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Optimal conditions for producing bactericidal sodium hyaluronate-TiO_2 bionanocomposite and its characterization

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ABSTRACT

In this research, the creation of optimum conditions for the formation of sodium hyaluronate-TiO_2 bionanocomposite and its antibacterial effect on gram positive and gram negative bacteria was evaluated. The Fourier transform infrared spectroscopy spectra, scanning electron microscopy images and energy dispersive X-ray spectroscopy pattern confirmed the formation of the bionanocomposite. Thermogravimetric analysis and differential thermal analysis indicated that the thermal stability rate had significantly improved with formation of the bionanocomposite. Nine experiments were designed based on the Taguchi method by applying different proportions of sodium hyaluronate biopolymer and TiO_2 nanoparticles at different stirring times. Bionanocomposite produced under conditions of experiment 5 (TiO_2 4 mg/ml, sodium hyaluronate 1 mg/ml and stirring time of 90 min) and experiment 9 (TiO_2 8 mg/ml, sodium hyaluronate 2 mg/ml and stirring time of 60 min) completely prevented the growth of *Staphylococcus aureus* and *Escherichia coli*. It can be concluded that sodium hyaluronate-TiO_2 bionanocomposite can be used as an effective antimicrobial compound in food, pharmaceutical, medical and environmental sectors.

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1. Introduction

Since the identification of bacteria, researchers have always been seeking for effective substances against them. During the years, bacteria have achieved effective mechanisms to counter antibiotics through chromosomal mutations and gene flow. Today, with the emergence of drug resistant pathogenic bacteria, including *Staphylococcus aureus* and *Escherichia coli*, confronting these kinds of bacteria is faced with many problems. The antibiotic resistant bacteria increased the risk of infectious diseases which threatens the human life [1,2]. During recent decades, the use of nanomaterials in various fields has quickly spread. In the meantime, pharmaceutical and medical industries have also benefited and the use of nanotechnology in these industries has led to the introduction of applied and new products to the market. Nanotechnology has come to the aid of people to reduce consumption of antibiotics day by day. The compounds containing nanoparticles can inhibit bacteria without developing drug resistance. The use of nanoparticles instead of larger particles increases the ratio of surface to volume. The increase in this ratio increases the penetration rate of nanoparticles and enhances their interaction with cells and tissues. The ultrafine size of these particles causes them to penetrate into tissue depth by increasing their cellular absorption [3,4].

In recent years, TiO_2 NPs have been widely used in various industries due to biocompatibility, strong oxidation potential, high stability, reasonable price, nontoxicity for humans and being ecofriendly. TiO_2 NPs have different phases such as rutile (tetragonal structure), anatase (tetragonal structure) and brookite (orthorhombic structure) [5,6]. Previous studies have shown that the two-phase composition of TiO_2 NPs (anatase and rutile) is better and more convenient for photocatalytic and antimicrobial purposes. Photocatalytic and antibacterial properties are the most important properties of TiO_2 NPs which are highly regarded [7,8].

Several studies have been conducted with the aim of increasing the efficiency of titanium dioxide by using it in the form of nanocomposite in combination with polymers and other metal nanoparticles [9–11]. The synthesis of biodegradable and biocompatible nanocomposites, consisting of inorganic and organic compounds, has been highly considered for use in various fields [12]. Hyaluronic acid and its sodium salt are biopolymers that are used for surface modification of various biomaterials. Sodium hyaluronate is a glycosaminoglycan which is the sodium salt of hyaluronic acid. This biopolymer is formed by repeating the

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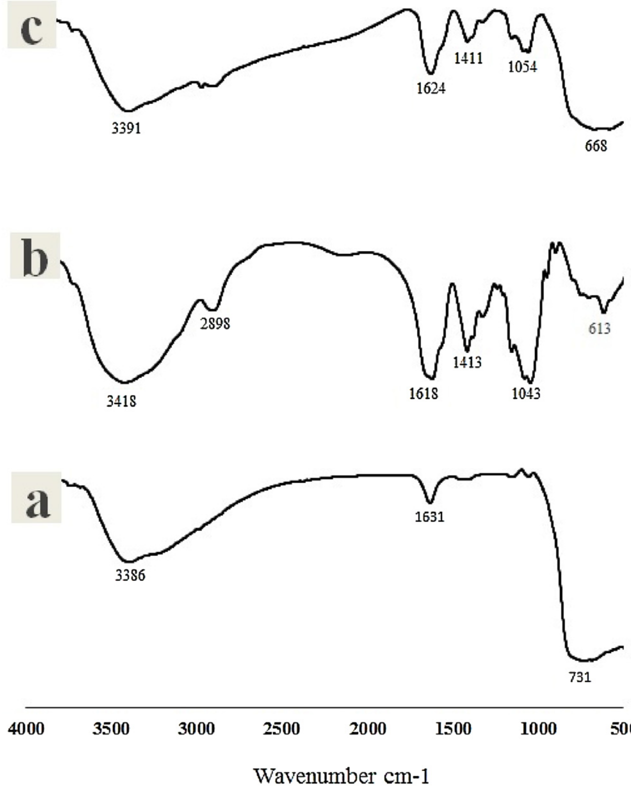

Fig. 1. FTIR spectra of (a) TiO_2 nanoparticle, (b) sodium hyaluronate biopolymer and (c) sodium hyaluronate-TiO_2 bionanocomposite.

units of disaccharide N-acetyl glucosamine and sodium glucoronate [13,14]. The nanocomposite, consisting of sodium hyaluronate biopolymer synthesized by biological method and TiO_2 NPs synthesized chemically, is considered due to the possibility of combining its characteristics. Therefore, the use of nanocomposite containing TiO_2 NPs as an alternative for antibiotics can be effective to deal with bacterial infections.

The aim of this study was to create optimal conditions for the synthesis of nanocomposites containing TiO_2 inorganic nanoparticles in the sodium hyaluronate biopolymer matrix and to examine its antibacterial properties. The properties of synthesized nanocomposites were studied by Fourier transform infrared spectroscopy (FTIR), high-resolution scanning electron microscopy (FESEM), energy dispersive X-Ray spectroscopy (EDX), differential thermal analysis (DTA) and thermogravimetric analysis (TGA). Utilizing colony forming unit (CFU) and disc diffusion, the antibacterial activity of SH-TiO_2 nanocomposites on gram-negative bacteria (*Escherichia coli*) and gram-positive bacteria (*Staphylococcus aureus*) were measured.

