RESEARCH ARTICLE

# Fabrication, characterization, and biological applications of $TiO_2$ nanoparticles coated by chitosan

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**ABSTRACT**

**Objective** This study aimed to investigate and compare the properties of titanium dioxide ($TiO_2$) and $TiO_2$/Chitosan ($TiO_2$/CS) NPs and their effect on wound healing and bone defect repair. $TiO_2$ NPs synthesized and modified using of CS.
**Methods** to evaluate the modification surface changes of NPs FTIR analyses were used. The size of NPs was evaluated by PCS and TEM. MTT assay was performed to compare the effects of various concentrations of NPs in cell culture. Here, the examination group was divided into two groups: the burn model treatment group and the bone defect treatment model. Histomorphologic observations were performed to evaluate and compare the results.
**Results** The TEM image showed a spherical shape and smooth surface with a particle size on a nanometric scale. The particle size of $TiO_2$ NPs modified with CS depicted in TEM images is in agreement with the results obtained by PCS results. Furthermore, the images show that the particles exhibit a spherical shape, and a matrix without aggregation. Cytotoxicity assay on mesenchymal stem cells (MSCs) approved nontoxic effects of $TiO_2$/CS with dose of 1 and 5 µg/ml. The data also demonstrated that coating the $TiO_2$ NPs with CS at higher concentrations significantly increases their biocompatibility. Histological investigations revealed that skin and bone regeneration in the experimental classes by $TiO_2$/CS was remarkably enhanced than other groups. The process of synthesis of NPs has effective influences on their bioactivity and cytotoxicity.
**Conclusion** Covering $TiO_2$ NPs with chitosan is highly suggested for tissue regeneration and engineering applications.

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## INTRODUCTION

Nanotechnology is an attractive field of research due to the production of nanoparticles (NPs) in different sizes, shapes, and chemical compositions and their many applications for humans. NPs are 3D materials with sizes ranging from 1 to 100 nanometers [1]. The fabrication, manipulation, and use of metal NPs are of great importance due to their reduced dimensions and consequently their unique thermal, optical, and electrical properties. The application of NPs in various fields of science, including agriculture, industry, and medicine is diverse and has attracted a lot of attention[2]. Passing from microparticles to NPs encounters changes in some of the physical properties including increasing the ratio of surface area to volume and entering the particle size into the realm of quantum effects. Increasing the ratio of surface area to volume, which gradually occurs with decreasing particle size, causes the behavior of atoms located at the particle surface to overcome the behavior of internal atoms. This phenomenon affects the physical and chemical properties of the particle[3].

Nanomaterials have many unique properties due to their great variety compared to other conventional chemicals. Physical properties (particle size, morphology, and solubility) and chemical properties (chemical composition and

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structure of nanoparticle coating type) are important and by changing each of these characteristics, it is predicted that the type and amount of biological effect will be different [4]. Other factors such as surface-to-volume ratio, phase transfer, chemical stability, and tendency to form mass may also be equally important. In this study, we have tried to examine some of the properties of titanium dioxide ($TiO_2$) NPs in the treatment and its relationship with bone and skin[5].

Due to the unique properties of $TiO_2$, it can be used in the production of paints, cosmetics, ceramics, photocatalysts, water and wastewater refinery, gas filtration, and many other industries. Titanium is present in almost all living organisms, waters and soils and is the ninth most abundant element in the earth's crust. Titanium nanostructures are important than others due to their chemical stability, non-toxicity and other beneficial properties [6]. Therefore, they are of special importance.

The human body accepts titanium without any adverse reaction and it is also more biocompatible and resistant than many other metals. Because titanium is corrosion-resistant, biocompatible, and has an innate ability to attach to human bone, it is considered as one of the most widely used metals in the medical field. In addition to titanium surgical equipment and titanium orthopedic rods, nails, and plates, titanium has become an essential material used in medicine[7].

There are various methods, including chemical and physical methods for the synthesis of metal nanoparticles in different sizes and shapes. The inorganic nanoparticles NPs such as silicon dioxide, zinc oxide and titanium dioxide were added during film formation, to form chitosan-based composite films, which could increase the physicochemical and biological properties[8].

$TiO_2$ has magnificent potential for use in medicine due to its unique nanostructures and properties such as capacity to elicit positive cell response, and establishment in body fluids. Biomedical purposes of $TiO_2$ can be classified into four central groups: drug delivery, antibacterial properties, biosensing, and implant administerings[9]. However, few subjects in the research adjust to the use of $TiO_2$ to promote cell supporting and bone regeneration[10].

Bone disorders are a significant concern and predicament that affects the middle-aged population, and this has led to the use of various methods to treat these disorders. The use of nanotechnology is a new method that has helped treat bone disorders. Today, the preparation and development of NPs in order to achieve physical and mechanical properties appropriate to the host tissue is increasing[11].

Also, About 300,000 people die every year due to skin injuries such as burns. Too many people hurt from social, emotional, and financial injuries affected by burning. Most burn deaths occur in third-world countries. Burns is one of the most important factors that damage the skin. In addition to causing the skin to be vulnerable to external factors, burns also lead to dehydration and the natural deformation of the skin. Given the absence of definitive treatment for burns, finding new solutions can be helpful [12-13].

