 mg/ml of titanium dioxide, 2 mg/ml of sodium hyaluronate and 60 min stirring time (experiment 9) have the highest inhibitory effect on cancer cell growth (68.89%) while the lowest effect (36.19%) is related to the nanocomposite synthesized in the conditions of experiment 3 (2 mg/ml of titanium dioxide, 2 of mg/ml sodium hyaluronate and 90 min stirring time).

The effect of  $\text{TiO}_2$  nanoparticles, sodium hyaluronate biopolymer and stirring time at different levels on the inhibition of cancer cell growth by sodium hyaluronate-titanium dioxide nanocomposite is displayed in Table 2. The second level in all three investigated factors shows the highest effect in inhibiting the cancer cell growth. The effect of  $\text{TiO}_2$  nanoparticles, sodium hyaluronate biopolymer and stirring time on the structure of sodium hyaluronate-titanium dioxide nanocomposite at the highest levels in inhibiting the cancer cell growth is 62.70%, 59.82% and 59.39%, respectively.

**Table 2.** Effect of different levels of factors on anticancer activity of sodium hyaluronate-titanium dioxide bionanocomposite

Factors	Level 1	Level 2	Level 3
$\text{TiO}_2$	42.02	62.70	61.33
Sodium hyaluronate	50.23	59.82	56.00
Stirring time	56.10	59.39	50.56

Table 3 reveals the interactions between  $\text{TiO}_2$  nanoparticles, sodium hyaluronate biopolymer and stirring time at different levels. The third level of sodium hyaluronate biopolymer and the second level of stirring time have the highest interaction (53.44%) on inhibiting the cancer cell growth. The  $\text{TiO}_2$  nanoparticles at the third level and the stirring time at the second level demonstrate a significant interaction (18.04%) in inhibiting the growth of MCF-7 cancer cells. The interaction between the third level of  $\text{TiO}_2$  nanoparticles and sodium hyaluronate biopolymer reveal the lowest effect on reducing the cancer cell growth (5.36%).

**Table 3.** Interaction of studied factors on anticancer activity of sodium hyaluronate-titanium dioxide bionanocomposite

Interacting factor pairs	Column	Severity Index (%)	Optimum conditions
Sodium hyaluronate × Stirring time	2×3	53.44	[3,2]
$\text{TiO}_2$ × Stirring time	1×3	18.04	[3,2]
$\text{TiO}_2$ × Sodium hyaluronate	1×2	5.36	[3,3]

Analysis of variance (ANOVA) of  $\text{TiO}_2$  nanoparticles, sodium hyaluronate biopolymer and stirring time for inhibition of cancer cell growth is shown in Table 4. The  $\text{TiO}_2$  nanoparticles and stirring time, respectively, have the most (69.60%) and least (7.47%) effects on inhibiting cancer cell growth. In addition, the sodium hyaluronate biopolymer

**Table 4.** Analysis of variance for anticancer activity of sodium hyaluronate-titanium dioxide bionanocomposite

Factors	DOF	Sum of Squares	Variance	F-Ratio (F)	Pure Sum	Percent (%)
$\text{TiO}_2$	2	802.83	401.41	21.44	765.39	69.60
Sodium hyaluronate	2	139.85	69.93	3.73	102.41	9.31
Stirring time	2	119.59	59.80	3.19	82.16	7.47

DOF - degree of freedom

is also effective in reducing the growth of MCF-7 cancer cells by 9.31%.

The optimal conditions for the production of sodium hyaluronate-titanium dioxide nanocomposite with the highest anticancer activity using the Taguchi method are predicted in Table 5. Based on the results, the second levels of  $\text{TiO}_2$  nanoparticles, sodium hyaluronate biopolymer and stirring time have the highest role in improving the inhibition of MCF-7 cell growth (with a contribution of 7.35%, 4.47% and 4.04%, respectively). Due to the average cell growth inhibitory level (55.35%) under different experimental conditions and its growth reduction in optimal conditions (15.86%), the cancer cell growth is expected to be inhibited by the nanocomposite synthesized in optimal conditions to 71.21%.