2. Materials and methods

2.1. Synthesis of titanium dioxide nanoparticles

The TiO_2 NPs were synthesized using sol-gel method. In this method, 10 ml of isopropanol was combined with 10 ml of double-distilled water. Then, 20 ml of titanium tetraisopropoxide (TTIP) solution was added to the solution dropwise while stirring it continuously. After continuous stirring of the resulted solution for one hour at a temperature of 60 °C, a yellow transparent gel was obtained. The resulted gel was placed in a hot air oven at 80 °C to form the yellow crystalline powder. Then, the obtained powder was calcined at 650 °C for 3 h to obtain TiO_2 NPs.

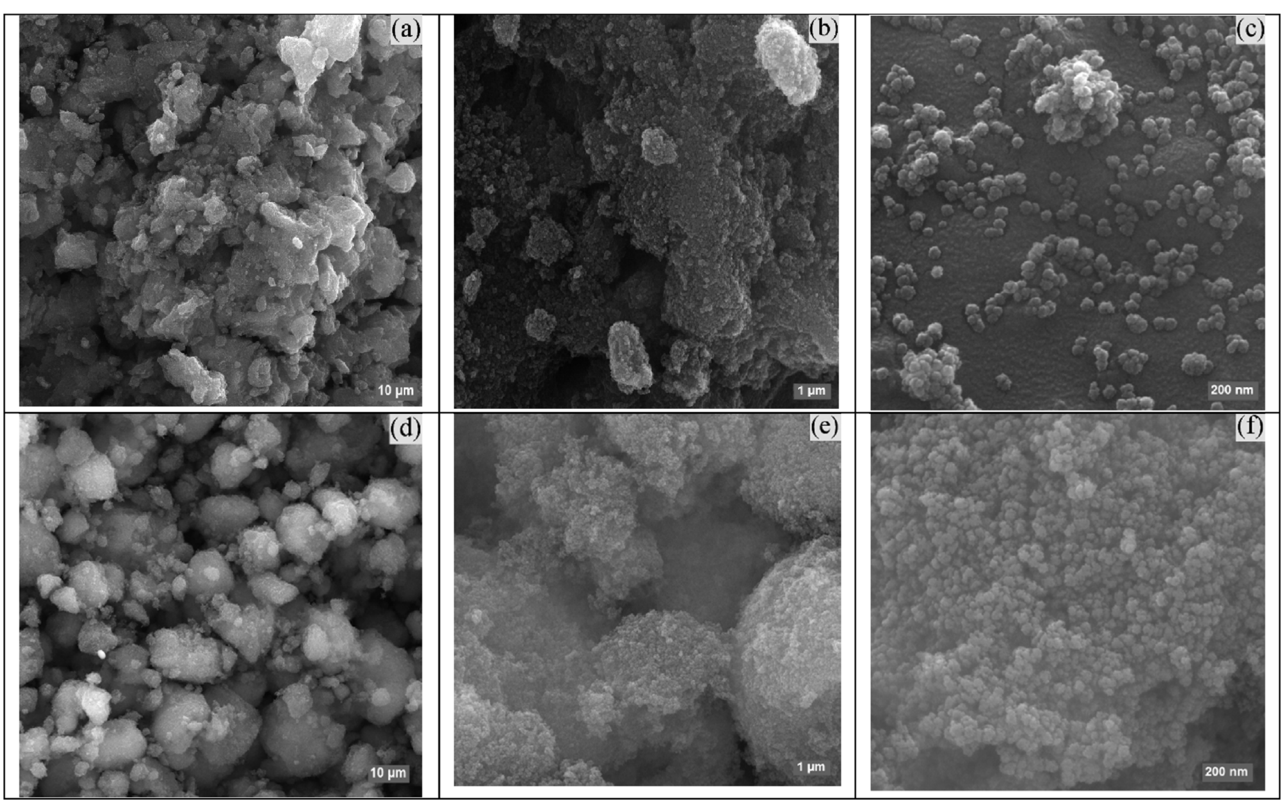

Fig. 2. SEM micrographs of (a–c) TiO_2 nanoparticle, (d-f) sodium hyaluronate-TiO_2 bionanocomposite at different magnification.

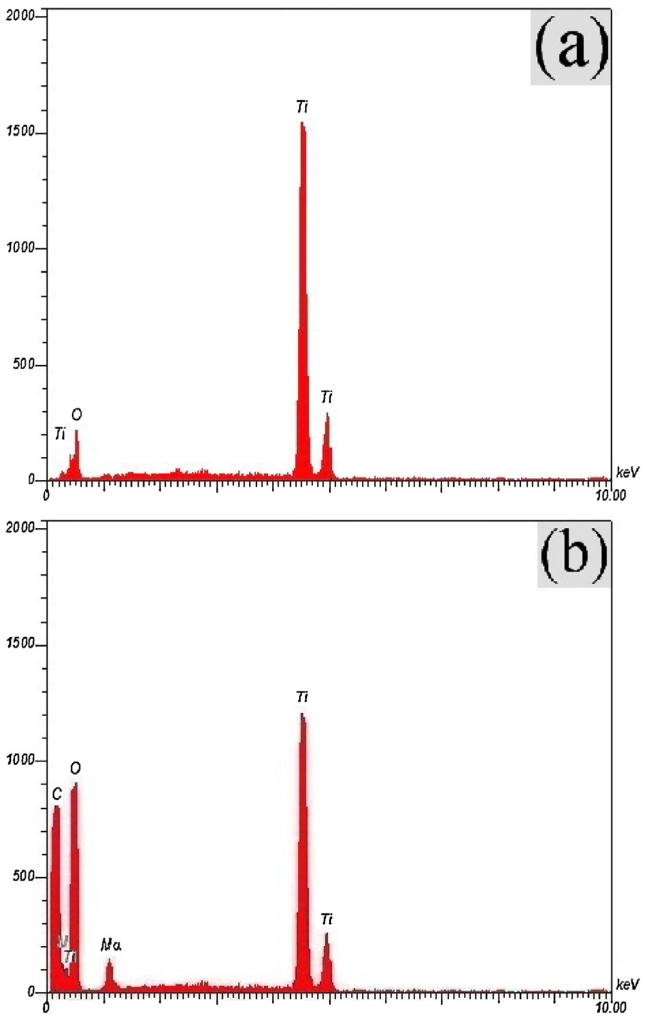

Fig. 3. Energy dispersive X-ray (EDX) of TiO_2 nanoparticle and sodium hyaluronate $-TiO_2$ bionanocomposite.

