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ORIGINAL RESEARCH PAPER

Preparation, characterization and antimicrobial property of ag^{+}- nano Chitosan/ZSM-5: novel Hybrid Biocomposites

Maasoumeh Khatamiana [1]; Baharak Divband [1,2]; Mahdieh Daryana [1]*

[1]Inorganic Chemistry Department, Faculty of Chemistry, University of Tabriz, Tabriz, Iran
[2]Research Center for Pharmaceutical Nanotechnology, Tabriz University of Medical Sciences, Tabriz, Iran

ABSTRACT

Objective(s): Binary hybrids of chitosan-zeolite have many interesting applications in separation and bacteriostatic activity.

Materials and Methods: Template free ZSM-5 zeolite was synthesized by hydrothermal method, physical hydrogels of nano chitosan in the colloidal domain were obtained in the absence of toxic organic solvent and then nano chitosan/ ZSM-5 hybrid composites with nano chitosan contents of 0.35%, 3.5%, 35% wt.% were prepared. The as prepared hybrid composites were ion-exchanged with Ag cations.

Results: XRD and FT-IR results revealed a good crystalinity of as synthesized template frees ZSM-5 with BET surface area of 307 m^2g^{-1}. Presence of chitosan in composites was confirmed by XRD patterns and FT-IR spectroscopic analysis, the chitosan content in composite was obtained with TG analysis. SEM analysis of composites shows that chitosan particles were dispersed within the nanometer scale. The antimicrobial activity of different samples was investigated and the results showed that the Ag^{+}-exchanged samples have the highest antibacterial properties. Cancer cell line A549 cell line were cultured in designated medium treated with Ag^{+}-exchanged samples at the concentration of 0.01 to 0.5 mg/ ml. After 24 and 48 hours incubation, the efficacy of Ag^{+}-exchanged samples to treat cancer cell lines were measured by means of cell viability test via MTT assay. Concentrations of 0.05 and 0.1 mg/ml of Ag^{+}-exchanged samples induced a very low toxicity.

Conclusion: These hybrid composite materials have potential applications on tissue engineering and antimicrobial food packaging.

Keywords: *Antibacterial properties, Chitosan, Cytotoxicity, Hybrid nanocomposite, Template free ZSM-5*

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INTRODUCTION

Zeolites are a class of crystalline aluminosilicates with uniform and ordered networks, which have laminar, fibrous cavities with nano metric dimensions, interconnected or not, in which they lodge ions, molecules, or salts. The exceptional applications of zeolites related to their hydrothermal stability, high Bronsted acidity and uniform micro pores of molecular dimensions (typically 0.25–1 nm). Zeolites are widely used as adsorbents, ion-exchangers, and catalysts [1-7]. Ion-exchange ability of zeolites leads to new applications, such as antibacterial properties [8-12].

The ZSM-5 has an MFI-type structure with medium pore size of 0.54–0.56 nm. This molecular sieves have suitable properties for catalysis [13-15], ion exchange, adsorption and membrane [16-18] applications. ZSM-5 has a very high temperature (>1000°C) and acid stability (down to pH=3) [19]. Commonly, ZSM-5 is synthesized by hydrothermal

*Corresponding Author Email: *divband@tabrizu.ac.ir*
Tel: (+98) *4133393129*
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method from hydrogels of silicon and aluminum [19-21]. Reports investigated that cations, temperature process, silica and alumina sources have important roles in the synthesis of template free ZSM-5 [22-28].

Chitosan, poly [ß-(1–4)-linked-2-amino-2-deoxy-d-glucose], is the N-deacetylated product of chitin that is the main component in the shells of crustacean. Chitosan is an antibacterial, biocompatible, environment friendly, non-toxic and biodegradable material. The amine and –OH groups of chitosan causes many special properties, applications and available for chemical reactions. Interaction of chitosan with poly anions form complexes. Chitosan has many utilization in various fields from fertilizers to pharmaceuticals which are based on its versatility, economical and easily availability [29-33]. Chitosan has a broad antimicrobial spectrum from gram-negative, gram-positive bacteria to fungi [34]. The antibacterial activity of chitosan is related to the species of bacteria, concentration, pH, polymeric molecular weight (MW) and degree of acetylation (DA) [35, 36]. Nano chitosan is a natural material which is biocompatible and bioactive [37]. So many Methods, such as the emulsion, ionic gelation, reverse micellar, self-assembling, etc., have been used to synthesize chitosan nanoparticles [31].

Because nano -sized composites (nano particles, nano materials) are able to attach more copies of microbial molecules and cells, they are expected to be more effective in penetrating and disrupting bacterial cell membranes, and nano chitosans are effective against a variety of organisms [35, 38] and if silver salts or other functional antimicrobial agents added to chitosan nanoparticle composites, antimicrobial activity enhances.Binary hybrids of chitosan-zeolite have many interesting applications in separation, bacteriostatic activity and etc. [39-44].In this research, template free ZSM-5 zeolite was successfully synthesized by hydrothermal method, then binary hybrid composites of Nano chitosan/ ZSM-5 with chitosan content of 35%, 3.5%, 35% and their Ag^+ changed forms are prepared. In addition, the antibacterial activity and MTT assay for cell viability of as-prepared samples have been studied.