To overcome these disadvantages, this study aimed to investigate and compare the properties of $TiO_2$ and $TiO_2$/CS NPs and their effect on wound healing and bone defect repair. A novel green surface modification was successfully carried out on $TiO_2$ NPs using chitosan (CS) to prevent nanoparticles aggregation and increase biocompatibility and bioavailability.

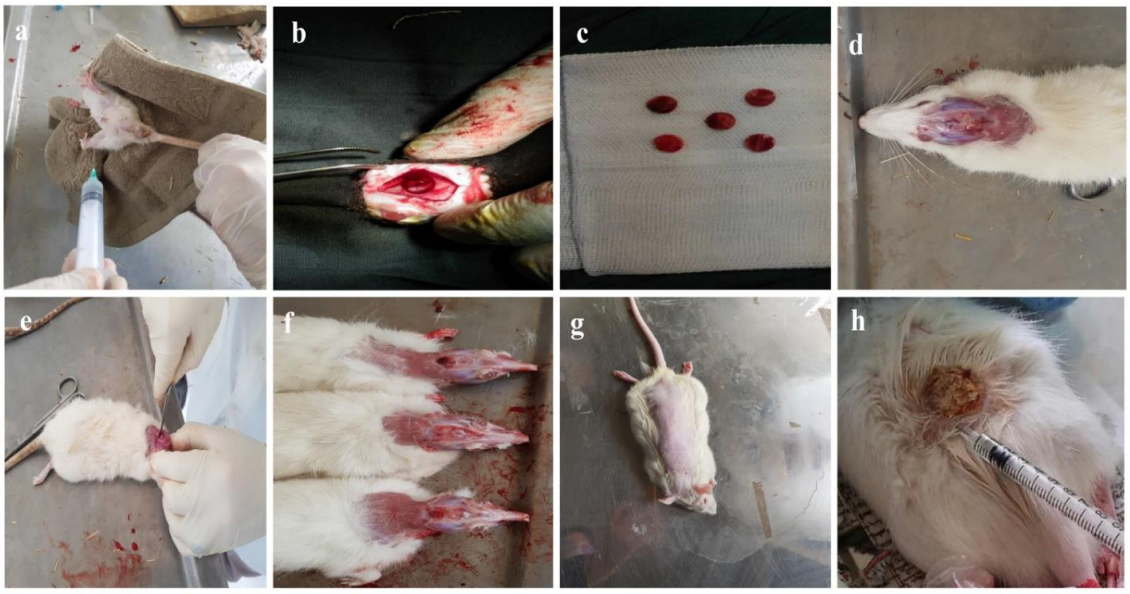
## METHODS

### *Materials*

Titanium Tetra Isopropoxide (TTIP, $C_{12}H_{28}O_4Ti$, 97%), Ethanol ($C_2H_5OH$, 96%), Methylene blue ($C_{16}H_{18}CIN_3S$), and distilled water was purchased from Merck India. Jasmine flowers were collected from the local market. The medium molecular weight CS with a molecular weight of 190000-310000 Da, a viscosity of 200-800 cPs, and a deacetylation degree of 75-85%, and also [3-(2, 3-epoxypropoxy) propyl] trimethoxysilane (EPO) were prepared from Sigma-Aldrich Company. Acetone (98%), ethanol (96%), and KBr (99%) were also supplied from Merck Company.

### *Synthesis of $TiO_2$ by hydrothermal method*

The slight modifications were made on the synthesis of $TiO_2$ NPs from the previously reported literature. Initially, 0.1 N of titanium tetra isopropoxide is dissolved in 20 ml of ethanol solution under continuous stirring for 30 min. After that, add a few drops of distilled water to form the dispersion medium. The product was placed on the ultrasonic bath for 20 min. After sonication, the solution was transferred into an autoclave at 150 °C for 3 h. Then the solution was




1. Schematic Fig. of the stages of establishing a cranial bone defect model (a-f) and a burn model (g and h).

cool to room temperature, and it was washed and centrifuged with deionized water to remove the impurities. Then it is filtered with Whatman No. 1 Filter paper. The filtered sample were dried oven at 110 °C for 5 h, and it is further annealed at 500 °C for 2 h. The resultant $TiO_2$ NPs was collected and processed with further characterization.

*Morphological characterization of TiO2 Nanoparticles*

The size was measured by photon correlation spectroscopy (Nano ZS4700 nano series, Malvern Instruments, UK). The transmission electron microscopy (TEM) (Zeiss- EM 10C- Germany) images were taken following staining of the isolated nanoparticles with 2% (w/v) phosphotungstic acid. Investigation of surface morphology of coated NPs was performed by scanning electron microscopy (SEM, KYKY-EM3200, China).

*FTIR characterization*

The chemical structure of CS, TiO2, CS/$TiO_2$ was analyzed by FTIR spectroscopy (VORTEX 80 spectrometer, Bruker Company, Germany). All samples were pressed into a pellet with KBr. The transmission spectra of the samples were obtained from 400 to 4000 $cm^{-1}$ with a resolution of 4 $cm^{-1}$.

