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In vitro antimicrobial activity of intercalated Modified Chitosan-Clay nanocomposite against different microorgamnisms

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Abstract

Unmodified sodium montmorillonite (Na-MMT) used to intercalate chitosan (CS) and modified chitosan. Their structures were characterized by XRD, TEM and IR techniques. The results showed that CS chains were inserted into silicate layers. The interlayer distance of the layered silicates in the nanocomposites enlarged as the amount of polymer increased. The thermal stability of the nanocomposites are characterized by TGA. In vitro antimicrobial assay showed that pristine montmorillonite could not inhibit the growth of bacteria. Chitosan was less effective in inhibiting bacterial growth than chitosan modified triphenyl phosphine (mod- CS). CS/MMT and mod-Cs/MMT nanocomposites had strong antimicrobial activity, particularly against Gram-positive bacteria. With the increase of the amount and the interlayer distance of the layered silicates in the nanocomposites, the nanocomposites showed a stronger antibacterial effect.

Keywords: Chitosan; Nanocomposite; Antimicrobial

1. Introduction

Microbial infection remains one of the most serious complications in several areas, particularly in medical devices. Recent headlines tell us that everything from our kitchen cutting boards, tupper ware and soda fountain delivery tubing are infected with everything from fecal matter to human pathogenes. Antimicrobial treatment is rapidly becoming a standard finish for some categories of textile products such as medical and hygienic uses (Shin et al.,1999). Furthermore antimicrobial agents are commonly used in coating of surfaces such as ship hulls, shower walls and many kinds of tubing. However, low molecular weight antimicrobial agents suffer from many disadvantages, such as toxicity to the environment and short-term antimicrobial ability (Jones et al.,2005; Sauvet et al.,2000). To overcome problems associated with the low molecular weight antimicrobial agents, antimicrobial functional groups can be introduced into polymer molecules. The use of antimicrobial polymers offers promise for enhancing the efficacy of some existing antimicrobial agents and minimizing the environmental problems accompanying conventional antimicrobial agents by reducing the residual toxicity of the agents, increasing their efficiency and selectivity, and prolonging the life time of the antimicrobial agents (Samour et al.,1978; Gebelein et al., 1982). Many studies have been performed on the antibacterial activity of low-molecular weight and polymeric quaternary ammonium salts (. Kanazawa et al., 1993; Cakmak et al., 2004; Zhu and Sun, 2004). The target size of the cationic biocides is the cell envelope of bacteria; thus, an increase in the molecular size due to polymerization, which may result in reduced permeability, is not regarded as a factor seriously affecting their activity. It is worth noting that polycationic biocides have been shown to possess a higher activity against bacteria(Ikedaet al.,1984). In addition, polymeric biocides are particularly important because they possess promising advantages over monomeric forms (Chen, 2004; Aian and Sun, 2004). Polycations with main chain or pendant quaternary ammonium salts show outstandingly high antibacterial activity against Gram-negative and Gram-positive strains and exhibit a wide spectrum of antimicrobial activity (Nonaka et al., 2000; Avram, 2001; Choi et al., 2001). Although many organic antimicrobials exhibit an efficacy, their temperature sensitivity does not allow an application and also their varying compatibility to different polymer matrices often lead to either too fast or too slow activity which imparts limitations to their use.

Chitosan (CS) is one of the most abundant biological polysaccharide derived from nature and is obtained by deacetylation of chitin present in crudtaceous. Chitosan is composed of linked 2-deoxy-2-amino-D-glucopyranose units and ß $(1\rightarrow4)$ linked 2-deoxy-2-acetamido-D-glucopyranose units. It has attracted considerable interest due to its biological activities such as antimicrobial (Jeon et al., 2001; No et al., 2002) antitumer and immune enhancing effects (Sugano et al., 1992). In addition to its unique properties such as biocompatibility, biodegrability and non toxicity, it is widely used in biotechnology, pharmaceutics, cosmetics, textiles agriculture fields due to its antifungal and antimicrobial activities (Yang, et al., 2005; Franklin and Snow 1982).

Unmodified chitosan is not antimicrobially active at pH 7, since it does not dissolve and does not contain any positive charge on the amino groups (Chung et al., 2005). The antimicrobial activity of chitosan also increases with increasing degree of deacetylation, due to the increasing number of ionizable amino group.

