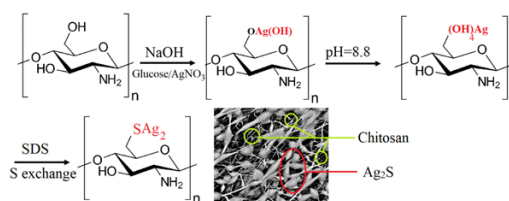


Preparation and Evaluation of Cross-Linked Chitosan/Silver Sulfide Luminescence Nanocomposites by Using Green Capping Agent Against Some Pathogenic Microbial Strains

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Abstract: In this paper, different silver sulfide (Ag₂S) nanoparticles were cross-linked with chitosan, as a biodegradable and bioactive polymer. Chitosan/Ag₂S nanocomposites were synthesized for the first time, using multistage distillation, with the micellization-assisted ultrasonic method. The designed nanocomposites system was evaluated, for *in vitro* efficient antibacterial activity. Various factors, including concentration of surfactants, pulse time, power irradiation, and interaction between these factors, will directly affect different properties of the cross-linked chitosan/Ag₂S nanocomposites. Results showed that cross-linked nanopolymer networks are capable of considerably inhibiting growth of antibiotic-resistant *Escherichia coli*, *Pseudomonas aeruginosa*, and *Serratia marcescens* as gram-negative bacteria, and *Bacillus subtilis* and *Micrococcus luteus* as gram-positive bacteria, between 64 up and 32 µg/mL. Antibacterial activity was conducted against 7 Gram-negative and Gram-positive bacteria using agar well diffusion assay and minimum inhibitory concentration (MIC), was determined. Samples were characterized by X-ray diffraction (XRD), scanning electron microscopy (SEM), transition electron microscopy (TEM), atomic force microscopy (AFM), Fourier transformed infrared spectrum (FT-IR), thermo-gravimetric analysis (TGA), and dynamic light scattering (DLS).



Keywords: crosslinking, chitosan/Ag₂S, nanocomposites, antibacterial activity, green capping agent.

1. Introduction

Nanotechnology as an interdisciplinary science has experienced a significant advancement in biomedical applications such as targeted antibacterial activity,¹ drug delivery,² hyperthermia,³ photoablation therapy,⁴ bioimaging,⁵ and biosensors.^{6,7} One of the recent efforts to address the challenge was the use of antimicrobial nanomaterials.⁸ Due to the presence and growth of numerous drug-resistant strains,⁹ an urgent need exists to develop novel antimicrobial agents.¹⁰ Among the most promising new antimicrobial agents, nanoparticles have shown high antibacterial activity in a large number of studies.¹¹ Nanoparticles and nanocomposites have shown wide applications in antibacterial properties.¹² The metal nanoparticles, because of their small size, have a large surface-to-volume ratio, which makes them have more contact with outside and thus have a significant antibacterial effect.¹³ The effects of nanoparticles on cells of living organisms depend on the diameter, size, and shape of nanoparticles.¹⁴ Antimicrobial changes that prevent the growth of pathogenic bacteria are subjects of intense interest.¹⁵ The cross-linking junction of the polymer chain is a bond that links one chain to

another and results in widespread and better performance of the particle size of structures. The improvement of mechanical properties and an increase in temperature stability and antibacterial activity are among the most important advantages of crosslinking structures. The biological polymer that have connection with micelles are extensively used to improve the antibacterial activity because of their significant raise surface-to-volume ratio. In general, there are many methods for the preparation of cross-linked nanocomposites such as free radical polymerization processes precipitation,¹⁶ sol-gel process,¹⁷ microwave method,¹⁸ and ultrasound methods.¹⁹ Over the past few years, several studies have been conducted on the use of nanostructures in antibacterial effects. The summary of these works is presented in Table 1. In the present study, Chitosan/Ag₂S nanocomposites are joined by radial emulsion structures using covalent joints. Hollow crosslinking chitosan nanocomposites with unique physicochemical properties entrapped Ag₂S in capillary network of interconnected structures. In this study, glucose structures were used as a green capping agent and reductant for preparation of Ag₂S Scheme 1. Antibiotic resistance pattern of bacteria determined 7 antibiotics: *E. coli* PTCC 1330, *K. pneumoniae* PTCC 1053, *S. marcescens* PTCC1621, *P. aeruginosa* PTCC1074, *S. aureus* PTCC. 1112, *M. luteus* PTCC. 1110, and *B. subtilis* PTCC. 1023. The results show that gram-positive and gram-negative bacteria except *P. aeruginosa* and *S. aureus* was inhibited between 32 µg/mL up to 64 µg/mL concentration of chitosan/Ag₂S nano-