*Surface modification of TiO2*

To synthesize $TiO_2$/CS, first, 1.5 g of CS were dispersed into 150 ml of dry dimethylformamide (DMF) and stirred. Then, 0.47 g of EPO was added and stirring was continued for 24 h at 80°C. Afterward, 2.5 g of $TiO_2$ were added and the mixture was stirred at the same temperature for another 24 h. $TiO_2$/CS NPs were separated by centrifuge and then washed several times using ethanol, DI water, and acetone. Finally, the modified nanoparticles were dried at room temperature for 24 h. Fig. 1 indicates the schematic of the surface modification procedure of $TiO_2$ NPs.

*Cell culture and MTT test*

Wharton's jelly mesenchymal stem cells (WJMSCs) were prepared from stem cell technology research center (BONYAKHTE company, Iran). Then the cells were seeded in a flask including DMEM (Gibco, USA) media supplemented with 10% of FBS (Gibco, USA), and incubated (Sina company, Iran) at 37 ˚C and 5% CO2. The cells were observed daily with an inverted microscope and cell division, density, and cell morphology were controlled. The supernatant media was changed every 72 h and the cells were separated by trypsin/EDTA (Gibco, USA).

To carry out the MTT assay, WJMSCs were cultured into steril plates at number of 1 × 104 /well and later cultured for 24h. The cells were treated with TiO2 and TiO2/CS with doses of 10, 50, and 100 µg/ml and maintained for 24h. In each well, 0.1 ml of MTT solution (0.5 mg/ml) was attached

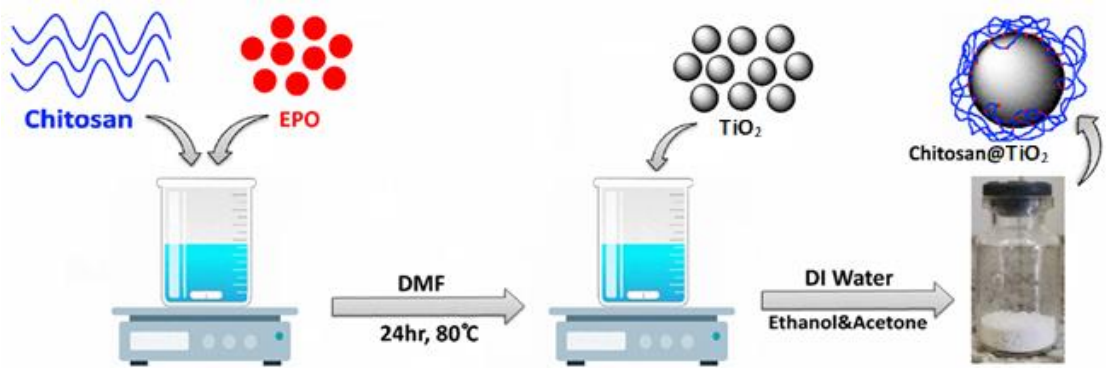



Fig. 1. The schematic image of preparation TiO2 modification with chitosan

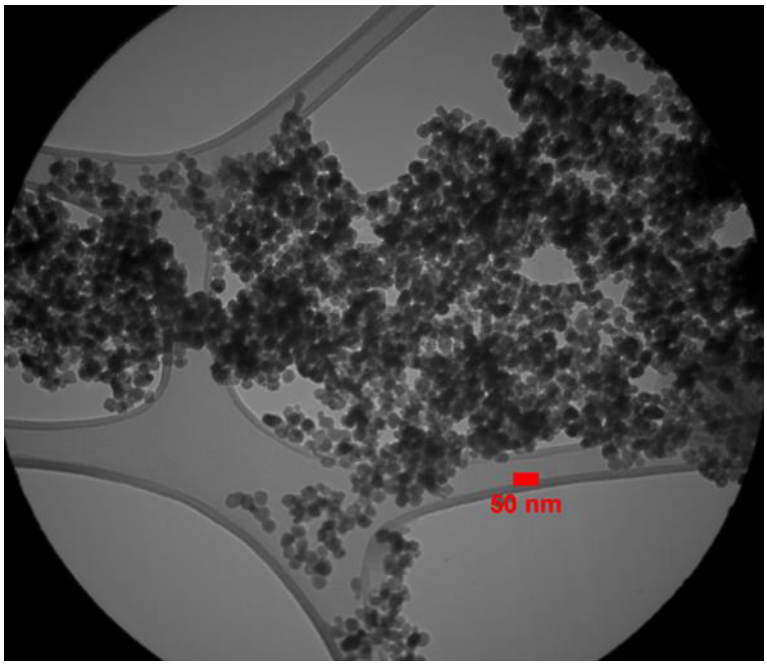



Fig. 2. TEM image of TiO2 nanoparticles

and the samples were maintained for 4 h. Then, the wells were poured and in all wells, 0.1 ml of DMSO was attached to separate the residue and shaken for 0.5 h. After that, the absorption of all wells was read by an ELISA reader at 570 nm (STAT, USA).