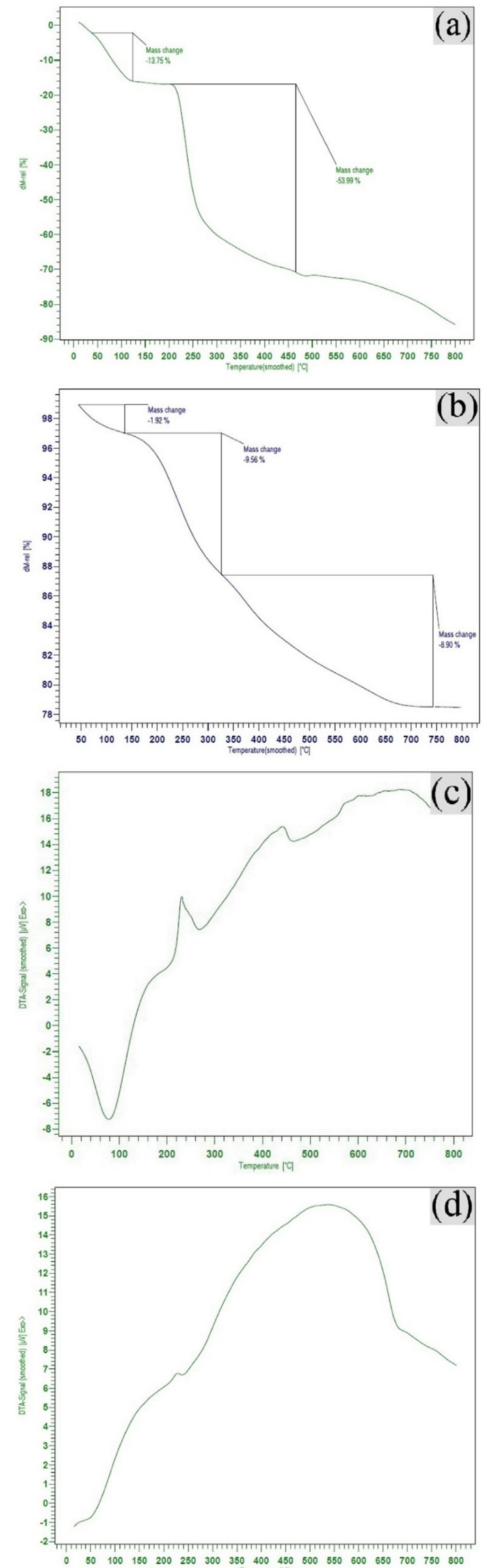

Fig. 4. Thermogravimetric analysis of (a) sodium hyaluronate biopolymer and (b) sodium hyaluronate $-TiO_2$ bionanocomposite; differential thermal analysis of (c) sodium hyaluronate and (d) sodium hyaluronate $-TiO_2$ bionanocomposite.

2.2. Synthesis of sodium hyaluronate biopolymer

Sodium hyaluronate biopolymer was synthesized using the *Streptococcus zooepidemicus* bacterium during a fed-batch fermentation process in a medium containing compounds of yeast extract, NaCl, $MgSO_4$. $7H_2O$, K_2HPO_4, casein enzyme hydrolysate and carbon source with the method described by Rangaswamy and Jain [15].

2.3. Synthesis of sodium hyaluronate-titanium dioxide nanocomposites

Applying the Taguchi method and Qulitek-4 software, 9 experiments containing different proportions of sodium hyaluronate biopolymer and TiO_2 NPs biopolymer at different stirring times were designed to determine the best conditions for the synthesis of SH-TiO_2 nanocomposites with the highest antibacterial activity (Table 1). To identify the optimal conditions, the values of 2, 4 and 8 mg/ml of TiO_2 NPs combined with the values of 0.5, 1 and 2 mg/ml of the sodium hyaluronate biopolymer were measured at stirring times of 30, 60 and 90 min using the in situ approach. The sodium hyaluronate biopolymer and TiO_2 NPs solutions were separately prepared at different concentrations based on the 9 experiments designed and sonicated for 10 min for better homogenization. The solutions containing sodium hyaluronate were then added dropwise to containers of TiO_2 NPs solutions while stirring

Table 1
Taguchi design of experiments and results of antibacterial activity of synthesized nanocomposites on gram-positive and gram-negative bacteria.

Experiment	TiO_2 (mg/ml)			Sodium hyaluronate (mg/ml)			Stirring time (min)			Bacterial survival (Log_{10} CFU/ml)	
	2	4	8	0.5	1	2	30	60	90	Gram positive	Gram negative
1		2			0.5			30		3.62	4.11
2		2			1			60		2.82	3.35
3		2			2			90		4.02	4.43
4		4			0.5			60		2.24	2.25
5		4			1			90		0.00	0.00
6		4			2			30		1.39	1.84
7		8			0.5			90		3.62	4.02
8		8			1			30		0.89	0.87
9		8			2			60		0.00	0.00

them continuously. After 30, 60 and 90 min of continuous stirring, some portions of the resulted solutions were stored at a 4 °C to assess the antibacterial properties, and the rest were transferred to a 40 °C oven; the resulted powder was used to evaluate characterization of the synthesized nanocomposites.