Two hypotheses for the mechanism of inhibition were reported. (i) The polycationic chitosan consumes the electronegative charges on cell surfaces and the cell permeability is changed, thus this interaction results in the leakage of intracellular electrolytes and proteinaceous constituents; (ii) chitosan enters fungal cells and then essential nutrients are adsorbed, which inhibit or slow down the synthesis of mRNA and protein (. Avadi et al.,2004) .

In an attempt to improve the activity of chitosan, the effect of modifying chitosan to obtain derivatives with higher activity such as N-sulphonated and N-sulfobenzoyl chitosan (Chen et al., 1998), carboxymethylchitosan (Chen et al., 2000), quaternary ammonium salt of chitosan (Jia et al., 2001) and form complexes of chitosan with materials, such as surfactants, essential oils, metals and organic acids (Hui et al., 2004).

Montmorillonite (MMT) clay is one of the smectite group, composed of silica tetrahedral sheets layered between an alumina octahedral sheets. The imperfection of the crystal lattice and the isomorphous substitution induce a net negative charge that leads to the adsorption of alkaline earthmetal ions in the interlayer space. Such imperfection is responsible for the activity and exchange reactions with organic compounds. MMT also contains dangling hydroxyl end-groups on the surfaces (Schell et al., 1993). MMT has large specific surface area; exhibits good adsorb ability, cation exchange capacity, standout adhesive ability, and drug-carrying capability. Thus, MMT is a common ingredient as both the excipient and active substance in pharmaceutical products (Herrera et al., 2000). The intercalation of organic species into layered inorganic solids provides a useful and convenient route to prepare organic – inorganic hybrids that contain properties of both the inor- ganic host and organic guest in a single material (Zhou et al., 2004).
Polymer-clay nanocomposites are materials of increasing interest because of their structural or functional behavior. The intercalation of the cationic chitosan into Na-MMT through a cationic exchange process provides nanocomposites with both interesting structural and functional properties. The focus of this paper is directed towards modified chitosan with chloactyle chloride and triphenyl phosphine and then interclacted into MMT with different ratio. It appears as an improved way of developing novel nanacomposite with phosphonium ion, which can inihibit the growth of bacteria at very low concentration. First. CS/MMT and mod-CS/MMT were prepared, XRD, TEM, FTIR were used to characterize their structures. At last this paper detailed the antimicrobial activity of the nanocomposites against microorganisms at different concentration.

2. Experimental

2.1.Materials

Chitosan from a shell of Chionoccetes opilio. It has 70 % degree of deacetylation with average molecular weight of 400.000 was purchased from Aldrich chemicals. Ibuprofen, triphenyl phosphine and chloroacetylchloride provided from Aldrich chemicals. The clay mineral used in this study was sodium montmorillonite (Colloid BP) from Southern Clay Products Inc (Gonzales, Texas, USA) with cation exchange capacity (CEC) of 114.8 meq/100g.

2.2. Chloroacetylation of chitosan.

Under magnetic stirring drops of pyridine (75.2 ml, 99.6 mmol) were added to 1 g chitosan. The mixture was cooled in an ice-salt bath and chloroacetyl chloride (7.965 ml, 99.6 mmol) was added drop wise with stirring. The reaction mixture was stirred overnight at 40 ºC for three days. The chloroacetylated chitosan was precipitated by addition of dilute HCl (1N), filtered off and washed with distilled water several times and the product was dried to give 0.95 g.

2.3. Synthesis of Triphenyl-chloro acetylated chitosan phosphonium salt (mod-CS).

2 g of chloro acetylatyed chitosan was dissolved in 20 ml dry DMF followed by addition of 5.33 g (20 mmol) triphenyl phosphine. The reaction mixture was stirred for four days at 80 ºC in an oil bath. The product was filtered off, washed with DMF to give 1.5 g.

2.4. Preparation of Triphenyl- (chloro acetylated chitosan) phosphonium salt-montmorillonite intercalates (mod-CS/MMT).

1 g of Na-MMT was swelled in 30 ml water by stirring several hours at 40°C. A solution of 1 g mod-CS dissolved in 10 ml DMF was added and stirred for 24 hr. The product (mod-CS/MMT (1:1)) was filtered, washed several times with water and collected by a filter press and dried at 80 ^{o}C in vacuum oven for 10 hr. The same procedure was done using 2.5 g and 5 g of mod-CS to prepare (mod-CS/MMT (2.5:1)) and (mod-CS/MMT (5:1)) respectively.