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Table 1. The summary of antibacterial properties of nanoparticles

Types of nanofibers	Synthesis approach	Application	Size of nanofibers	References
Aluminium oxide (Al_2O_3)	Chemical	Clinical isolates of <i>Escherichia coli</i>	<50 nm	[26]
Titanium dioxide (TiO_2) NPs	Sol-gel	Showed zero viability on <i>P. aeruginosa</i> at 40 mg/30 mL and on <i>S. aureus</i> and <i>E. coli</i> at 60 mg/30 mL	22-30 nm	[27]
Chitosan/silver-NPs/polyvinyl alcohol	Reduction with glucose	aerobic bacteria	150 nm	[28]
Ag@Co magnetic nanocomposites	Chemical reduction	<i>Pseudomonas aeruginosa</i> , <i>Klebsiella pneumonia</i>	<45 nm	[29]
CeO_2 NPs	Precipitation	<i>Escherichia coli</i>	35 nm	[30]

**Scheme 1.** Summary of experimental route for the synthesis of Chitosan/ Ag_2S nanocomposites.

composites. Chitosan is a weak base and is insoluble in water but with becoming nanostructures because of their high surface-to-volume ratio, solubility and penetration increases and destroy cell wall bacteria.²⁰ It is irrefragable that chitosan molecules in bacteria surrounds might complex metals and blockage some essential nutrients to flow, contributing to cell death.²¹ Add the Ag_2S nanoparticles to chitosan as a substrate help significantly to increase antibacterial properties.

The as-prepared products were analyzed by XRD, EDAX, SEM, TGA, and FT-IR.

2. Experimental

2.1. Materials and methods

All the primitives used in this work such as AgNO_3 , chitosan, sodium dodecyl sulfate (SDS), NaOH and dimethyl sulfoxide were of analytical grade and without further purification. They were obtained from Merck or Sigma-Aldrich Company and used without further purifications. Also, deionized water used in this research work was provided in the laboratory. The method used in this research work included micellization-assisted microwave irradiation. XRD patterns were recorded by a Rigaku D-max C III, X-ray diffractometer using Ni-filtered Cu-K α radiation. The morphologies of the samples were investigated by scanning electron microscopy (SEM, JXA-8100, and JSM-6700F). Transmission electron microscopy (TEM) images were obtained on a Philips EM208 TEM with an accelerating voltage of 200 kV in University of Shahid Bahonar Kerman, Iran. The Fourier transform infrared spectroscopy (FTIR) spectra were recorded on Magna-IR, spectrometer 550 Nicolet in KBr pellets in the range of 400-4000 cm^{-1} . Thermal stability of the nanostructures formulations was examined by thermogravimetry analysis (TGA) curves with specifications from room temperature to 800 °C with a heating rate of 10 °C/min under an argon atmosphere using SCINCO thermal gravimeter S-100 (Seoul, Korea).

2.2. Muller Hinton Agar Cultivation

The culture medium used in this study was Hellenic Agar, which was prepared and autoclaved in 18 mL test tubes. The solid medium was melted. Each dilution of the sample is 2 mL with a melting tube and spread in a plate so that the final dilutions were 64, 32, 16, 8, 4, 2, 1, and 0.5 micrograms per milliliter. Positive and negative control plates were also considered in this work. The distribution of the antimicrobial agent should be uniform in the agar medium. Wells in 6 mm diameter were punctured in the media by sterile cork borers and filled with 20 μL of the Chitosan/ Ag_2S nanostructures. The first concentration used was 80 mg/mL. The plates were incubated at 37 °C for 24 h.