*Preparation and maintenance of animals*

Here, 30 adult rats (male, Wistar) registering 250-300 g were obtained from the center of Shahid Chamran University of Ahvaz. The animals were held for 7 days in approved conditions to adjust to the unknown condition with a free permit for meals and with periods of 12 h of sunlight and 12 h of night.

*Burning model*

The deep burn was caused by a metal pin with a diameter of 10 mm, that was heated in bubbling water for 5 min [14]. The rats were anesthetized and the hair on the rat's body was gently shaved and cleaned with iodine. The heated pin was put on the back of the rats for 0.5 min. After causing the burn, the animals were aimlessly sorted into 2 classes of examination and control. The examination rats were parted into two classes. In one, animals were intraperitoneally insinuated with TiO2 NPs at a dose of 5 µg/ml, in the other group the rats received TiO2/CS NPs at a dose of 5 µg/ml. The rats were individually kept in cells with formal requirements until the completion of the time (for 40 days).

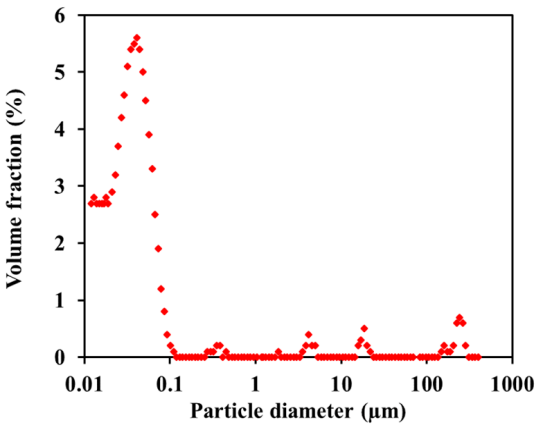



Fig. 3. Particle size distribution of TiO2 nanoparticles

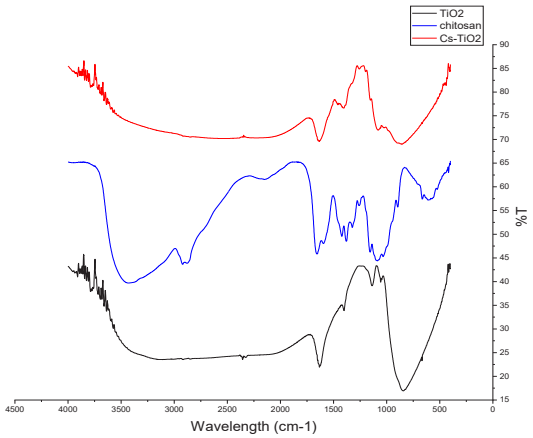



Fig. 4. The FTIR spectra obtained from TiO2/CS (red line), CS (blue line), and TiO2 (black line)

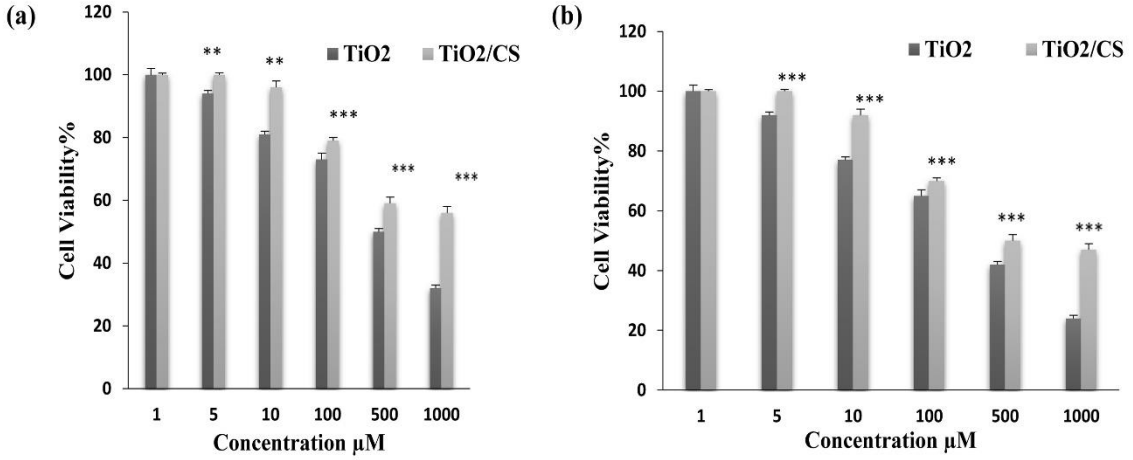



Fig. 5. MTT method for analogizing cell viability of mesenchymal stem cells exposed to 1, 5, 10, 100, 500, and 1000 µg/mL doses of TiO2 and TiO2/CS at (a) 1 day (b) 3 days after treatment (n = 3). *** shows a marked difference at the trust class of P<0.001.

*Modeling of Critical defects*

At first, the rats were anesthetized using 0.3 ml/200 gr of a mixture of ketamine and xylazine. Then the area of the surgery in the forehead was provided for sterile surgery and performed a 0.8 cm hole with 0.8 cm trephine and air-motor division. Following cleaning the defect produced with sterilized PBS, the defects were administered as follows: Group 1: Critical bone sit without any treatment as a control, Group 2: Critical bone defects treated with TiO2, and Group 3: Critical bone defects treated with TiO2/CS. Then the cut-off areas were sutured in a couple of layers later each surgery. To limit contamination after the operation, enrofloxacin was utilized as an antibiotic in 500 µl in 500 ml of drinking water for 4 days following the operation. Furthermore, 3 mg/kg morphine was was prescribed to decrease pain after the operation.