2.4. Antibacterial activity

The antibacterial activity rate of SH-TiO_2 nanocomposite was investigated by using two methods of colony forming units (CFU) and disc diffusion under light condition. The colony forming units (CFU) approach was used to determine the best ratio for biopolymer to nanoparticle and the stirring time with the highest antibacterial activity. In this method, a suspension of gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) bacteria with an approximate concentration of 10^8 CFU/ml was prepared. Then, the solutions containing the bacteria and different concentrations of nanocomposites were shaken at 37 °C and 140 rpm for 6 h. After shaking, the resulted solutions were diluted 10-fold using serial dilution, and the dilutions were cultured on nutrient agar medium for 24 h in an incubator at 37 °C. All dilutions had three replications for each of the 9 experiments units designed by the Taguchi method. The growth rates of colonies were calculated for each experimental unit after counting based on their replicates mean values. After determining optimal conditions for the synthesis of SH-TiO_2 nanocomposites with the greatest antibacterial activity, the antibacterial activity rate of the synthesized nanocomposite was compared with its components using two colony forming units (CFU) and disc diffusion methods.

In the disk diffusion method, after culturing *Staphylococcus aureus* and *Escherichia coli* bacteria on nutrient agar medium, 3 discs containing sodium hyaluronate biopolymer, TiO_2 NPs and SH-TiO_2 nanocomposite were placed on the culture medium, and the plates were then incubated for 24 h at a temperature of 37 °C. The diameter of inhibition zone was then measured for each disc.

2.5. Characterization

The FTIR spectra of sodium hyaluronate biopolymer, TiO_2 NPs and SH-TiO_2 nanocomposite were prepared by alpha spectrometer (Bruker, Germany) with the preparation of KBr tablet in the 500–4000 cm^{-1} range. Microscopic images of TiO_2 NPs and SH-TiO_2 nanocomposites were provided by a high-resolution scanning electron microscope (TESCAN, Czech Republic) in three magnifications of 10 μ, 1 μ and 200 nm. In addition, the energy dispersive X-ray (EDX) detector was used on FESEM to determine the constituent elements of the samples. The disruptive behaviors of sodium hyaluronate biopolymer and SH-TiO_2 nanocomposite were analyzed by thermal analysis (TGA, DTA) of samples by means of Linseis STA PT1000 thermal analysis device. To this end, about 0.3 g of each sample was prepared and measured in nitrogen atmo-

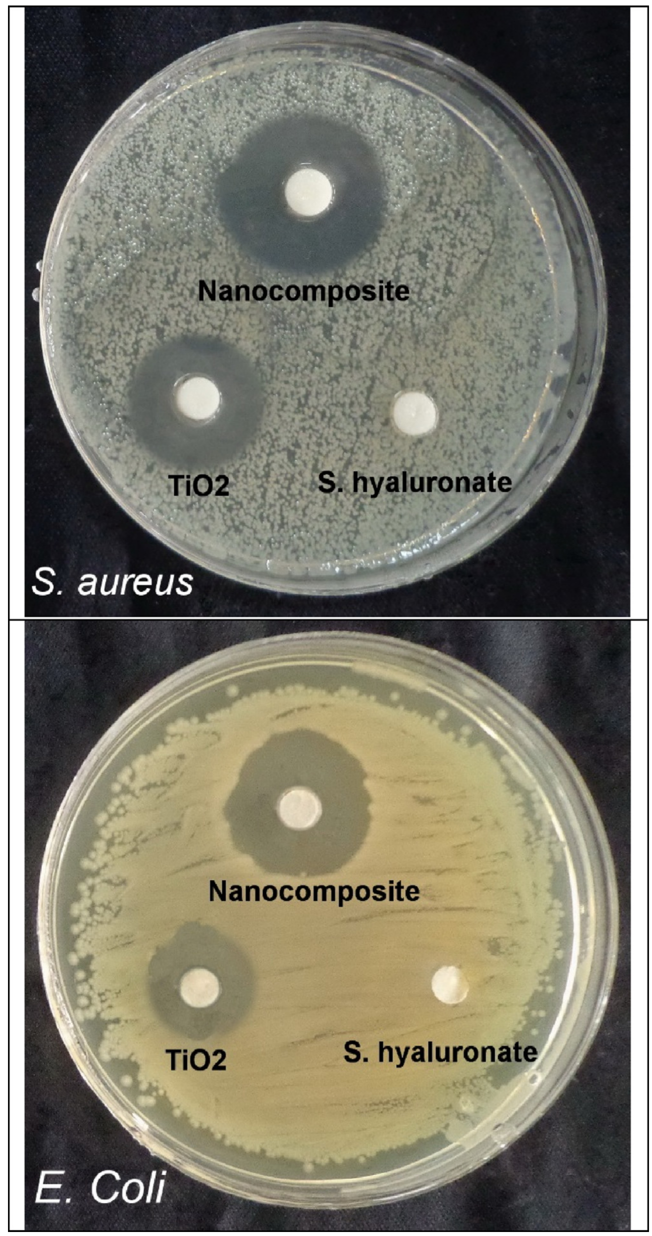

Fig. 5. Comparison of antibacterial activity of sodium hyaluronate-TiO_2 bio-nanocomposite, Tio_2 nanoparticle and sodium hyaluronate biopolymer against gram-positive and gram-negative bacteria.

sphere with heat amount of 10 °C/min in the temperature range of 0–800 °C.

3. Results and discussion

3.1. FTIR analysis

The FTIR spectra of TiO_2 NPs, sodium hyaluronate biopolymer and SH-TiO_2 nanocomposite were in the range of wave number of 500–4000 cm^{-1}, shown in Fig. 1. The peaks in the range below 800 cm^{-1} referred to the binding of oxygen and titanium. The bands of TiO_2 at 1631 cm^{-1} and 3386 cm^{-1} were attributed to surface O—H groups and adsorb water [16]. The peaks observed in the range below 1000 cm^{-1} in the sodium hyaluronate biopolymer referred to the stretching of C—O—C group in its structure. The broadband seen in the range of 1043 cm^{-1} was attributed to the stretching caused by C—O—C, C—O and C—O—H groups. The sharp bands observed in the ranges of 1413 cm^{-1} and 1618 cm^{-1} were caused by the stretching created by C—O group with C=O combination and amid II group, respectively. The band in the 2898 cm^{-1} area was due to C—H stretching and the sharp peak at 3418 cm^{-1} area was related to NH stretching and OH stretching [17].