2.3. Transmission of microbial suspension

A number of sampler heads were sterilized by autoclaving with the time of transfer of microbial suspension. To transfer microbes to the surface of the plates, a 2.5- μL capacity sampler was used for transferring each germ to a separate sampler head. For this purpose, at least 2.5 μL of microbial suspension was transferred to the agar surface. In this study, 7 microorganisms were identified, which indicated the transfer location of 7 germs. After absorbing the microbial suspension to the surface of the agar plates, it was reversed in the incubator and placed at 37 °C for 24 h. The appearance of each plate was studied in terms of the growth or lack of growth of the tested microbes. The lowest concentration of the active ingredient that can inhibit the growth of the microbe MIC is against microbes

3. Results and discussion

Microwave irradiation power through the creation of radical species in the solvent phase provides primary energy for the preparation of nanostructures. For the Preparation of Chitosan/ Ag_2S nanostructures at the first, 0.41 g AgNO_3 was dissolved in 20 mL of deionized water (solution A) and then 5mL NaOH 2M was added dropwise to the solution. The pH was adjusted with digital pH meter in the range of 7.5 to 8. After that, 0.07 g of SDS was dissolved in 10 mL of DMSO and added into the solution A. The mentioned mix of AgNO_3 and SDS dissolved in DMSO was continuously put into reflux system and stirred with 400 rpm for 2 h at 50 °C. In the next step, 0.05 g of chitosan ($\text{C}_6\text{H}_{11}\text{NO}_4$)_n as stabilizer and substrate reagent was dissolved in 10 mL of deionized Water: DMSO with the ration of 1:2 and then stirred at 300 rpm for 30 min at 50 °C (solution B) and added to reflux system. Afterward, the mixture was loaded into a beaker and

Table 2. Reaction conditions for synthesis of Chitosan/Ag₂S nanocomposites

Sample no.	Power (W)	Time (min)	Solvent ratio DI Water: DMSO	Morphology
1	600	10	1:2	Agglomerated
2	750	10	1:2	Agglomerated
3	900	10	1:2	Agglomerated
4	600	5	1:2	Cross-link
5	750	5	1:2	Cross-link + NPs
6	900	5	1:2	Cross-link + NPs

*Deionization: DI

put into the microwave Teflon container, and the reactions were performed in a microwave digestion system under various conditions. Preparation conditions and the morphology for the Chitosan/Ag₂S the synthesized nanocomposites are presented in Table 2. The white precipitates obtained were centrifuged at 4000 rpm for 5 min and then washed with alcohol and distilled water for several times. The products were collected by filter paper and dried in vacuum at 70 °C for 4 h. It was found that the dimensional stability of the formulation increases with an increase in its concentration.

3.1. Crystallography properties

To investigate crystallinity phase structure of the as-synthesized Chitosan/Ag₂S nanocomposites, we used XRD pattern analysis within the scan range of $10 < 2\theta < 80$. First, it was observed that the prepared samples were not crystalline well and were composed of amorphous phase Figure 1(a). To obtain the crystalline structure, the obtained products were put in a boat-shaped crucible and then placed in the furnace and heated at 120 °C for 4 h. The obtained XRD pattern of the Chitosan/Ag₂S nanocomposites (JCPDS14-0072) is illustrated in Figure 1(b). Diffraction peaks can be indexed to the tetragonal structure (space group Fd-3m) placed at 26°, 27°, 31°, 32°, 34°, 35°, 41°, 44°, 46.5°, 53° and 55° that can be assigned to (101), (110), (121), (213), (102), (200), (112), (300), (110), (322), and (221) Miller indices.

Broaden peaks in the XRD pattern show that the particle size of the product is in the range of nanometer. The crystallite size of the product can be estimated from the diffraction patterns from the full width of the half maximum (FWHM) with the Debye-Scherrer equation (Eq. (1)), which was examined to be about 80–110 nm.

$$D_{\text{XRD}} = 0.9\lambda (57.3)/W_{\text{size}} \cos \theta \quad (1)$$

$$\text{Where } W_{\text{size}} = (W_b^2 - W_s^2)^{1/2} \quad (2)$$

$$W_{\text{size}} = \text{FWHM} \quad (3)$$

$$\text{for sample } W_{\text{size}} = \text{FWHM} \quad (4)$$

For further demonstration, EDAX technique as the compositional and the elemental analysis or chemical characterization of the samples was accomplished using energy-dispersive x-ray spectroscopy (EDS, EDX, EDXS) as a non-destructive technique that can be virtually analytical supplement of SEM. The EDAX spectrum of Chitosan/Ag₂S nanocomposites in Figure 1(c) shows the presence of elements such as C and O, and also S, Na, and N, which are related to sodium dodecyl sulfate and chitosan structures, respectively.