2.5 Antimicrobial activities

Na-MMT, CS, mod-Cs and their nanocomposites were screened for their antimicrobial activity against the gram-negative bacterium *(Escherichia coli, Klebsiella, Serratia sp., Salmonela, and Pseudomonas*), the gram-postive bacterium (*Staphylococcus aureus, B. subtillus* and *Pernela sp*) and the fungi (*C. albicans, Cryptococcus neoformans, Aspergillus flavus* and *fusarium*). The bacteria strains were maintained on nutrient agar and nutrient broth, while the fungi were maintained on sabouraud agar. All media were sterilized in autoclave before experiments. 20 mg of powdered samples were placed onto bacteria strains maintained on nutrient agar plate (3g peptone; 5g NaCl; 5g beef extract; 20 g agar per liter) and fungi that were placed onto sabouroud (10 g peptone, 20 g glucose). After 24h of incubation at 37 °C, the diameters of inhibition zones were measured. The reaction of the microorganisms with the nanocomposites was determined by the size of inhibitory zone.

2.6 Minimal inhibition concentrations (MICs).

The minimal inhibitory concentration of the nanocomposite against the tested microorgamisms was deteremined by agar dilution method and colony forming units/ml (CFU/ml). Each culture medium was enriched with different concentration (0, 2.5, 5, 10 and 20 mg/ml) of the nanocomposite. About 0.5 ml of each standerd organism's suspension was mixed with 9.0 ml of diluted corresponding media. The number of bacteria and fungi was counted before adding the nanocomposite. The results were recorded after 48 hrs. Three replicate experiments were performed. Number of living colonies or colony forming unit (CFU/ml) was counted using the bioassay method and compared with the controlled microorganisms. The sub inhibitory concentration (sub MICs) was used in studying the same action of the nanocomposite.

2.7. Effect of Cs –MMT and mod-Cs –MMT on the morphology of the tested organisms.

The tested organisms were treated with the sub leathal dose of Cs –MMT and mod-Cs –MMT nanocomposite. The morphology of these organisms was examined microscopically after staining with Gram stain (crystal violet, iodine and safranine). Ethyl alcohol was used to decolorize the smear. The slide was examined under oil immersion lens and photographed using photo Auto MPS 45.

2.8. Characterization

Infrared absorption spectra were carried out on a Perkin Elmer 1420 spectra-photometer using KBr disc technique in the wavelength range of 4000-400 cm^{-1}. Wide Angle X-ray diffraction (WAXD) measurements were recorded; using a Phillips powder-Diffractometer equipped with a Ni-filtered Cu-K_α (λ=1.5418 Å); at a scanning speed of 2 °/s. The samples were dried in a vacuum oven at 80 ^oC for 12 h, and then mounted on a sample holder with a large cavity. A smooth surface was obtained by pressing the powder samples with a glass plate. Bragg´s Law ($n\lambda=2d\sin\theta$) was used to compute the crystallographic spacing. Thermogravimetric analysis (TGA) was carried using Perkin-Elmer thermal analyzer system at a heating rate of 10˚C/ min from 30 to 800 ˚C under nitrogen atmosphere. REMI (Bombay-India-400002) centrifuge was used to collect the cells. Micrographs were taken for microorganisms before and after treatment with the nanocomposite with a Photo automat MPS 45 Light microscope using an oil immersion lens at magnification 100.