EXPERIMENTAL

Materials

Sodium aluminate, silicic acid, sodium hydroxide pellets, acetic acid, $NaH_2PO_4.2H_2O$, $Na_2HPO_4.12H_2O$ and $AgNO_3$ were obtained from Merck co. Chitosan (medium molecular weight) was obtained from Alderich.

Measurements

A Siemens D500 diffractometer with Cu kα radiation (λ = 1.5418 A and 2θ = 4–80°) was used to collected X-ray diffraction patterns (XRD) at room temperature. The nitrogen adsorption/desorption isotherms were measured at 77.46°K using a micromeritics ASAP 2020 instrument. The specific surface area was obtained using the BET (Brunauer-Emmett-Teller) method. Micropore volume of the template free ZSM-5 was obtained from the t-plot method. FT-IR spectra were obtained with a Bruker Tensor 27 Fourier Transform Infrared spectrometer with the KBr pellet technique. Scanning electron microscope (MIRA3 TESCAN) equipped with energy dispersive X-ray (EDX) facility was used to capture SEM images and to perform elemental analysis. The SEM sample was gold coated prior to examination and SEM was operated at 15 kV. TGA analysis was carried out on the chitosan nano particles/ZSM-5 hybrid composite in air up to 900 °C with a heating rate of 10 °C min^{-1} using a STAR SW 8.10 instrument to determine the chitosan content.

Materials preparation

Preparation of template-free ZSM-5 zeolite (Z):

50 mmol sodium hydroxide and 6 mmol sodium aluminate were dissolved in deionized water. The mixture were mixed and a proper amount of silica sol (25wt. %) was slowly added to the above solution under vigorous stirring. After stirring for 12h, 0.2% seed was added and then hydrothermally treated for 32 h in an 180°C oven.

Preparation of chitosan **nanoparticles** *emulsion (nCS):*

Chitosan was ionically cross-linked with sodium tripolyphosphate (STPP), was used to synthesize the nanoparticles in dilute solution. So, first of all STPP was prepared by mixing 1.6 g ($NaH_2PO_4.2H_2O$) and 7.4 g ($Na_2HPO_4.12H_2O$) and then the mixture was heated in a laboratory furnace at 560°C for 1h.

0.26 g chitosan was dissolved in 20 ml acetic acid (0.5% dilute), then 130 ml deionized water was added. The STPP (sodium tripolyphosphate) solution (95.4 ml, 1.45 mg/ml) was slowly dropped into a chitosan solution. After stirring (1500 rpm/min) for 20 min, a milky emulsion was obtained and named as nCS.

Preparation of binary hybrid composite of nano chitosan/ZSM-5 (nCSZ):

Different amounts of chitosan nano particles emulsion was stirred with 1g ZSM-5, and then ultrasounded, so the binary hybrids are prepared as illustrated in Table 1.

Table 1. Experimental details as stirring and ultrasonic duration used for preparation of nCSZ composites

Nano chitosan/ZSM-5 (%)	Stirring duration (day)	Ultrasonic time (minute)
35	6	60
3.5	3	40
0.35	1	15

Preparation of Ag^+- exchanged nano chitosan /ZSM-5 hybrid composite (nCSAZ)

All of the nCSZ composites were ion-exchanged with silver ions so the composites were stirred with aqueous solution of $AgNO_3$ with the Ag: nCSZ mass ratio of 1:25.

Antimicrobial testing

The antimicrobial activity of CS, nCS, nCSZ, CSA, nCSA and nCSAZ nano composites were tested qualitatively. Three different microbe including, Escherichia coli 25922, Staphylococcus aureus 25923, and Candida albicans 0231 were used for testing the antimicrobial activity of the samples. The cells of E. coli, S. aureus and c. albicans were grown on nutrient agar and incubated at 37 °C for 48h, and the antimicrobial activity was tested using modified agar diffusion assay (Well diffusion method). The plates were examined for possible inhibition zone after 48 h incubation.

The presence of any clear zone around samples on the plates was recorded as an inhibition against the microbial species. Finally, the microbial activities of each sample were repeated three times.

MTT assay for cell viability

A549 alveolar adenocarcinoma cells (9×10^3 cells/well) were incubated in 96-well plates. 200 µL of cell culture media was added to each plate and incubated for 24 hours at 37°C and 5% CO_2.

The cells were divided in 5 groups in triplicates: blank, nCSAZ nanocomposite (different concentrations: 0.05, 0.1, 0.25, 0.5 mg/ml). After 24 h incubation, the media were removed and the washed with Phosphate-buffered solution. Briefly, 50 µL of 2 mg/mL MTT (3-(4, 5-dimetylthiazol-2-yl)-2, 5-diphenyl- trazolium bromide) and 150 µL culture medium of was added to each well and incubated at 37°C for 4 hours and then the media was removed and dimethyl sulfoxide and Sorenson buffer was added to each well.

Finally by using an ELISA plate reader (BioTeck, Bad Friedrichshall, Germany) the absorbance was read at 570 nm wavelength.

RESULTS AND DISCUSSION

XRD analysis

The XRD pattern of synthesized template-free ZSM-5 powders can be indexed from the ASTM date [45]. Fig. 1a and b indicate XRD patterns of samples that were crystallized without and with seed respectively by changing crystallization time from 24 to 32 h.

The crystallinity of template-free ZSM-5 powders, which was crystallized at 32 h, showed the highest crystallinity.