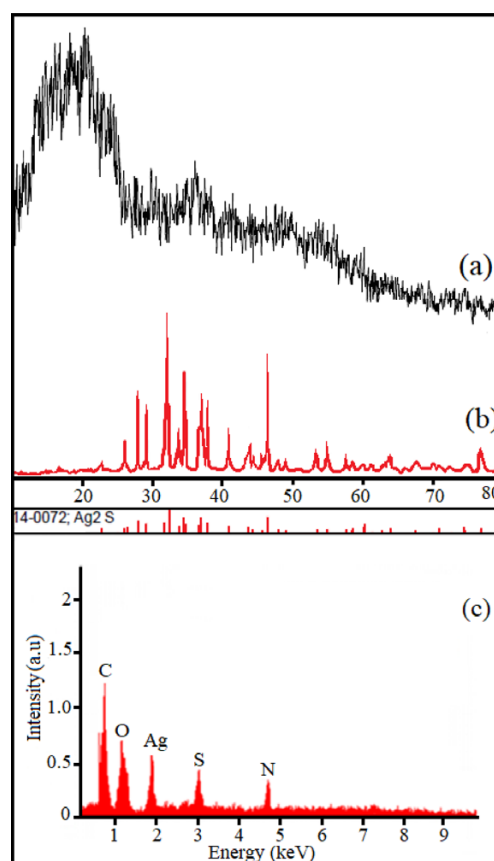


Figure 1. The XRD pattern of the Chitosan/Ag₂S nanocomposites in room temperature (a) and after 120 °C (b). EDAX pattern of synthesized Chitosan/Ag₂S nanocomposites.

3.2. Morphological definition

The surface morphological properties and the nanostructure were studied by a SEM device. The typical SEM images of the Chitosan/Ag₂S nanocomposites prepared at 600, 750, and 900 watts in 10 min are presented in Figure 2(a)–2(c), respectively. The SEM image of the product by increasing the irradiation power from 600 to 900 in 10 min due to the high energy applied in the high powers shows that the primarily formed nuclei stick together and form a hunch. With increasing the microwave time to 10 min, more energy is used to form nanostructures. *In vitro* studies in this work showed that with increasing the power of the device, nanocomposites are agglomerated, irregular structures are created, and the particle size of the products is increased.

SEM images of samples prepared at 600, 750, and 900 watts in 5 min are shown in Figure 3(a)–(c), respectively. The mor-

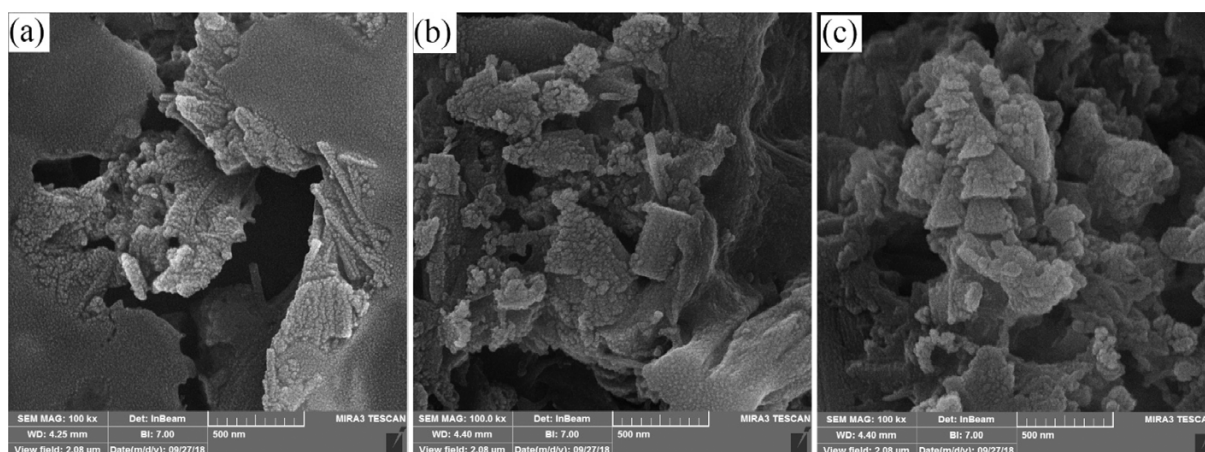


Figure 2. SEM images of the Chitosan/Ag₂S nanocomposites prepared at 600 (a), 750 (b) and 900 (c) watt in 10 minutes.