FTIR spectra indicated different capacities of absorption bands which described the interaction between the biopolymer and the nanoparticle in the nanocomposite structure. The FTIR spectrum of SH-TiO_2 nanocomposite showed a combination of characteristics of sodium hyaluronate biopolymer and TiO_2 NPs bands. The absorption band in the range of 3391 cm^{-1} showed the creation of hydrogen bonds between hydroxyl groups of sodium hyaluronate biopolymer and TiO_2 nanoparticles. Other changes in wave numbers and size of the peaks in the spectrum of SH-TiO_2 nanocomposite indicated the creation of a strong bond between sodium hyaluronate biopolymer and TiO_2 NPs through hydrogen bonds that acknowledged the formation of the nanocomposite.

3.2. SEM analysis

The structure, morphology and size of nanoparticles of titanium dioxide and hyaluronate sodium-TiO_2 nanocomposite were examined at different magnifications using the FESEM (Fig. 2). Morphology analysis of synthesized TiO_2 NPs showed that these nanoparticles were spherical and homogeneous with relatively rough surface (Fig. 2a–c). The desirable morphology of synthesized TiO_2 NPs increased the amount of their interaction with the sodium hyaluronate biopolymer. FESEM observations suggested that the size of synthesized nanoparticles was favorable. Their average size was calculated using X-ray diffraction to be approximately 23 nm. Fig. 2d–f shows the distribution of TiO_2 nanoparticles in sodium hyaluronate biopolymer matrix at different magnifications. The images indicate the optimal coating of nanoparticles on sodium hyaluronate biopolymer and formation of SH-TiO_2 nanocomposites. Several studies reported that the formation of polymer-based nanocomposites with TiO_2 nanoparticles improved the properties of these nanoparticles, including their antibacterial properties [18–20].

3.3. EDX analysis

Appling the energy dispersive X-ray (EDX) detector on FESEM, the constituent elements of TiO_2 NPs and SH-TiO_2 nanocomposites were evaluated whose results are presented in Fig. 3. The EDX result graph of TiO_2 suggests that the synthesized nanoparticles are only composed of the elements Ti and O as 80.95% and 19.05%, respectively. The results showed no peak indicating the presence of another element, which suggested the purity of the synthesized nanoparticle. The EDX pattern of SH-TiO_2 nanocomposites contained titanium (39.26%), oxygen (28.34%), carbon (26.72%), sodium (4.21%) and nitrogen (1.47%), which confirms the existence of these elements in the nanocomposite. Sodium hyaluronate biopolymer is rich lateral groups, which can easily bind with TiO_2 nanoparticles and form a strong and lasting nanocomposite.

3.4. Thermal analysis

Fig. 4 shows the sodium hyaluronate biopolymer and the SH-TiO_2 nanocomposite thermal analysis according to thermogravimetric analysis and differential thermal analysis. Mass loss of the sodium hyaluronate biopolymer occurred at two stages. The first step was in the area under 150 °C as 13.75%, while the second phase was at approximately 220 °C at a rate of 53.99%. In the first stage, mass loss occurred due to dehydration while the second step happened due to degradation in the structure of the sodium hyaluronate biopolymer. Differential thermal analysis of sodium hyaluronate polymer showed an endothermic peak at 78 °C and two endothermic peaks in the range of 220–500 °C. Matching the results of two spectra (TGA/DTA) showed that the endothermic peak at 78 °C and the endothermic peaks in the range of 220 and 500 °C in the DTA curve corresponded with mass loss of sodium hyaluronate biopolymer in the range under 150 °C and the range of 220–460 °C, respectively in the TGA curve.

The TGA curve of SH-TiO_2 nanocomposite showed three stages of mass loss in the temperature ranges less than 140 °C, 140–328 °C and 328–742 °C at a rate of 1.92%, 9.56% and 8.90%, respectively. In agreement with the TGA results, the DTA curve displayed the endothermic and exothermic peaks in the mass loss ranges of the SH-TiO_2 nanocomposite. The mass loss observed in the range of 140 °C was due to dehydration while the mass loss in the second stage (140–328 °C) occurred as a result of the decomposition and evaporation of a part of the biopolymer structure and the loss of volatile products. The third mass loss in the range of 328–742 °C occurred due to complete removal of organic compounds and phase change of TiO_2 nanoparticles to anatase. Comparing the (TGA/DTA) curves of sodium hyaluronate biopolymer and SH-TiO_2 nanocomposite revealed that the sodium hyaluronate polymer lost 67.74% of its mass at a temperature range of 0–800 °C due to heating, while the nanocomposite weight loss was 20.38%. Therefore, one can conclude that the thermal stability rate has significantly increased with formation of the nanocomposite. It is known that the rate of thermal degradation in nanocomposites is influenced by the composition of their organic and inorganic materials at the surface. The surface bonds in SH-TiO_2 nanocomposite can be resulted from the interaction between COO— and NH groups on the biopolymer surface with Ti atoms on the nanoparticle surface. Reduced size of nanoparticles, and thus, their increased area, can increase the interaction of the nanoparticles with the biopolymer which creates more sustainable complexes and improve thermal properties of the nanocomposites [21,22]. The formation of nanocomposites increases the surface of nanoparticles and enhances thermal stability of biopolymer. Therefore, better stability and antibacterial activity can be observed in synthesized nanocomposite rather than its components in different environmental conditions.

3.5. Antibacterial analysis

By using the Taguchi method, 9 experiments were designed to predict the best conditions for the synthesis of SH-TiO_2 nanocomposites with the highest antibacterial effect. For this purpose, the antibacterial effects of synthesized nanocomposites on *Staphylococcus aureus* and *Escherichia coli* bacteria were investigated by using different levels of TiO_2 NPs and sodium hyaluronate biopolymer with different stirring times. The results of antibacterial

Table 2
Antibacterial activity of Tio_2 nanoparticle, sodium hyaluronate biopolymer and sodium hyaluronate-TiO_2 bionanocomposite against *Staphylococcus aureus* and *Escherichia coli.*

Factors	Gram positive bacteria (*Staphylococcus aureus*)		Gram negative bacteria (*Escherichia coli*)	
	Bacterial survival (Log_{10} CFU/ml)	Zone of inhibition (mm)	Bacterial survival (Log_{10} CFU/ml)	Zone of inhibition (mm)
Tio_2	3.64	17	3.98	14
Sodium hyaluronate	8.03	0	7.92	0
Sodium hyaluronate-TiO_2	0	21	0	19

Table 3
Main effects of different factors on the survival rate of *Staphylococcus aureus* and *Escherichia coli* bacteria.