According to the report of Y. Cheng et al. [22], the synthesized ZSM-5 with 24 hour hydrothermal time was the best time, that we saw an excess peak in XRD pattern which they did not investigate it. As shown in Fig. 1a, there was an excess peak around 2θ= 4.39, that by using seed (0.2%) this peak disappeared.

From these results we can conclude that, by using seed and proper time for conventional hydrothermal process, template free ZSM-5 synthesized with the best crystalinity.

When the relative intensity of the peaks of angel 2θ = 22-25° is higher than peaks of angel 2θ= 7-9°, the crystal structure is orthorhombic, while when the intensity of the peaks of angel 2θ =7-9° is higher, the crystal structure is monoclinic.

The N_2 adsorption/desorption isotherm of ZSM-5 zeolite were measured at 77.46°K.The parameters, including the BET surface area, micropore Area, micropore volume, and External Surface, obtained

Table 2. Surface texturing data of template free ZSM-5 zeolite

Sample	BET Surface Area (m^2/g)	Micropore Area (m^2/g)	Micropore Volume (cm^3/g)	External Surface (m^2/g)
ZSM-5	307.58	131.60	0.068	175.97

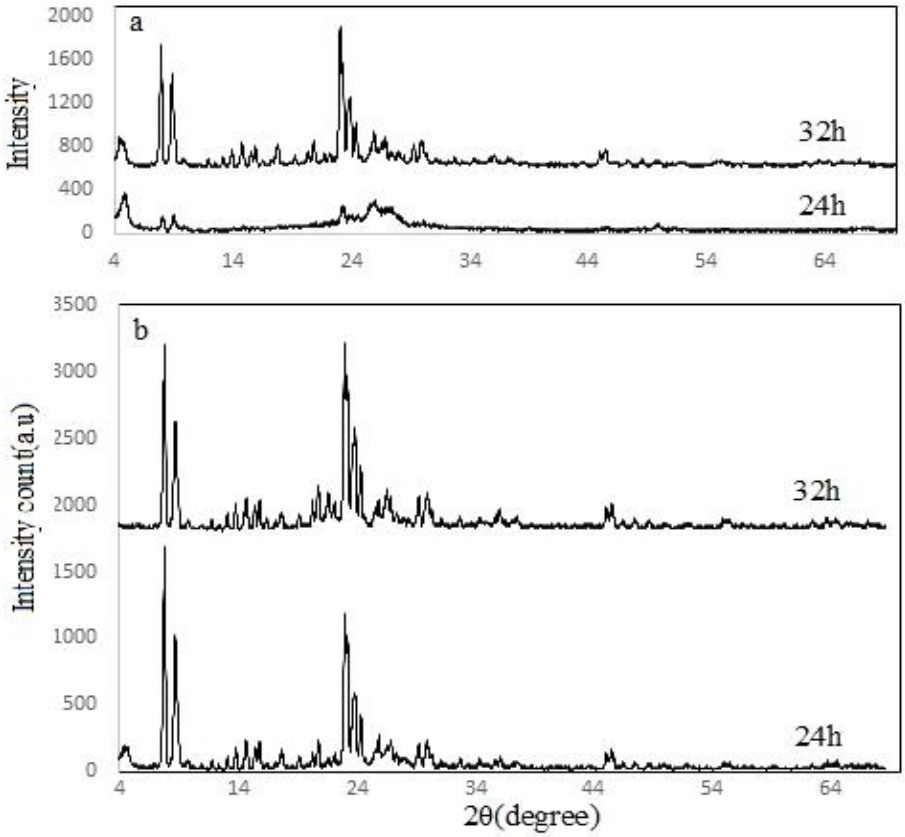

Fig. 1. Effect of crystallization without seed (a), with seed (b) on crystallinity of as-synthesized ZSM-5

from BET equation and t-plot method, are summarized in Table 2.

Fig. 2 shows XRD patterns of the binary hybrid composites of nano chitosan/ ZSM-5 with chitosan content of 35%, 3.5%, 0.35%, 0%. By increasing the amount of chitosan nano particles, the intensity of ZSM-5 peaks were decreased, which was according to Sun *et al.,*'s results [46].

FT-IR spectra analysis

The FT-IR transmission spectra for template free ZSM-5 zeolite are shown in Fig. 3. The infrared range features absorption peaks of ZSM-5 at 452 cm^{-1} (T-O bend, T=Al, Si), 542 cm^{-1} (double ring vibration), 794 cm^{-1} (external symmetric stretch), 1092 cm^{-1} (internal asymmetric stretch), 1222 cm^{-1}, (external linkages between TO_4 tetra hedral), 1635, 3483 cm^{-1}, respectively (bending and stretch vibration of H_2O molecules) [45].The absorption bands around 542 and 452 cm^{-1} are related to the ZSM-5 crystalline structure and from the intensities' ratio of these two peaks; the degree of crystallinity of zeolite sample can be estimated.FT-IR spectra of (a) chitosan and (b) chitosan nano particles are shown in Fig. 5. According to the FTIR spectra of chitosan, a characteristic band at 3500-3900 cm^{-1} is attributed to –NH and –OH groups stretching vibration. And the peak at 1604 in chitosan is an $-NH_2$ absorption peak.