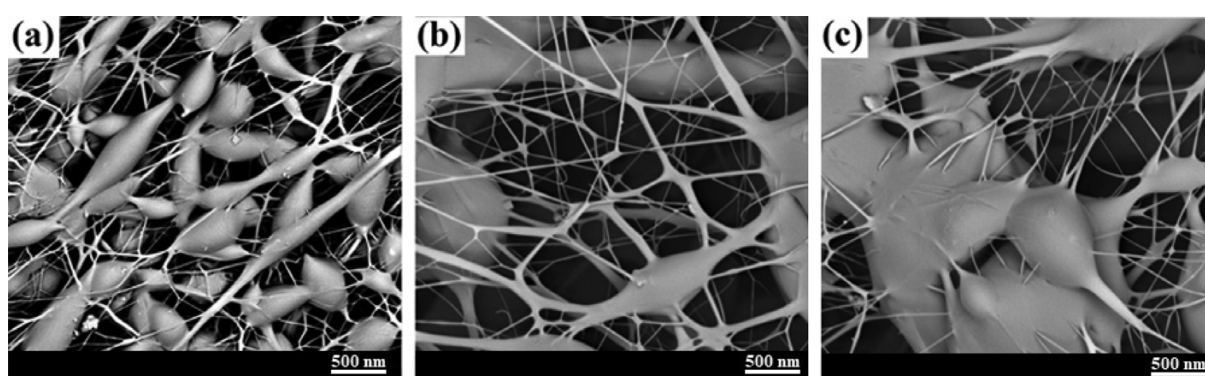


Figure 3. SEM images of the Chitosan/Ag₂S nanocomposites prepared at 600 (a), 750 (b) and 900 (c) watt in 5 minutes.

phology of all samples showed that chitosan can be responsible for the longitudinal growth and also creating crosslinking between particles. Hence, various crosslinking in addition to increasing the thermal stability of nanostructures improved their antibacterial effect as the integrated network. However, by increasing the irradiation power from 600 to 900 watts, chitosan polymer chains become thinner and the particle size of the Ag₂S nanoparticles increased gradually.

To further investigate the details of chemical bonds, FT-IR spectra were used. Results of Chitosan/Ag₂S nanocomposites and Chitosan composites samples in the wavenumber range of 400–4000 cm⁻¹ are given in Figure 4(a). The broad absorption peak at 3500–3100 cm⁻¹ is related to OH and NH₂ bands²² and the peak at 1652 cm⁻¹ is attributed to the presence of binding of Ag₂S nanoparticles to N-H bond of chitosan.²³ Moreover, a band at 2927 cm⁻¹ is attributed to C-H stretching and the band at 548 cm⁻¹ confirms the presence of Ag-S binds. A few weak peaks appeared at 615–793 cm⁻¹ are attributed to stretching vibration of the C-H band of sodium dodecyl sulfate molecules that were absorbed on the surface of the nanoparticle. The thermal behavior of Chitosan/Ag₂S nanocomposites was studied by thermogravimetric analysis (TGA) and differential thermal analysis (DTA) in the temperature range of 50 to 350 °C at a heating rate of 10 °C/min under the nitrogen atmosphere. TGA and DTA curves of Chitosan/Ag₂S nanocomposites are shown in Figure 4(b). TGA analysis curve of nanocomposites shows a one-step separated region, where the most significant weight loss occurs between the temperature