Factors	Gram positive bacteria (*Staphylococcus aureus*)			Gram negative bacteria (*Escherichia coli*)		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
TiO_2	3.37	1.08	1.61	3.96	1.36	1.63
Sodium hyaluronate	3.03	1.33	1.70	3.46	1.41	2.09
Stirring time	1.88	1.63	2.54	2.27	1.87	2.82

Table 4
Effects of interacting factor pairs on survival rate of gram-positive and gram-negative bacteria.

Type of bacteria	Interacting factor pairs	Column	Severity Index (%)	Reserved column	Optimum conditions
Gram positive bacteria (*Staphylococcus aureus*)	S. hyaluronate × Stirring time	2 × 3	31.90	1	[2,3]
	TiO_2 × Stirring time	1 × 3	21.92	2	[2,3]
	TiO_2 × S. hyaluronate	1 × 2	19.21	3	[2,2]
Gram negative bacteria (*Escherichia coli*)	S. hyaluronate × Stirring time	2 × 3	48.98	1	[2,3]
	TiO_2 × S. hyaluronate	1 × 2	16.81	3	[2,2]
	TiO_2 × Stirring time	1 × 3	13.20	2	[2,3]

Table 5
ANOVA test for factors affecting the growth reduction of *Staphylococcus aureus* and *Escherichia coli* bacteria.

Type of bacteria	Factors	DOF	Sum of Squares	Variance	F-Ratio (F)	Pure Sum	Percent (%)
Gram positive bacteria (*Staphylococcus aureus*)	TiO_2	2	8.64	4.32	2.34	4.94	26.77
	S. hyaluronate	2	4.81	2.41	1.30	1.11	6.03
	Stirring time	2	1.31	0.66	0.36	0.00	0.00
Gram negative bacteria (*Escherichia coli*)	TiO_2	2	12.28	6.14	2.72	7.77	31.44
	S. hyaluronate	2	6.56	3.28	1.45	2.05	8.29
	Stirring time	2	1.36	0.68	0.30	0.00	0.00

Table 6
Predicted the optimum conditions of sodium hyaluronate-TiO_2 bionanocomposite synthesis with the highest antibacterial activity.

Factors	Gram positive bacteria (*Staphylococcus aureus*)		Gram negative bacteria (*Escherichia coli*)	
	Level	Contribution	Level	Contribution
TiO_2	2	0.94	2	0.96
Sodium hyaluronate	2	0.69	2	0.91
Stirring time	2	0.39	2	0.45
Total contribution from all factors		−2.02		−2.32
Current grand average of performance		2.02		2.32
Bacterial survival at optimum condition		0.00		0.00

activity rates of synthesized nanocomposites on gram-positive and gram-negative bacteria are presented in Table 1, indicating that the experiments 5 and 9 have the most favorable conditions. The bionanocomposites produced under conditions of experiment 5 (TiO_2 4 mg/ml, sodium hyaluronate 1 mg/ml and stirring time of 90 min) and experiment 9 (TiO_2 8 mg/ml, sodium hyaluronate 2 mg/ml and stirring time of 60 min) completely prevented the growth of *Staphylococcus aureus* and *Escherichia coli*, and no bacteria grew in their presence. Appling the colony forming units (CFU) method, the SH-TiO_2 antibacterial activity was compared with its components and it was observed that the antibacterial activity of the synthesized nanocomposite was significantly more than the sodium hyaluronate biopolymer and TiO_2 NPs (Table 2). The use of disc diffusion method to compare the SH-TiO_2 nanocomposite antibacterial properties with its components also showed similar results (Fig. 5, Table 2). SH-TiO_2 nanocomposite showed inhibition zones of 21 mm and 19 mm against gram-positive and gram-negative bacteria, respectively, which were significantly higher compared with sodium hyaluronate biopolymer (0 mm, 0 mm) and TiO_2 NPs (17 mm, 14 mm).

The use of various combinations inside the sodium hyaluronate biopolymer matrix to produce nanocomposites with antibacterial properties was reviewed in previous studies, which reported that synthesized nanocomposites had desirable antibacterial activity [23–25]. In this research, the structure of nanocomposite was surveyed by FTIR, FESEM and EDX analysis the results of which confirmed the formation of nanocomposites. By comparing the antibacterial properties of nanocomposites containing TiO_2 nanoparticles with their components, several researchers announced that the formation of nanocomposite improved its antibacterial properties; this is in line with the results obtained in this study [26–28]. The efficiency of nanoparticles in the nanocomposite structure is influenced by their characteristics such as particle size, morphology and surface area, light absorption capacity, etc. Therefore, by reducing the size of nanoparticles and increasing area, their antimicrobial activity would increase. Furthermore, the type of bacteria also has an effect on their sensitivity to nanoparticles and their antibacterial activity [29]. Sodium hyaluronate $-TiO_2$ nanocomposite can exert their antibacterial effects through two ways of influencing the cell wall and affecting the internal parts of the bacterial cell. This compound affects the bacterial cell wall through different mechanisms and disrupts its activities. After attaching to the bacteria walls, SH-TiO_2 nanocomposite disrupts its electrolyte balance. It can also impact the function of efflux pumps. These actions impair the bacterial activity and ultimately lead to its death. On the other hand, the SH-TiO_2 nanocomposites can affect the enzymes and proteins of the respiratory cycle through binding to their sulfhydryl, carboxyl and phosphate and changing their conformations; this causes disruption of cellular respiration and leads to the death of bacterial cell. After entering into the bacterial cell, SH-TiO_2 nanocomposite can affect its activity through various mechanisms. By binding to proteins and enzymes that play a vital role in the life cycle of cells, this nanocomposite can interfere with their activities. The synthesized nanocomposite can also affect functions such as bacterial replication and transcription by binding to bacterial DNA. Another mechanism of the SH-TiO_2 effect of nanocomposites within the cell is through H_2O light oxidation and generation of reactive oxygen species, such as radical peroxide and hydroxyl (•O2, •OH), which cause bacterial cell death with the destruction of vital cell components such as DNA, proteins and enzymes [30–32].