In the FT-IR spectra of chitosan nano particles, the intensity of the peak at 3500-3900 cm^{-1} is lower than chitosan because of vibration limitation of OH groups, the peak at 1604 cm^{-1} was disappeared and two new peaks at 1564 and 1683 cm^{-1} was appeared. This indicated that the phosphate groups of STPP linked to the amino groups of chitosan and formed strong intermolecular hydrogen bond. The interaction between STPP and Chitosan was shown in Fig. 4 [30, 37, 47-48].

As shown in Fig. 6, in nano chitosan/ZSM-5 composites (from a to c) band at 3500–3800 cm^{-1}

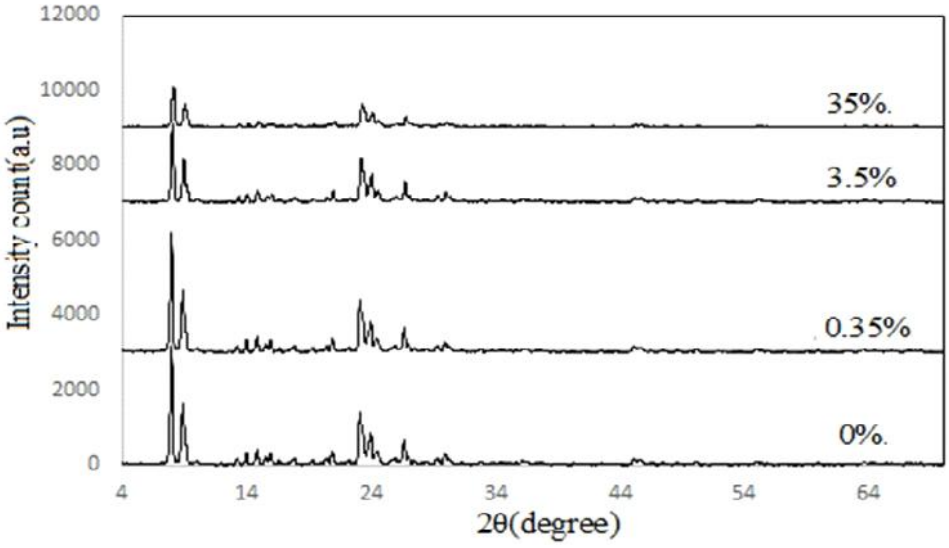

Fig. 2. XRD pattern of binary hybrid composites of nano chitosan/ ZSM-5 with chitosan content of 35%, 3.5%, 0.35%, 0%

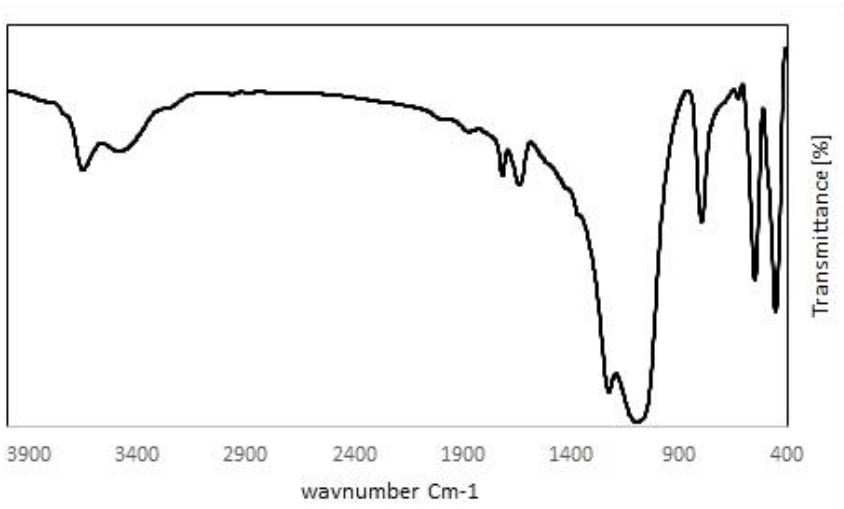

Fig. 3. The FT-IR transmission spectra for ZSM-5 zeolite without organic template

which is attributed to the hydroxyl groups stretching vibrations become weaker and wider by increasing the amount of chitosan nano particles, which indicates formation of hydrogen-bonding interaction between the hydroxyl groups of ZSM-5 and chitosan nano particles [41]. In the case of nano chitosan/ ZSM-5 (35%), although the vibration of hydroxyl groups are limited, because chitosan nano particles have free OH groups itself, the intensity of this peak is stronger than nano chitosan/ZSM-5 (3.5%) but weaker than bare ZSM-5.

The characteristic band at 1550 cm^{-1} is assigned as amino groups which apparently decrease after the incorporation with ZSM-5 [46].

The multiple peaks between 1200 and 1000 cm^{-1} in pure chitosan nano particles is belong to stretching vibrations of C–O groups of chitosan. In the case of ZSM-5 T–O groups have the same frequency of C–O stretching, and in composites, the intensity of these peaks are stronger [41].