3. Characterization of organo-clay/drug.

3.1 XRD spectra.

An XRD is a powerful and straight forward technique for monitoring the formation of intercalated or exfoliated nanocomposite. It is observed from Fig. 1 that the peak characteristic to the basal spacing of Na-MMT appears at 2 θ =9.6 º corresponding to d_{001} is 9.6 Aº. The XRD of CS (Fig. 2 a) shows the characteristic crystalline peaks around 2θ = 10, 20, 22 º. The peaks around 10 and 20 are related to crystal (1) and crystal (2) in chitosan respectively. The unit cell of crystal (1) is characterized by a=7.76, b=10.91, c=10.30 Aº and β=90 º and it is larger than that of crystal (2), whose unit cell is characterized by a=4.4, b=10.0, c=10.3 Aº and β=90 º [21, 22]. After modification of chitosan, mod-CS showed two sharp peaks at 2θ = 12, 15 º and abroad peak at ~ 22 º. This indicates that mod-CS formed a new crystallite. In mod-CS/MMT (1:1) the d_{001} peak of the clay has been disappeared and a broad peak at 2θ = 4º, appeared corresponding to an increase in d-spacing. This increase in basal spacing is attributable to the intercalation of polymer chain inside the clay layers. The low peak intensity in the nanocomposite is because of the decrease in coherent layer scattering. However in mod-CS/MMT (2.5:1) a shoulder is observed. This indicates that a substantial part of clay mineral is only exfoliated. No distinguishable peak could be observed in mod-CS/MMT (5:1). This indicates that these silicate layers dispersed in mod-CS matrix lose their structural registry due to exfoliation.

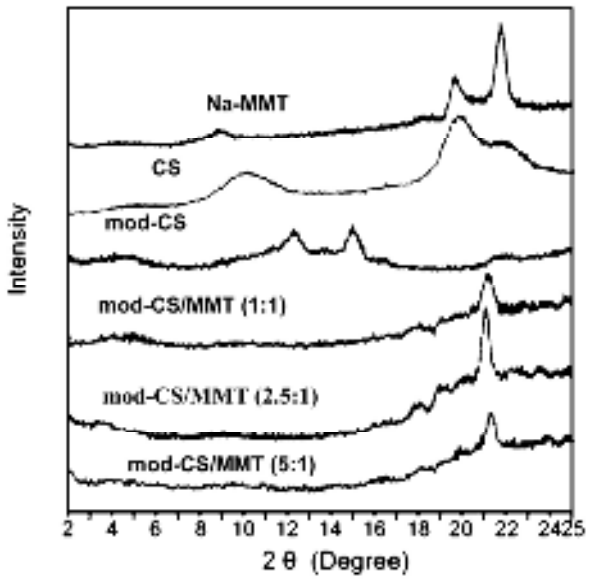

Fig. 1. X ray diffraction pattern of Na-MMT, CS, mod-CS, mod-CS/MMT (1:1), mod-CS/MMT (2.5:1) and mod-CS/MMT (5:1).

3.2. IR specrum.

IR spectrum of Na-MMT Fig. 2 show that characteristic absorption band at 3632 cm^{-1} [ν (O-H)] is assigned to the stretching vibration of Al-OH. The symmetrical Si-O-Si band [ν (Si-O-Si)] is characterized by the stretching band at 1160 cm^{-1}. Other characterstic adsorption bands of pure clay mineral are at 914 [δ (Al-Al-O)], 886 [δ (Al-Fe-O)] and 848 cm^{-1} [δ (Al-Mg-O)]. IR spectrum of CS shows a broad band at 3462 cm^{-1} corresponding to the stretching vibration of N-H. The peak at 2937 and 2857 cm^{-1} are typical of C-H stretch vibration, While peaks at 1660, 1551 and 1309 cm^{-1} are characteristic of amides I, II and III respectively. The sharp peaks at 1442 and 1374 cm^{-1} are assigned to the CH_3 symmetrical deformation mode and 1036 cm^{-1} is indicative of C-O stretching vibration [ν (C-O-C)]. The small peak at ~798 cm^{-1} corresponds to wagging of the saccharide structure of CS. The IR spectrum of mod-CS shows a band at 1740, 1615 cm^{-1} corresponding to C=O stretching vibration, NH- respectively. The bands at 1421 (m), 1164 (m) and 1063 cm^{-1} (w) corresponding to phosphonium salt attached to Ph group is shown in Fig.2.