*Histological studies*

The rats were killed after 40 days. Skin pieces were removed in 1×1 dimensions at the burn site and fixed in formalin 10%. For bone defects, the rats were killed by chloroform after eight weeks, and then the defective sites were dismissed with some bone nearby and fixed by 10% formalin. Histotechnical steps were performed and incisions were stained using Hematoxylin & Eosin (H&E). Histological examination was done by Image J software.

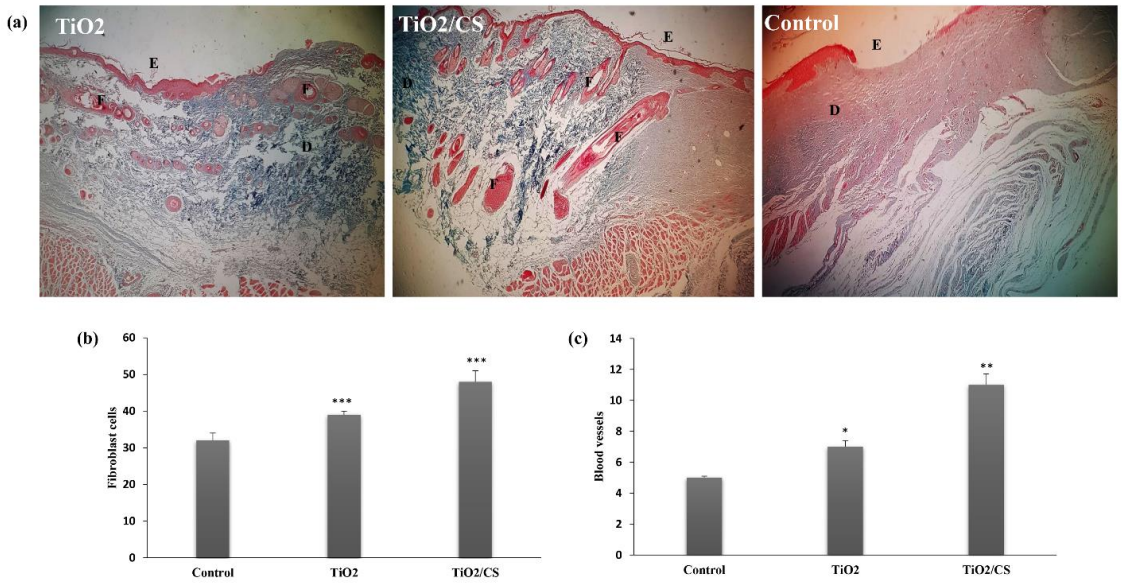



Fig. 6. (a) Morphological study of dermis and epidermis after 8 weeks of treatment by H&E staining (images are taken with 4X magnification). (b) Observation of fibroblastic cells 40 days after therapy. (n = 5). *** demonstrates that there is a considerable contrast at the trust level of P<0.001. (c) Observation of blood vessels on days 40 after therapy: (n = 5). * and ** demonstrate that there is a considerable contrast at the trust level of P<0.05 and P<0.01 respectively. E: Epidermis, D: Dermis, and F: Follicle

*Alkaline phosphate (ALP) analysis*

ALP activity was measured by the Pars Azmoon Company's ALP kit. Blood was obtained from rats in each group after 8 weeks and centrifuged at 2200 rpm for 25 min. Then the serum was isolated and frozen. To measure ALP activity 20 µL of serum and 200 µL of kit solutions were added to each well of a 96-plate (n=3). Absorption was measured 405 nm.

*Statistical surveys*

The results were investigated using SPSS (Ver.18) software and ANOVA tests (Mean ± SEM). The graphs were planned in the Excel 2016 software. Variations with P<0.05 were estimated as significant.

## RESULTS

*Transition electron microscopy studies*

Analysis using TEM revealed that the modified TiO2 NPs were successfully dispersed into the chitosan matrix and that the roughness of the chitosan-$TiO_2$ nanocomposites was significantly reduced. Moreover, FTIR analyses indicated that the chitosan interacted with $TiO_2$ NPs and possessed good compatibility. The TEM image (Fig. 2) showed a spherical shape and smooth surface with a particle size on a nanometric scale. The particle size of $TiO_2$ NPs modified with chitosan depicted in TEM images agrees with the results obtained by PCS (Fig. 3) results. Furthermore, the images show that the particles exhibit a spherical shape and a matrix without aggregation.

*FTIR results*

The infrared spectrum of chitosan can be seen. N-H and O-H stretching, as well as intramolecular hydrogen bonding, are illustrated by a strong band in the area 3291–361 cm 1. C-H symmetric and asymmetric stretching are relevant for the absorption bands at roughly 2921 and 2877 cm1, respectively. The vibration of the Ti-O-O bond is responsible for the peak detected at about 590 cm1. The presence of Ti-O bonds is clearly indicated by the FTIR spectrum (Fig. 4).