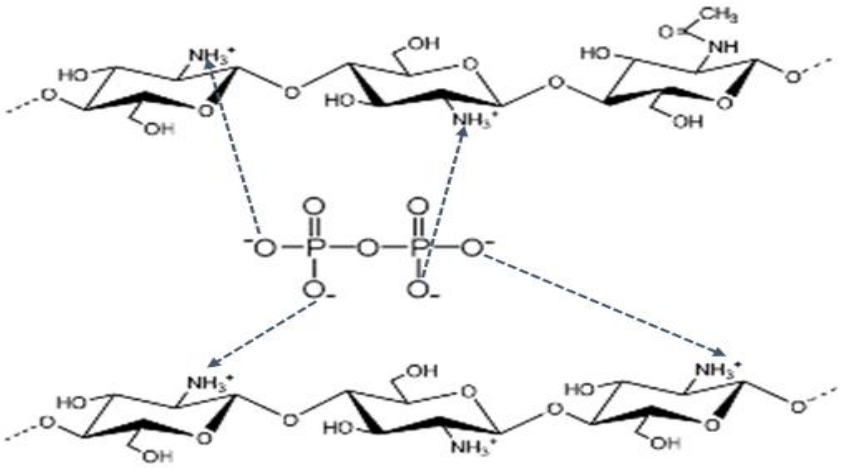

Fig. 4. Interaction between STPP and Chitosan

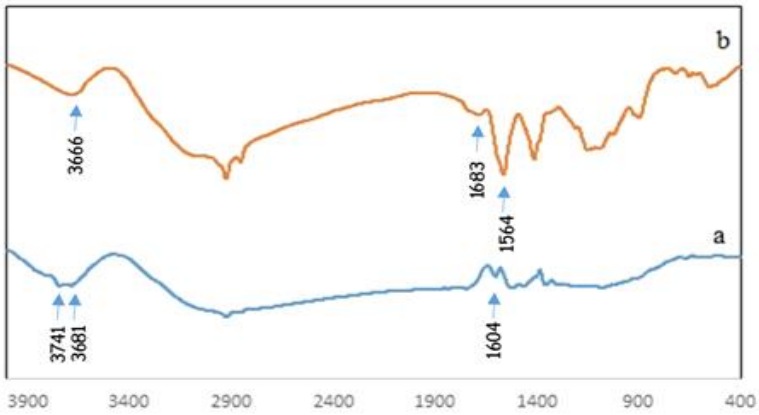

Fig. 5. FT-IR spectra of (a) chitosan and (b) chitosan nano particles

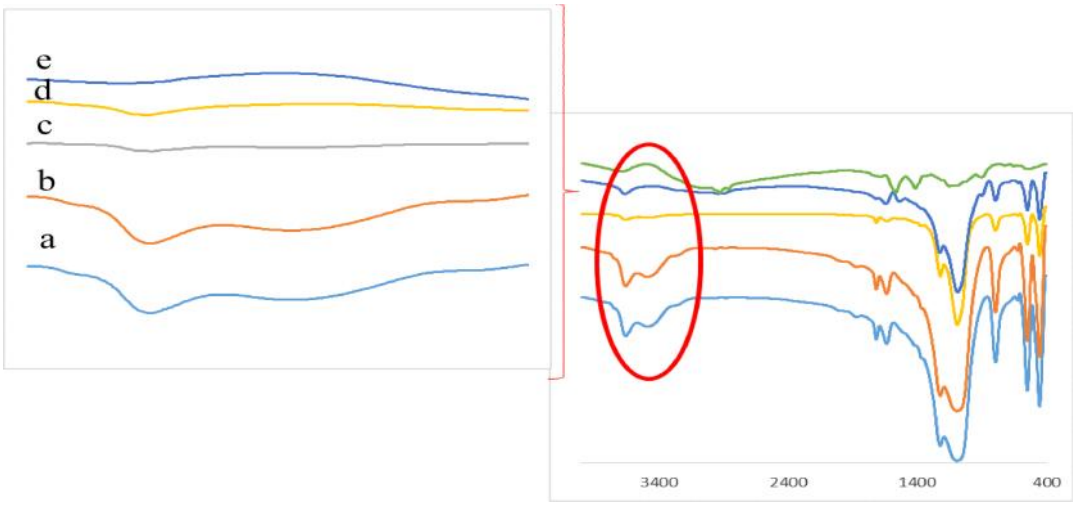

Fig. 6. The FT-IR transmission spectra for (a) Z, (b) nCSZ(0.35%), (c) nCSZ(3.5%), (d) nCSZ(35%), (e) pure nCS

TG analysis

To measure the exact contents of nCS in the hybrid composites, TG analyses were conducted.

As shown in Fig. 7, The TG curve of nCS exhibits that the first thermal event occurs at 50-140°C with a weight loss of 5.04% which may be due to the loss of residual water. Two steps in 230-500°C with a weight loss of 52.93%, and 500-870°C with a weight loss of 36.86%, that according to literature data, pyrolysis of chitosan starts with breaking of the glycosidic bonds, then decompose to acetic, butyric and lower fatty acids. It can be assumed that in the thermal degradation steps, cross-linked membranes small molecular degradation products and crosslinking agents are liberated [49] and thermal degradation of the nCS, up to 800°C the total weight loss of nCS was found to be 94%. The TG curve of nCSZ (35%) is almost the same as that of chitosan nano particles. The first stage of weight losses occurred at 50-170°C (water loss (0.42%)) and the steps of 200-450°C (13.24%), 450-600°C (5.15%), and 600-800°C (5.08%) ascribed to decomposition of nCS and impurity in ZSM-5 crystal. From the weight loss at the stages, we can calculate the real nCS contents in the designated 35% nCSZ hybrid composite is about 18.5 wt.%.