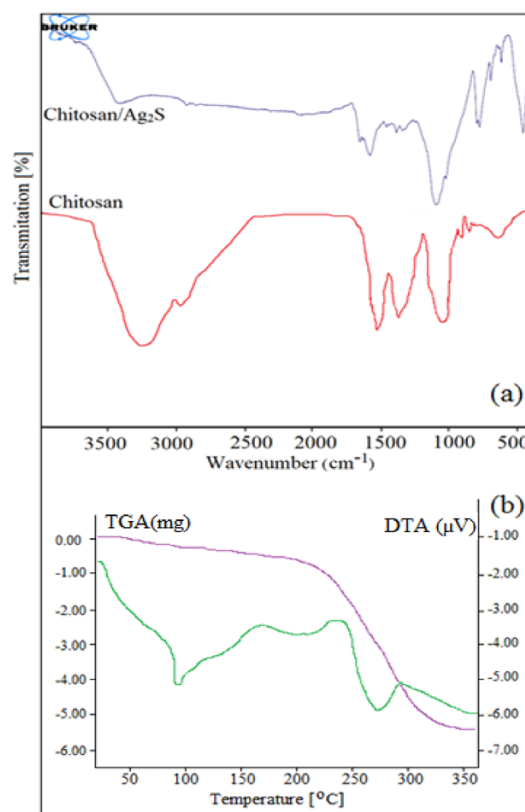


Figure 4. FT-IR spectrum of Chitosan, Chitosan/Ag₂S nanocomposites (a) and TGA/DTA curve analysis as-synthesized Chitosan/Ag₂S nanocomposites.

range of 235–250 °C, which is related to the structural degradation of nanoparticles entrapped in the chitosan polymer profile structures. From 300 °C to 400 °C, Chitosan/Ag₂S nanocomposites are wrinkled, followed by the complete structural destruction.

3.2. Luminescence property

In this work, the optical properties were studied through Uv-vis spectroscopy to investigate targeted drug delivery through optical properties of nanocomposites and this can be useful in tracking and controlling the amount of antibiotic. The behavior of different materials with visible light rays is known as the optical properties of materials. Nanoparticles have the electrons and holes position which show strong translates photoluminescence (PL) in the Uv-visible range. The emission wavelength in the Chitosan/Ag₂S nanocomposites is directly related to the particle size as larger particles which will absorb lower energy. Figure 5(a) shows ultraviolet-visible spectroscopy of the Chitosan/Ag₂S nanocomposites obtained at 435–447 nm excitation wavelength. Based on the previous literature the PL mechanism of Ag₂S can be explained in five categories. I. The near UV emission with 330–370 nm wavelength can be attributed to band edge or edge to edge transitions.²⁴ II. The UV emission with 380–400 nm wavelength range is attributed to the deep levels like dislocations,

inter lattice atoms and vacancy states of Ag²⁺.²⁵ III. Blue emission with 430–470 nm wavelength that is related to trapping states caused by Ag²⁺ and S²⁻ vacancy and surface states. Investigation of surface morphological properties of Chitosan/Ag₂S nanocomposites measured with AFM images is shown in Figure 5(b). It can be seen that nanocomposites are formed with a uniform special cross-section. The surface and morphological properties of the Chitosan/Ag₂S nanocomposites were estimated quantitatively by the root mean-squared roughness (R_{rms}) which obtained from the AFM data image and determined according to the equation in follows (Eq. (5)):

$$R_{rms} = \sqrt{\frac{\sum_{n=1}^N (z_n - \bar{z})^2}{N-1}} \quad (5)$$

That in this equation z_n represents the height of the n th data, $\bar{z}$ is equal to the mean height of Z_n in AFM topography, and N is the number of data. For an area of 67.17 pm² the roughness average is 1.94 nm.

Dynamic light scattering (DLS) as a non-destructive analysis calculated the hydrodynamic diameter of the samples. The size of a particle is calculated from the translational diffusion coefficient by using the Stokes Einstein equation (Eq. (6));

$$d(H) = kT/3\pi\eta D \quad (6)$$

where $d(H)$, D , k and T are hydrodynamic diameter, translational diffusion coefficient and Boltzmann's constant and absolute temperature respectively, also η is viscosity.

DLS diagrams of the as-synthesized Chitosan/Ag₂S nanocomposites after a day and after a month were displayed in Figure 6. The results show that the nanocomposites exhibit uniform