*The results of the study of cell viability*

Several concentrations of $TiO_2$ and TiO2/CS were applied for examination groups, and had no toxic effect in concentrations of 1 µg/ml. The survival percentage of cells in the examination group with concentrations of $TiO_2$/CS was extremely more increased than further classes after 24 h (p<0.05). Then the $TiO_2$ group in doses of 5 and 10 µg/ml did not show a considerable difference analogized to the control. The cell viability was remarkably reduced in both $TiO_2$ and

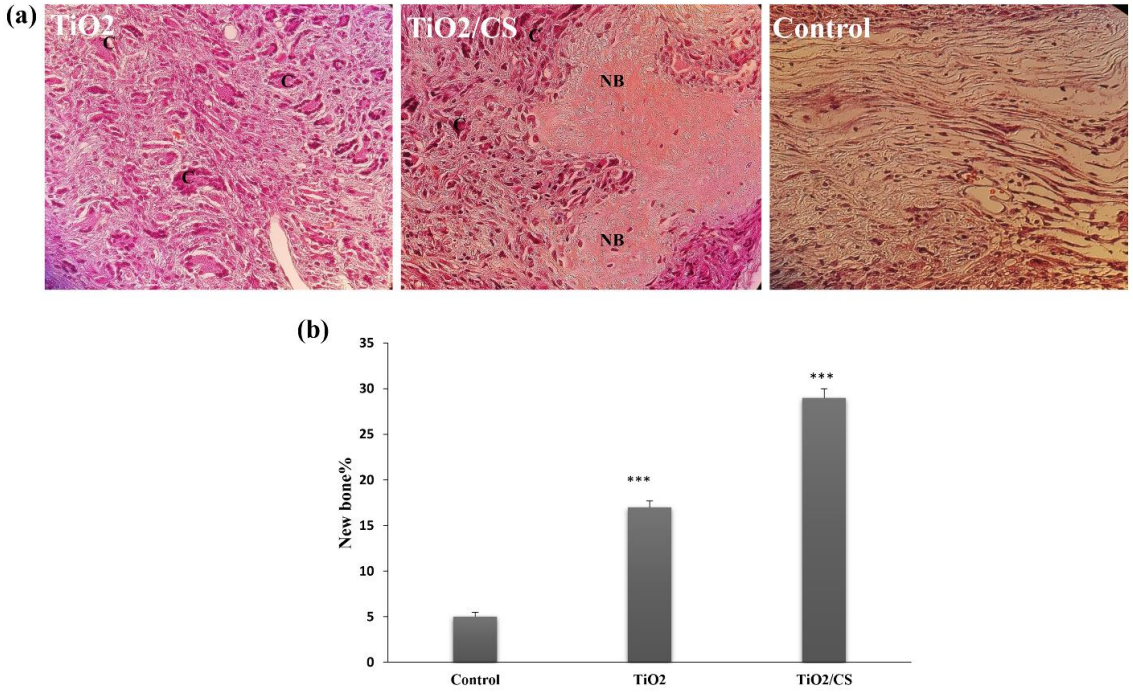



Fig. 7. (a) Morphological study of bone defects after 8 weeks of treatment by H&E staining. (images are taken with 40X magnification). NB: new bone and C: callus bone. (b) A comparison diagram of the mean renewed bone mineralization in various classes eight weeks after therapy. (n = 5). *** demonstrates that there is a considerable contrast at the trust level of P<0.001.

$TiO_2$/CS groups analogized to the untreated cells in the dose of 100, 500, and 1000 µg/ml. Of course, this decrease in the $TiO_2$ class was extremely more increased than in the TiO2/CS class (Fig. 5).

*Skin histomorphological results*

Microscopic examinations in groups with H&E staining after 40 days explained that the diameter of the dermis and epidermis in the examination classes was markedly longer than in the untreated samples (Fig. 6). The results showed the formation of collagen in the examination classes was quicker than in the control. Also, the medium estimate of blood vessels and fibroblasts was markedly higher in experimental than in untreated classes after 40 days of treatment ($p<0.05$).

*Bone histomorphological results*

Histomorphological examinations for bone were carried out after 8 weeks in various samples. As shown in Fig. 7, we recognized the loose fibrosis tissue in the deficient area in the untreated samples (class 1). Furthermore, no bone regeneration was recognized. In class 2 ($TiO_2$): the callus bone was recognized nearby the defects. Blood vessels and cells were also observed in the defect areas. The ratio of renewed bone resurrection was nearly 17% which was remarkably enhanced analogized to the untreated samples. In class 3 ($TiO_2$/CS): The new bone was recognized and many lacunae holding osteocytes were seen. Furthermore, a notable amount of blood vessels were observed and the ratio of renewed bone resurrection was nearly 29% which was remarkably enhanced analogized to the control samples and the $TiO_2$ class.

*Serum ALP activity*

ALP activity was estimated eight weeks after treatment. The outcomes revealed a considerable growth in ALP activity in the treatment classes analogized to the untreated example. The most ALP activity was marked in group TiO2/CS followed by the TiO2 group. The increase in enzyme activity in group TiO2/CS was quite significant than the other groups (Fig. 8).