It was concluded that the nano chitosan was found to be thermally more stable and this was confirmed from the initial decomposition temperatures which was matched up with Sudha. P.N. et al's report [49]. The decomposition of the nCS in the composites occurred at 200°C and that is lower than decomposition temperature of the pure nCS. It indicates that the thermal stability of the composite is not substantially improved after incorporating ZSM-5[46].

SEM analysis

The scanning electron micrographs of nCS, Z, nCSZ (35%, 3.5%) are shown in Fig. 8 which shows that chitosan is in nanometer scale with the thickness of 35-45 nm. ZSM-5 zeolite crystalized in hexagonal

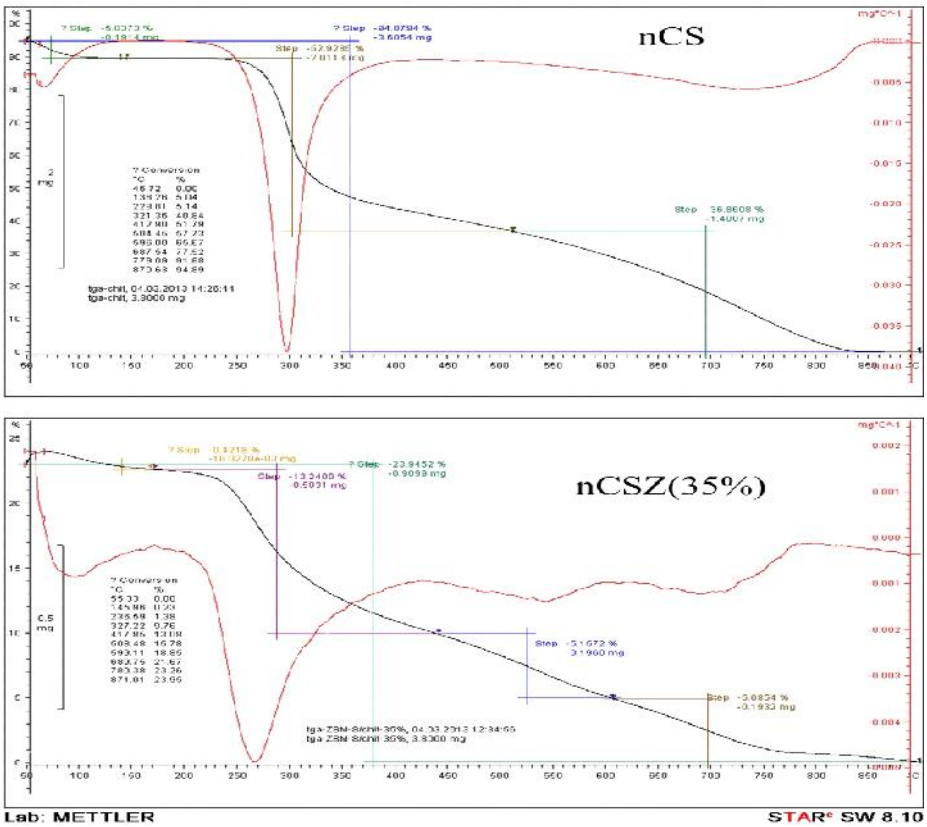

Fig. 7. TGA thermogram details of nCS, nCSZ (35%)