**Table 5.** Prediction of optimal conditions for the synthesis of sodium hyaluronate-titanium dioxide bionanocomposite with maximum anticancer activity

Factors	Level	Contribution
$\text{TiO}_2$	2	7.35
Sodium hyaluronate	2	4.47
Stirring time	2	4.04
Total contribution from all factors		15.86
Current grand average of performance		55.35
Cell growth inhibition at optimum condition		71.21

## DISCUSSION

In the present study based on the Taguchi method, some nanocomposites were synthesized using different amounts of  $\text{TiO}_2$  nanoparticles, sodium hyaluronate biopolymer and different stirring times and were exposed to MCF-7 breast cancer cell lines in order to detect the strongest anticancer composition. The evaluation of the morphological characteristics and size of  $\text{TiO}_2$  nanoparticles and sodium hyaluronate-titanium dioxide nanocomposite with SEM confirms the synthesis of the nanoparticle and nanocomposite. The MTT test results indicated that the anticancer activity level of the studied nanocomposites varied from 36.19% to 68.89%, and the nanocomposite produced in experiment 9 had the highest inhibitory effect on the cancer cell growth. Previous studies reported anticancer activity of  $\text{TiO}_2$  nanoparticles alone and in the structure of nanocomposites, which is consistent with the results of this study [18-20]. In addition, former researches have shown that the application of  $\text{TiO}_2$  nanoparticles in the form of nanocomposite can improve its anticancer activity [21,22]. Ren *et al.* [18] synthesized a nanocomposite consisting of doxorubicin as an anticancer drug and  $\text{TiO}_2$  nanoparticles to overcome breast cancer cells with multiple drug resistance. The electrostatic interactions caused the stability of the synthesized nanocomposite. The anticancer effects of nanocomposite on resistant MCF-7 cancer cell lines were investigated using MTT assay. Based on the results, the use of nanocomposite showed a 2.4-times more anticancer effect than doxorubicin. They stated that the doxorubicin-titanium dioxide nanocomposite at a controlled pH in releasing the drug could be used to overcome breast cancer cells exhibiting multiple drug resistance.

The effective properties of nanoparticles have increased their use in cancer treatment. Nanoparticles can be applied to treat cancer through passive or active processes. A passive process has the advantage of enhanced permeability and retention (EPR). The nanoparticles are easily released through the damaged vascular system in cancer tissues and cause cancer cells to die. In addition to cancer cells, the inflamed tissue also has damaged vessels that easily are affected by the nanoparticles. Therefore, the passive activity of nanoparticles is not very accurate. In an active process, nanoparticles are functionalized to target precisely the cancer cells. In the research on the anticancer therapy, metal oxide nanoparticles were used directly against tumor cells *in vitro* and *in vivo* [23,24]. Despite various studies conducted in this regard, the mechanism of action of the nanoparticle effect on the cancer cells is unclear so far.

Gopinath *et al.* [25] examined the mechanism of the nanoparticle effects in inducing cell death in cancer cells and reported that the nanoparticles damage the membrane integrity of the cancer cells when bound to the membrane, and activate the p53 protein. The p53 protein is a known agent for the activation of proapoptotic genes, which then mobilize the bax, bad and bak proteins. These proteins cause leakage of the mitochondrial membrane and release of cytochrome c, which switches on Caspase 3 in a cascade reaction. Eventually, the Caspase 3 disrupts the nuclear membrane and causes DNA fragmentation. Simultaneously, the upregulation of Cmyc greatly enhances the apoptotic signals and down-regulates antiapoptotic genes of bcl2 and bclXL, which increases the incidence of apoptosis and thus the destruction of cancer cells.

## CONCLUSION

The results showed that the TiO<sub>2</sub> nanoparticles synthesized by the sol-gel method and sodium hyaluronate biopolymer produced by *Streptococcus zooepidemicus* were well combined together and the sodium hyaluronate-titanium dioxide nanocomposite was constructed with an optimal anticancer activity. The comparison of the anticancer activity level of synthesized nanocomposites using MTT assay showed that the nanocomposite synthesized in the conditions of experiment 9 (8 mg/ml of TiO<sub>2</sub> nanoparticles, 2 mg/ml of sodium hyaluronate biopolymer and 60 min stirring time) had the maximum anticancer activity against the Michigan Cancer Foundation-7 (MCF-7) cell line. Due to the increasing incidence of cancer risk and the lack of effective anticancer compounds, we recommend evaluating the cytotoxicity of the synthesized nanocomposite in the conditions proposed in this study on different cancer cell lines and in normal cells.

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