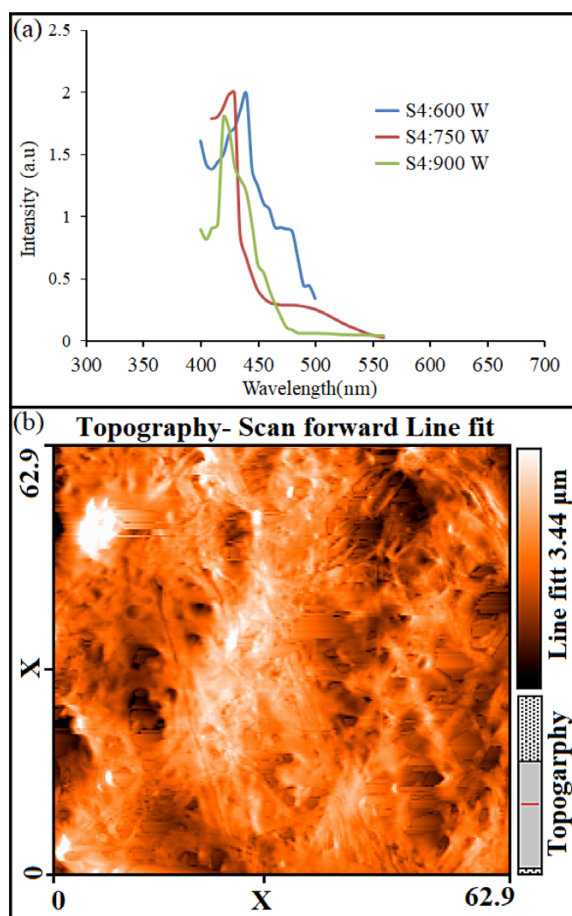


Figure 5. UV-Vis spectra Chitosan/Ag₂S nanocomposites prepared by microwave method (a), AFM image of the Chitosan/Ag₂S nanocomposites (b).

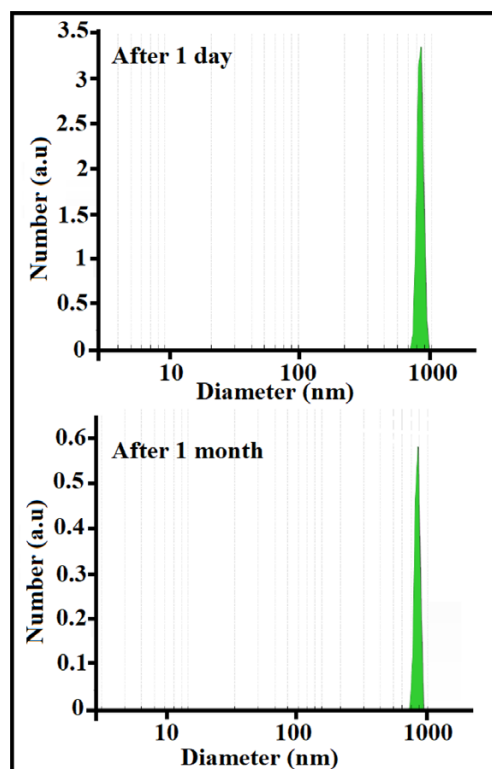


Figure 6. DLS analysis of the Chitosan/Ag₂S nanocomposites after a day (top diagram) and after a month (down diagram).

particle size distribution with less aggregation after a month and intensity diameter calculated about $DI=780$ nm. The size distribution obtained is a plot of the relative intensity of light scattered by particles in various size classes and is therefore known as an intensity size distribution.

3.4. Antibacterial activity

In agar well diffusion assay, which is used to evaluate the antimicrobial activity, the prevention of bacterial growth was determined in culture media loaded with different concentrations of Chitosan/ Ag_2S nanocomposites such as 64, 32, 16, 8, 4, 2, 1, and 0.5 $\mu\text{g/mL}$ and an inhibition zone diameter was evaluated in constant time periods. Gram-negative bacteria showed a higher sensitivity to Chitosan/ Ag_2S nanocomposites at the lower concentration in comparison with gram-positive bacteria. Antibacterial activity of Chitosan/ Ag_2S nanocomposites in terms of MIC is shown in Table 3.

Antibacterial activity of Chitosan/ Ag_2S nanocomposites against 4 Gram-negative and 3 Gram-positive bacteria using agar well diffusion with nanocomposites prepared under different conditions is shown in Figure 7. The results showed that the size of the inhibition zone increases linearly with an increase in Chitosan/ Ag_2S nanocomposites concentration from 0.5 $\mu\text{g/mL}$ up to 64 $\mu\text{g/mL}$. The size of the inhibition zone varied with the type of bacterial strain and concentration of nanocomposites. MIC method is a useful study to determine the lowest concentration of antibiotic which could completely inhibit the visible growth of bacterial strains. Among bacterial strains, the maximum sensitivity was observed in *B. subtilis* with the MIC of between 16 and 8 $\mu\text{g/mL}$. There are reactions between Chitosan/ Ag_2S nanocomposites and macromolecules of living bacteria. The positive bulk of the nanoparticle acts as an electromagnetically absorbent microbe and destroyed the surface of the microbial cells, eventually. Due to the lack of space on the plates, the names of germs were written briefly such as *Escherichia coli* (E), *Klebsiella pneumonia* (K), *Micrococcus luteus* (L), *Bacillus subtilis* (B.S), *Staphylococcus aureus* (S.U), *Serratia marcescens* (Sera), *Pseudomonas aeruginosa* (P.S).