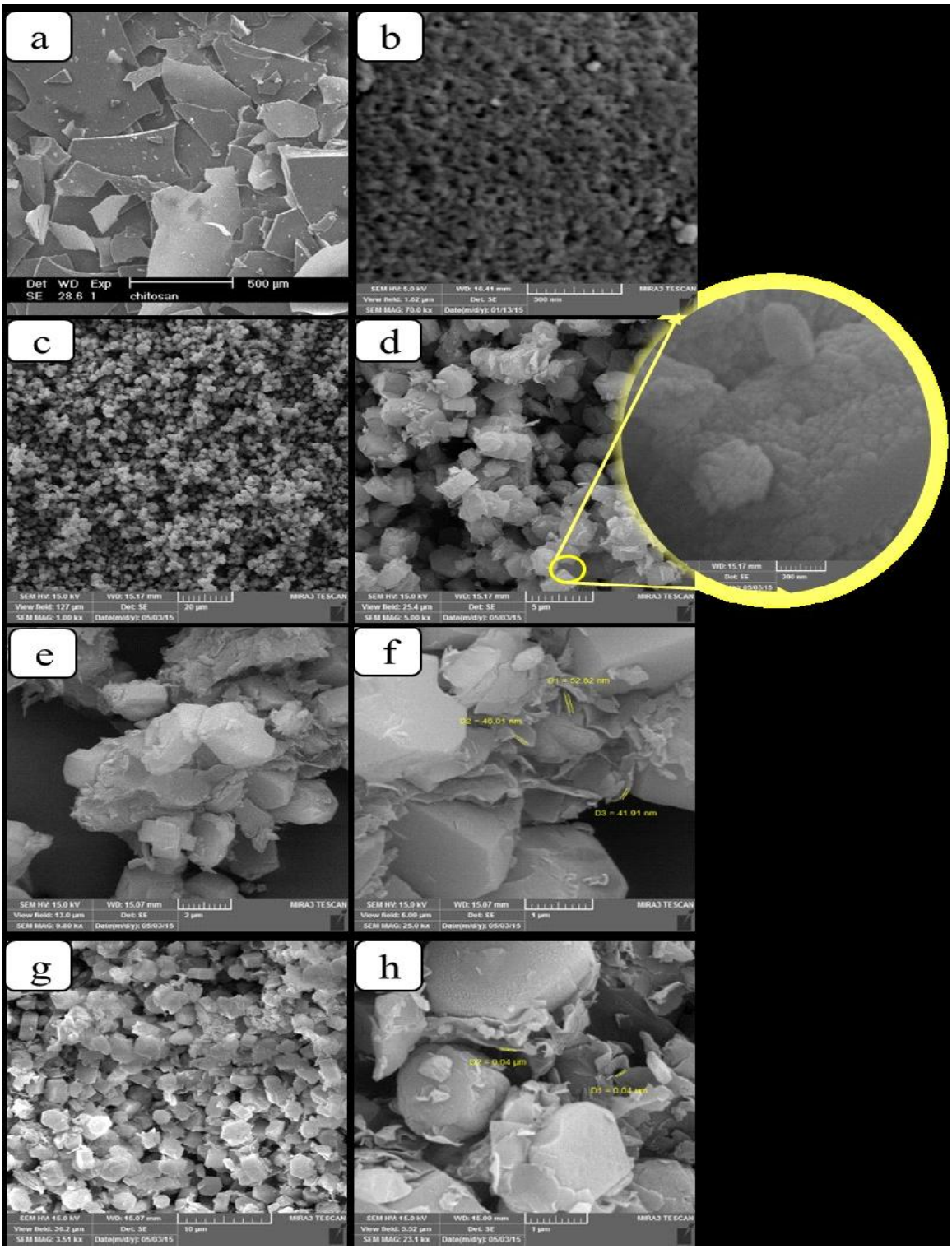

Fig. 8. SEM images of nCS(a,b), template free ZSM-5 (c,d), nCSZ (35%) (e,f), nCSZ(3.5%) (g,h)

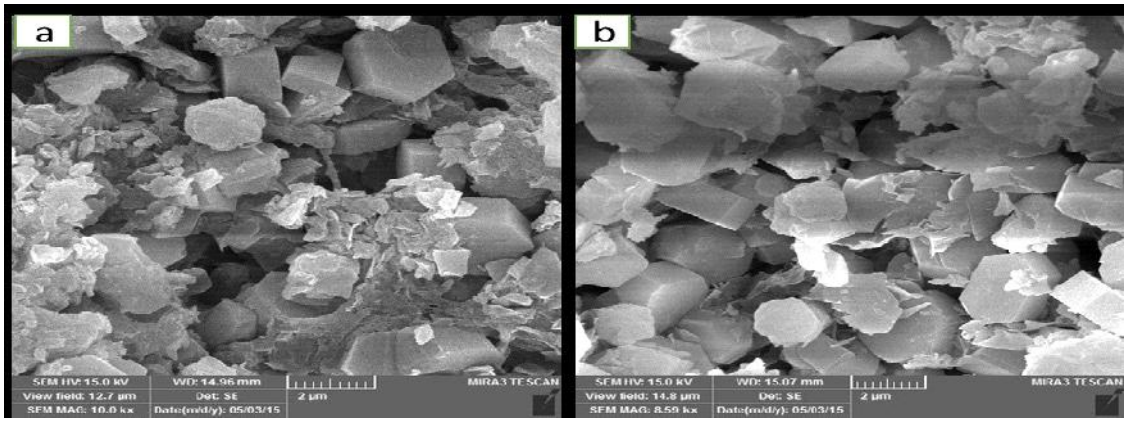

Fig. 9. SEM images of nCSZ (35%) (a), nCSZ(3.5%) (b) with a magnification of 2µm

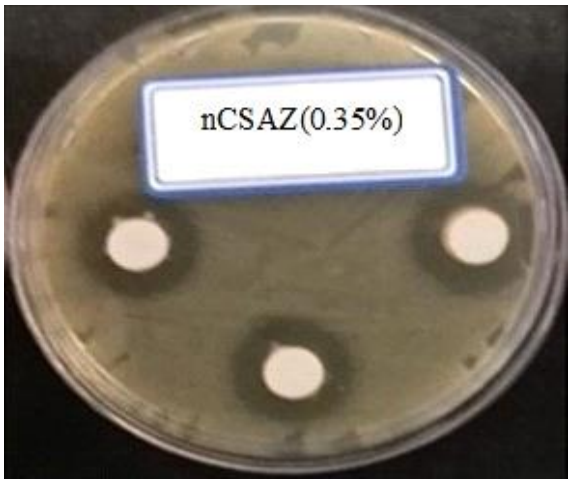

Fig. 10. Photograph of antimicrobial test results of nCSAZ (0.35%) against E.coli

shape with a diameter of 2-2.5 µm, with also some smaller particles, that these spherical particles gather together and forming ZSM-5 zeolite, as shown in Fig. 8d.

SEM analysis shows that chitosan particles were dispersed within the nanometer scale. As shown in Fig. 9, when the amount of nano chitosan is low, chitosan nano particles is inside of the ZSM-5 cavities, while in nCSZ(35%) the amount of chitosan nano particles is much more than nCSZ(3.5%), chitosan nano particles fill the cavities and also outside of the ZSM-5.

Antimicrobial activity

Fig. 10 shows a typical antimicrobial test result of nCSAZ (0.35%) against E.coli. The tests on all samples were repeated using two other microorganisms (S. aureus, c. albicans) and the results are shown in Table 3.