The main effects of factors of TiO_2 NPs, sodium hyaluronate biopolymer and stirring time on the viability of gram-positive and gram-negative bacteria are shown in Table 3. All three studied factors showed favorable effect on the second level in reducing the growth of *Staphylococcus aureus* bacteria. Factors of TiO_2 NPs, sodium hyaluronate biopolymer and stirring time reduced the viability of this bacterium up to 1.08, 1.33 and 1.63 (Log_{10} CFU/ml), respectively. The studied factors showed similar results on *Escherichia coli* bacteria, and all three factors had the best performance at their second level. TiO_2 NPs, sodium hyaluronate biopolymer and stirring time reduced the viability of *Escherichia coli* up to1.36, 1.41 and 1.87 (Log_{10} CFU/ml), respectively.

Table 4 shows the interaction of the studied factors on survival rate of *Staphylococcus aureus* and *Escherichia coli* bacteria. The second level of sodium hyaluronate biopolymer and the third level of stirring time had the highest interaction influence on reducing the growth of gram-positive and gram-negative bacteria as 31.90 and 48.98, respectively. The lowest interaction effect on *Staphylococcus aureus* belonged to the interaction of second levels of TiO_2 NPs and sodium hyaluronate biopolymer as 19.21, while on *Escherichia coli*, it belonged to the interaction of TiO_2 NPs at the second level and the stirring time at the third level as 13.20.

Analysis of variances of factors effective in reducing growth of the studied bacteria are presented in Table 5. The results indicated that TiO_2 NPs and sodium hyaluronate biopolymer had the greatest impact on reducing the growth of gram-positive bacteria as 26.77 and 6.03, respectively. Like in *Staphylococcus aureus*, in gram-negative bacteria, TiO_2 NPs (31.44) and sodium hyaluronate biopolymer (8.29) had the highest impact on reducing the growth of *Escherichia coli*. The stirring time factor showed no significant effect on the reduction of *Staphylococcus aureus* and *Escherichia coli* bacteria growth rates.

After evaluating the effect of the studied factors and their interaction, the best conditions for producing SH-TiO_2 nanocomposites with the highest antibacterial activity were determined using the Taguchi method (Table 6). The results showed that factors of TiO_2 NPs, sodium hyaluronate biopolymer and stirring time, respectively, had the most important role in reducing the survival rate of gram-positive and gram-negative bacteria. The second level was introduced to be the optimum level for all three factors and it was predicted that the nanocomposite synthesized in these conditions can completely prevent the growth of the bacteria.

4. Conclusion

Based on the results of this study, the synthesis of SH-TiO_2 bionanocomposite in optimum conditions can completely prevent the growth of *Staphylococcus aureus* and *Escherichia coli* bacteria. In addition, comparing the SH-TiO_2 nanocomposite with its components by FTIR, FESEM, EDX, TGA and DTA analyses indicates the formation of the nanocomposites and differences in their structure, properties, morphology and antibacterial properties. SH-TiO_2 nanocomposite has high and sustainable antibacterial activity and can be used as a green alternative to conventional antibacterial ingredients to confront pathogenic bacteria drug resistance and increased risk of outbreaks of infectious diseases.

References

[1] L. Garcia-Sureda, C. Juan, A. Domenech-Sanchez, S. Alberti, Role of Klebsiella pneumoniae LamB Porin in antimicrobial resistance, Antimicrob. Agents Chemother. 55 (2011) 1803–1805.
[2] P. Nordmann, Global spread of carbapenemase-producing enterobacteriaceae, Emerg. Infect. Dis. 17 (2011) 1791–1798.
[3] E. Taylor, T.J. Webster, Reducing infections through nanotechnology and nanoparticles, Int. J. Nanomed. 6 (2011) 1463–1473.
[4] M. Taran, S. Etemadi, M. Safaei, Microbial levan biopolymer production and its use for the synthesis of an antibacterial iron (II, III) oxide–levan nanocomposite, J. Appl. Polym. Sci 134 (2017) 44613.
[5] S. Dai, Y. Wu, T. Sakai, Z.H. Du, M. Sakai Abe, Preparation of highly crystalline TiO2 nanostructures by acid-assisted hydrothermal treatment of hexagonal-structured nanocrystalline titania/cetyltrimethyammonium bromide nanoskeleton, Nanoscale Res. Lett. 5 (2010) 1829––1835.
[6] B. Li, Y. Zhang, Y. Yang, W. Qiu, X. Wang, B. Liu, Y. Wang, G. Sun, Synthesis characterization, and antibacterial activity of chitosan/TiO2 nanocomposite against Xanthomonas oryzae pv. oryzae, Carbohydr. Polym. 152 (2016) 825–831.
[7] S. Yeniyol, I. Mutlu, Z. He, B. Yuksel, R.J. Boylan, M. Urgen, Z.C. Karabuda, C. Basegmez, J.L. Ricci, Photocatalytical antibacterial activity of mixed-phase TiO2 nanocomposite thin films against aggregatibacter actinomycetemcomitans, BioMed Res. Int. 2015 (2015) 705871.
[8] W. He, H. Huang, J. Yan, J. Zhu, Photocatalytic and antibacterial properties of Au-TiO2 nanocomposite on monolayer graphene: from experiment to theory, J. Appl. Phys. 114 (2013) 204701.
[9] S.S. Behera, U. Das, A. Kumar, A. Bissoyi, A.K. Singh, Chitosan/TiO2 composite membrane improves proliferation and survival of L929 fibroblast cells: application in wound dressing and skin regeneration, Int. J. Biol. Macromol. 98 (2017) 329–340.
[10] M. Montazer, A. Behzadnia, M.B. Moghadam, Superior self-cleaning features on wool fabric using TiO2/Ag nanocomposite optimized by response surface methodology, J. Appl. Polym. Sci. 125 (2012) 356–363.
[11] K. Kavitha, S. Sutha, M. Prabhu, V. Rajendran, T. Jayakumar, In situ synthesized novel biocompatible titania–chitosan nanocomposites with high surface area and antibacterial activity, Carbohydr. Polym. 93 (2013) 731–739.
[12] S. Kango, S. Kalia, A. Celli, J. Njuguna, Y. Habibi, R. Kumar, Surface modification of inorganic nanoparticles for development of organic–inorganic nanocomposites–a review, Prog. Polym. Sci. 38 (2013) 1232–1261.
[13] B.S. Anisha, R. Biswas, K.P. Chennazhi, R. Jayakumar, Chitosan–hyaluronic acid/nano silver composite sponges for drug resistant bacteria infected diabetic wounds, Int. J. Biol. Macromol. 62 (2013) 310–320.
[14] G. Kogan, L. Soltes, R. Stern, P. Gemeiner, Hyaluronic acid: a natural biopolymer with a broad range of biomedical and industrial applications, Biotechnol. Lett. 29 (2007) 17–25.
[15] V. Rangaswamy, D. Jain, An efficient process for production and purification of hyaluronic acid from Streptococcus equi subsp. zooepidemicus, Biotechnol. Lett. 30 (2008) 493–496.
[16] A. Leon, P. Reuquen, C. Garin, R. Segura, P. Vargas, P. Zapata, P.A. Orihuela, FTIR and raman characterization of TiO2 nanoparticles coated with polyethylene glycol as carrier for 2-methoxyestradiol, Appl. Sci. 7 (2017) 49.
[17] J.A. Alkrad, Y. Mrestani, D. Stroehl, S. Wartewig, R. Neubert, Characterization of enzymatically digested hyaluronic acid using NMR, Raman, IR, and UV–Vis spectroscopies, J. Pharm. Biomed. Anal. 31 (2003) 545––550.