Chitosan/ Ag_2S nanocomposites applied the antibacterial effect in two viewpoints. First, these nanocomposites can reduce adenosine triphosphate (ATP) synthase activities with changing the membrane potential, which leads to a decrease in the metabolism process. Secondly, collapsing biological mechanism of bacteria through refused the subunit of the ribosome binding. At

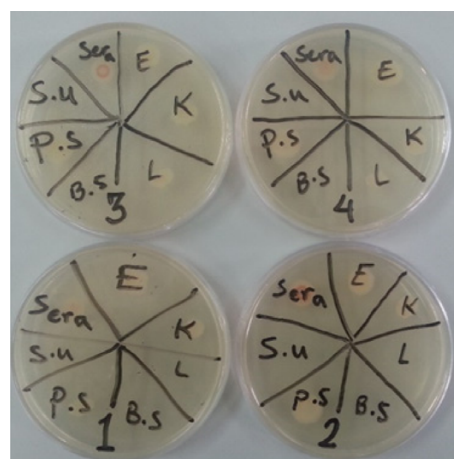


Figure 7. Antibacterial activity of Chitosan/ Ag_2S nanocomposites against 4-gram negative and 3 gram positive bacteria.

the same time, they proved to be less toxic to mammal cells. Chitosan/ Ag_2S nanocomposites because of their high surface-to-volume ratio, can easily react with phosphorus or sulfur in DNA molecules at cell wall bacteria. The Ag_2S nanoparticles (NPs) bounded with thiol groups of enzymes through the release of oxygen species and disrupted their respiratory chains. Therefore, damage occurred in the cell structures and finally led to cell death mechanism.

4. Conclusion

The present study deals with the synthesis, characterization, and antibacterial activity of cross-linked Chitosan/ Ag_2S nanocomposites. Due to the positive charge on the surface of the nanoparticles, they can be effective as an alternative to antibiotics. Nanoparticles can inhibit germs without increasing drug resistance. It was found that the cross-linked Chitosan/ Ag_2S nanocomposites showed suitable antibacterial effects on the bacterial isolates. Our results also show that using glucose as a green capping agent and reductant as a new capping agent leads to produce nanostructures with suitable size distribution. More importantly, the antibacterial activity of the Chitosan/ Ag_2S nanocomposites illustrates a suitable growth inhibition performance of antibiotic-resistant *Escherichia coli*, *Pseudomonas aeruginosa*, and *Serratia marcescens* as gram-negative bacteria and *Bacillus subtilis* and *Micrococcus luteus* as gram-positive bacteria between 64 and 32 $\mu\text{g/mL}$.

Table 3. Antibacterial activity of Chitosan/ Ag_2S nanocomposites in terms of MIC

Bacteria	Concentration ($\mu\text{g/mL}$)	64 $\mu\text{g/mL}$	32 $\mu\text{g/mL}$	16 $\mu\text{g/mL}$	8 $\mu\text{g/mL}$	4 $\mu\text{g/mL}$	2 $\mu\text{g/mL}$	1 $\mu\text{g/mL}$	0.5 $\mu\text{g/mL}$
<i>E. coli</i> PTCC 1330		-	-	+	+	+	+	+	+
<i>K. pneumoniae</i> PTCC 1053		-	-	+	+	+	+	+	+
<i>S. marcescens</i> PTCC1621		-	-	+	+	+	+	+	+
<i>P. aeruginosa</i> PTCC1074		+	+	+	+	+	+	+	+
<i>S. aureus</i> PTCC. 1112		+	+	+	+	+	+	+	+
<i>M. luteus</i> PTCC. 1110		-	+	+	+	+	+	+	+
<i>B. subtilis</i> PTCC. 1023		-	-	-	-	+	+	+	+

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