Although it has been proved that chitosan has an antimicrobial activity, its mechanism is still not fully understood. It seems that chitosan acts differently against gram-positive and gram-negative bacteria. Chitosan itself has antimicrobial activity due to (1) the main reason is interaction between available positively charged chitosan molecules in chitosan acetic solution and negatively charged microbial cell membranes, (2) the binding of chitosan with microbial DNA, (3) the chelation of metals to nutrients which are essential to microbial growth [34]. Chitosan nano particles are more effective in penetrating and disrupting bacterial cell membranes [38].

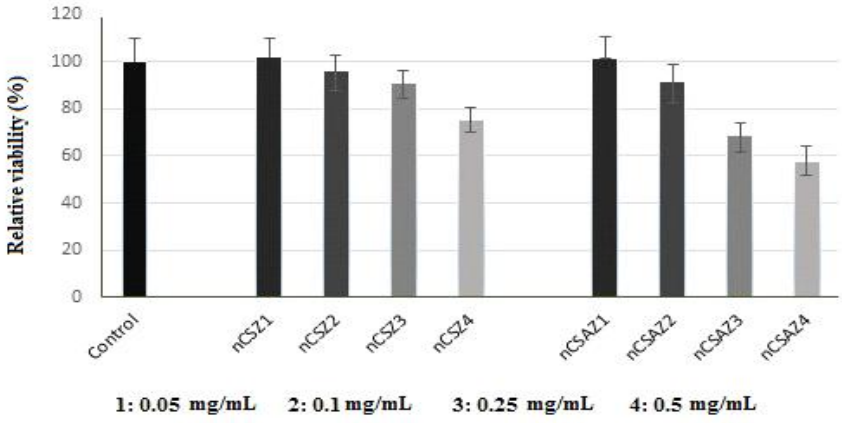

Fig. 11. Relative viabilities of A549 alveolar adenocarcinoma cells after being incubated with various concentrations of nCSZ and nCSAZ

Table 3. Antimicrobial Activity of CS, nCS, nCSZ(35%, 3.5%, 0.35%), CSA, nCSA, nCSAZ(35%, 3.5%, 0.35%) against E.coli, S.aureus, C.albicans

test organism / sample	E. coli	S. aureus	C. albicans
CS	+	-	-
nCS	+	+	-
nCSZ(35%)	-	-	-
nCSZ(3.5%)	-	-	-
nCSZ(0.35%)	-	-	-
CSA	+	+	-
n\CSA	+	+	-
nCSAZ(35%)	+	++	++
nCSAZ(3.5%)	++	+	++
nCSAZ(0.35%)	++	+	+

The bacteriostatic activity of chitosan and nano chitosan against bacteria was low, and they didn't show any activity against fungi, because when chitosan is in powder form (not polycationic form) the main reason of bacteriostatic activity of chitosan does not exist. After Ag- exchanging of samples the activity was increased Composites of nCSZ didn't act against bacteria and fungi because nano chitosan was encapsulated in the pores of zeolite, interestingly, nCSAZ (35%,3.5%,0.35%) exhibited microbial inhibition zones against E.coli. The antimicrobial activity of nCSAZ (0.35%) was the best since, with the increasing of zeolite amount, exchanging of Ag^+ with Na cations of zeolite was increased so the antimicrobial activity was improved.

Cytotoxicity of the nCSZ and nCSAZ

The cytotoxicity of the nCSZ and nCSAZ were investigated in the A549 cell line. Fig. 11 shows the relative cell viability ($[C_r/C_0]$ 100%) vs. different concentration of nCSZ and nCSAZ, determined by the MTT assay. Here, C_o is the viable cell numbers in the control sample, and C_r is the viable cell numbers treated with the nanocomposites. The error bars are the calculated standard deviation. The relative viability of cells treated with 0.05 mg/ml of nCSZ or nCSAZ is about 100±5%. The relative viabilities (%) of cells treated with higher concentrations of nCSZ and nCSAZ (0.1, 0.25 and 0.5 mg/ml) are 96, 90 and 75% and 91, 68 and 57%, respectively after 24-h incubation. The results indicate nanocomposites are safe up to 0.25 mg/mL of nCSZ and 0.1 mg/ml of nCSAZ.

CONCLUSION

By using seed and proper time for conventional hydrothermal process, template free ZSM-5 with the best crystalinity was synthesized. The BET surface area of as synthesized ZSM-5 was obtained 307 m^2g^{-1}. Binary hybrid composites of Nano chitosan/ ZSM-5 and their Ag^+ changed forms are prepared successfully. The FT-IR of nano chitosan/ ZSM-5 indicates formation of hydrogen-bonding interaction between the hydroxyl groups of ZSM-5 and chitosan nano particles. SEM analysis of composites shows that chitosan particles was dispersed within the nanometer scale. The antimicrobial activity of different samples was

investigated and the results shows that the Ag^+-exchanged samples have the better properties. So the A549 cell line was used to evaluate the cytotoxicity of nCSZ and nCSAZ. Briefly, nanocomposites exhibit dose-dependent toxicity to cells and very low toxicity was observed up to 0.25 and 0.1 mg/mL concentrations for nCSZ and nCSAZ, respectively. The results provide primary information about the potential use of nCSAZ in various medical applications such as tissue engineering and industrial applications such as antimicrobial food packaging.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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