## DISCUSSION

In this study, $TiO_2$ NPs were modified using chitosan coating and used to repair bone defects and wound healing. The new green surface modification was successfully carried out on $TiO_2$ NPs using chitosan. This modification prevented

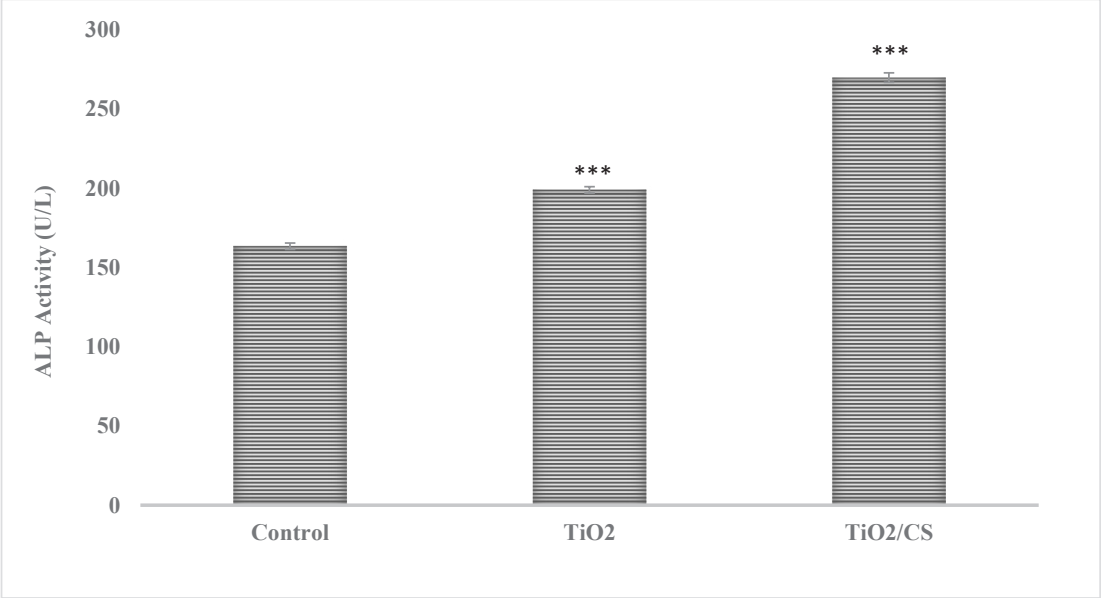



Fig. 8. Measurement of serum ALP activity. The most ALP activity was marked in group TiO2/CS followed by the TiO2 group. (n=3). *** demonstrates that there is a considerable contrast at the trust level of P<0.001.

nanoparticle aggregation and added their biocompatibility and bioavailability. To evaluation, the surface changes of NPs FTIR and TEM analyses were used. The particle size of $TiO_2$ NPs modified with chitosan has been measured to be 30 nm using photon correlation spectroscopy and transmission electron microscopy. Also, our results showed that these modified NPs made from $TiO_2$ are promising candidates for biomedical purposes, bone repair, and wound healing.

Bone is one of the gold-standard transplant tissues. There are three basic strategies for treating bone defects. The first strategy is an autologous transplant. In the second strategy, which is an allografts transplant, another person's body tissue is used for treatment[15]. Each of these methods has disadvantages that have led researchers to seek a third solution, which is the use of synthetic and laboratory materials to repair bone. This method is rapidly growing and developing due to its many advantages[16]. There are remarkable agents regarding the unification of implants and synthetic materials by bone tissue, such as surface characteristics and structure. For this reason, bioactive, biocompatible, and manufactured implant elements were invented by researchers[17].

The preparation of nanomaterials for osteointegration is one of the fundamental objections in orthopedic research. An important policy to be considered in bone regeneration is to support cell viability and growth[18, 19].

Osteogenesis is one of the most important properties of materials that should be considered in bone repair. Titanium is a non-allergenic metal meaning that the immune system does not attack it. Titanium compounds have been applied as the central substance in bone repair and implants because of their special strength, low flexible modules, competent stability, and capacity to create a light and stable layer that is stable to corrosion[20, 21].

Among metals, TiO2 NPs advance to be regarded as one of the most exciting materials due to their excellent features, such as great tensile strength, adaptability, high corrosion resistance, and low toxicity[22, 23]. However, there were limitations involving diminished interaction with surrounding tissue and reduced bioactivity. One of the strategies to succeed in these obstacles is to cover the surface of this metal group with biocompatible compounds. In 2015, Chung et al., covered the titanium surface with hydroxylapatite and bone morphogenetic protein-2 (BMP2) to create an identical nanoporous fabrication. BMP-2 is a growth portion that is incomparable because of its effects on cell induction to osteoblasts. Moreover, $TiO_2$/ hydroxylapatite, with a fit porous composition, promotes cell adhesion and growth. All of these points direct a decrease in bone regeneration[24].