[18] N. Erdem, U.H. Erdogan, A.A. Cireli, N. Onar, Structural and ultraviolet-protective properties of nano-TiO2-doped polypropylene filaments, J. Appl. Polym. Sci. 115 (2010) 152–157.
[19] P.A. Charpentier, K. Burgess, L. Wang, R.R. Chowdhury, A.F. Lotus, G. Moula, Nano-TiO2/polyurethane composites for antibacterial and self-cleaning coatings, Nanotechnology 23 (2012) 425606.
[20] B. Li, Y. Zhang, Y. Yang, W. Qiu, X. Wang, B. Liu, Y. Wang, G. Sun, Synthesis characterization, and antibacterial activity of chitosan/TiO2 nanocomposite against Xanthomonas oryzae pv. oryzae, Carbohydr. Polym. 152 (2016) 825–831.
[21] P. Coimbra, P. Alves, T.A.M. Valente, T.R. Santos, I.J. Correia, I.P. Ferreira, Sodium hyaluronate/chitosan polyelectrolyte complex scaffolds for dental pulp regeneration: synthesis and characterization, Int. J. Biol. Macromolec. 49 (2011) 573–579.
[22] Y. Li, S. Qing, J. Zhou, G. Yang, Evaluation of bacterial cellulose/hyaluronan nanocomposite biomaterials, Carbohydr. Polym. 103 (2014) 496–501.
[23] A.M. Abdel-Mohsen, R. Hrdina, L. Burgert, R.M. Abdel-Rahman, M. Hasova, D. Smejkalova, M. Kolar, M. Pekar, A.S. Aly, Antibacterial activity and cell viability of hyaluronan fiber with silver nanoparticles, Carbohydr. Polym. 92 (2013) 1177–1187.
[24] E. Montanari, A. Gennari, M. Pelliccia, C. Gourmel, E. Lallana, P. Matricardi, A.J. McBain, N. Tirelli, Hyaluronan/Tannic acid nanoparticles via Catechol/Boronate complexation as a smart antibacterial system, Macromol. Biosci. 16 (2016) 1815–1823.
[25] P. Sacco, A. Sechi, A. Trevisan, F. Picotti, R. Gianni, L. Stucchi, M. Fabbian, M. Bosco, S. Paoletti, E. Marsich, A silver complex of hyaluronan–lipoate (SHLS12): synthesis, characterization and biological properties, Carbohydr. Polym. 136 (2016) 418–426.
[26] M. Shakir, M.S. Khan, S.I. Al-Resayes, U. Baig, P. Alam, R.H. Khan, M. Alam, In vitro DNA binding, molecular docking and antimicrobial studies on a newly synthesized poly (o-toluidine)–titanium dioxide nanocomposite, RSC Adv. 4 (2014) 39174–39183.
[27] L. Karimi, M.E. Yazdanshenas, R. Khajavi, A. Rashidi, M. Mirjalili, Using graphene/TiO2 nanocomposite as a new route for preparation of electroconductive self-cleaning, antibacterial and antifungal cotton fabric without toxicity, Cellulose 21 (2014) 3813–3827.
[28] S. Parham, S. Chandren, D.H. Wicaksono, S. Bagherbaigi, S.L. Lee, L.S. Yuan, H. Nur, Textile/Al2O3?TiO2 nanocomposite as an antimicrobial and radical scavenger wound dressing, RSC Adv. 6 (2016) 8188–8197.
[29] V. Mayur, P. Jayshree, M. Mukta, T. Sonal, Morphology and antibacterial activity of carbohydrate-stabilized silver nanoparticles, Carbohydr. Res. 345 (2010) 1767–1773.
[30] N. Beyth, Y. Houri-Haddad, A. Domb, W. Khan, R. Hazan, Alternative antimicrobial approach: nano-antimicrobial materials, Evid. Based Complement. Alternat. Med. 16 (2015) 246012.
[31] A. Kubacka, M.S. Diez, D. Rojo, R. Bargiela, S. Ciordia, I. Zapico, J.P. Albar, C. Barbas, V.A. dos Santos, M. Fernandez-Garcia, M. Ferrer, Understanding the antimicrobial mechanism of TiO2-based nanocomposite films in a pathogenic bacterium, Sci. Rep. 4 (2014) 4134.
[32] X. Pan, I. Medina-Ramirez, R. Mernaugh, J. Liu, Nanocharacterization and bactericidal performance of silver modified titania photocatalyst, Colloids Surf. B 77 (2010) 82–89.