Mohammadi et al., experimented to compare the influence of $TiO_2$, $SiO_2$, and SiC on the improvement of controlling conditions, mechanical power, and the hydraulic resistance of calcium phosphate bone cement. Their outcomes showed that $TiO_2$ NPs are of great interest to improve the mechanical power of cement in a short time. In addition to the effect of dose, the size of nanoparticles is also involved in influencing cell behavior[25]. Alves et al., administered to make a collection of 50–90 nm diameter $TiO_2$ nanotube, which induced the form of micron fabrication of actual bone and supplied them with bioactive components (phosphorous and calcium) to create a more effective useful implant[26].

In a study conducted by Hermenean et al. in 2017, chitosan was used together with graphene oxide nanoparticles to treat bone defects. Their results confirmed the significant role of these compounds in the healing process, as they reported that the amount of new bone formation after 18 weeks in the group receiving chitosan was about 15% and in the group receiving chitosan and nanoparticle it was about 60%. This finding was consistent with the results of the present study so our results also showed that after 8 weeks, the amount of new bone formation in the group receiving nanoparticles was about 17% and in the chitosan/nanoparticle group it was about 30% [27].

Also, in Hermenean's study, the activity of alkaline phosphatase enzyme was investigated, and according to their results, the activity level of this enzyme in the groups receiving chitosan and chitosan/nanoparticles was reported to be about 120 and 250 U/L, respectively. Our results also confirmed the increase in the activity of this enzyme in the nanoparticle and nanoparticle/chitosan groups, about 199 and 269, respectively.

Also, the hypotheses regarding the possible harms of nanotechnology have led to their prudent use. Therefore, in this study, we investigated the toxicity effects of these nanoparticles in *in vitro*. Our results showed that $TiO_2$ NPs at concentrations 1 and 2 have no toxic effects in endometrial cells. Our results also showed that coating these NPs with chitosan significantly reduces their toxicity effects.

In 2013, Kavitha et al. optimized titanium nanoparticles with chitosan and investigated their effects on the survival of AGS cells, and their results showed that the cell survival rate in these cells increased from 100 to 150 after 24 hours in the group of titanium nanoparticles compared with titanium nanoparticles/chitosan at a concentration of 5 μM. Our results also showed an increase in cell survival in the optimized group, so that at a concentration of 5 μM, cell survival increased from 92% in the titanium nanoparticles group to 100% in the titanium/chitosan group [28].

In addition, consistent with our results, another study by Zafar et al. showed that coating titanium nanoparticles using chitosan increases the biocompatibility of these nanoparticles, so cell survival increased from 90% to 120% due to coating.

Previous studies have shown that the use of chitosan increases the antimicrobial properties[29]. Studies have also shown that the use of NPs including $TiO_2$ due to their antimicrobial properties or increased angiogenesis can accelerate the healing process of skin wounds[30].

Our analysis of specimens with H&E staining demonstrated that the thickness of the dermis and epidermis in the experimental classes was remarkably more increased than the untreated class. Also, the amount of collagen formation, fibroblasts, and the number of blood vessels in the experimental classes were significantly more increased than the untreated class. In all restored samples, the $TiO_2$/CS group performed significantly better than the $TiO_2$ group. These results definitely show the significant effect of modified TiO2 NPs on wound healing.

In 2019, Nikpasand et al. used titanium nanoparticles along with hydrogel gelatin to repair skin burns, and their results showed a significant effect of titanium nanoparticles, especially in the modified form, compared to the control sample. Their results showed that after 14 days, titanium nanoparticles caused a remarkably more increased in fibroblasts compared to the untreated class, and the cells in this group increased to about 46 and reached about 68 in the modified nanoparticles group. Also, their data demonstrated that the number of blood vessels in the modified nanoparticle group increased significantly compared to the control sample, although no increase in the blood vessels was observed in the unmodified class [31]. Our results also showed that in the modified titanium nanoparticle group, the number of blood vessels was significantly higher compared to the control and unmodified ones (about 11/$mm^2$).

## CONCLUSION

This study confirmed that $TiO_2$ NPs exhibit a promising attitude for bone regeneration and wound healing. The new green surface modification was successfully carried out on $TiO_2$ NPs using chitosan. This modification prevented nanoparticle aggregation and added their biocompatibility and bioavailability. Physiological responses can be managed by modifying construction methods, e.g., by covering NPs using natural proteins. Results have shown that $TiO_2$/CS NPs could induce the formation of new bone in the defect area. Furthermore, in vivo experiments have established the biocompatibility and loss of inflammatory interaction of $TiO_2$/CS NPs, which is a NPs with a high capacity for utilization in bone regeneration and wound healing.

Moreover, our results showed that $TiO_2$ NPs are more secured for biomedical applications. Though, complete more studies of $TiO_2$ is expected before using it in clinical training.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AVAILABILITY

Available upon the request.

## AUTHOR CONTRIBUTION

E. H; responsible for the overall supervision and carry out in vivo experiments. E. R; contributed to data and statistical analysis and drafted the manuscript. All authors read and approved the final manuscript.

## ETHICS APPROVAL

The animal experiment was approved by the Animal Ethics Committee of Shahid Chamran University (Approval No. EE/1400.2.24.14804/scu.ac.ir & Approval No. SCU.SC1400.31316). This article does not contain any studies with human participants.

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