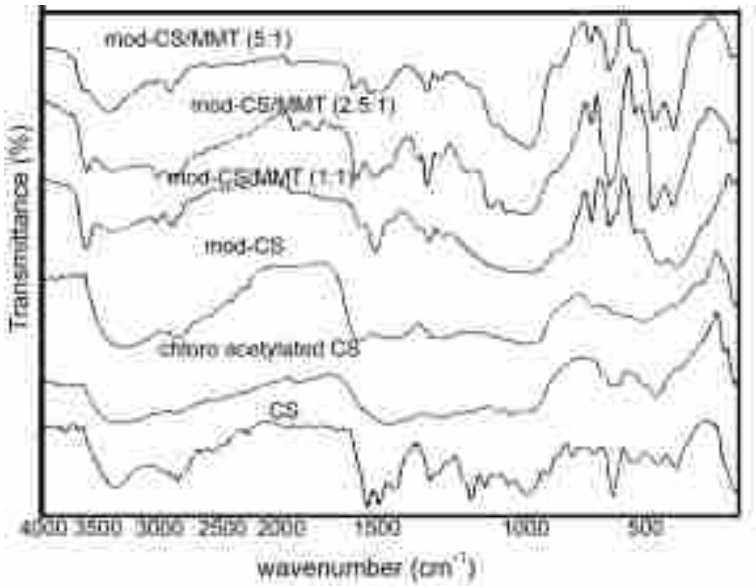

Fig. 2. IR. Spectra of CS, Na-MMT, mod-CS, mod-CS/MMT(1:1), mod-CS, mod-CS/MMT(2.5:1) and mod-CS, mod-CS/MMT(5:1) in the region 4000-400 cm^{-1}.

IR is an appropriate technique to study Polymer-clay interaction. It is suggested that when chelation of transition metal ions by CS occurs there is a shift in N-H vibration (Peppas, Khare; 1993). In this regard, the small peak at 1551 cm^{-1} corresponding to the deformation vibration [ν (N-H)] amide II of the amine group shifted to lower frequency at 1530 and 1490 cm^{-1} in the mod-CS/MMT indicating possibility of an electrostatic interaction between the negatively charged structure of clay and amine groups of CS. Additionally, compared with CS, there were three peaks at ~612, 520 and 458 cm^{-1} in mod-CS/MMT (5:1). These peaks were of low intensity in CS and suggest the possibility of a strong interaction between CS and clay.

3.3. TEM analysis

TEM of a thin film of mod-CS/MMT (5:1), mod-CS/MMT (1:1) are shown in Fig.3 respectively. Image A show that silicate layers remain in exfoliated structure in mod-CS/MMT (5:1). However, it is observed that few layers remain in intercalated structure in mod-CS/MMT (1:1), the over whelming majority of silicate layers are in intercalated structure. This is consistent with the conclusion drawn from XRD.

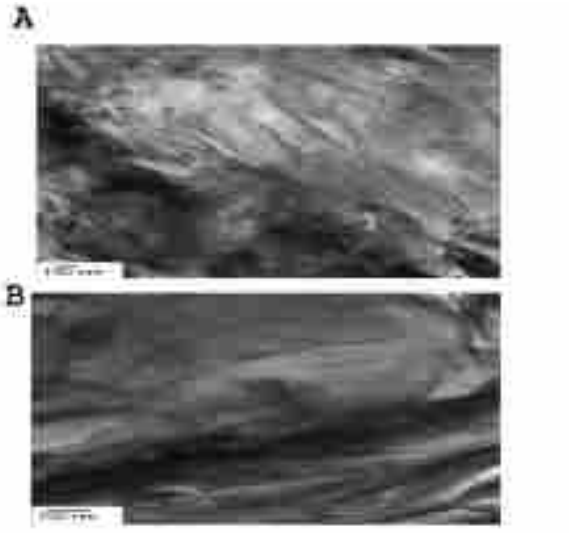

Fig.3. TEM images of A) mod-CS/MMT (5:1), B) mod-CS/MMT (1:1)

3.4. Thermal stability.

TGA profile of mod-CS Fig. 4 show two steps for weight loss at the temperature around 100 °C and 200-450 °. The first weight loss was due to the free water evaporation. The second weight loss corresponding to the decomposition of chitosan. The TGA curves of mod-CS/MMT at different concentrations show superior thermal stability. This behavior is expected because the clay platelets protect and delay the intercalated chains from undergoing a degradation process. As the amount of mod-CS increased the total weight residue decreased. This enhanced thermal stability was believed to be ascribed to the MMT nanolayers acting as barriers for the degradation of mod-CS in the interlayer spacing and partially related to the hindered diffusion of a volatile decomposition product within the nanocomposites and the shielding effect induced by the existence of the silicate nanolayers became more dominant with the increase of MMT content in the nanocomposites.

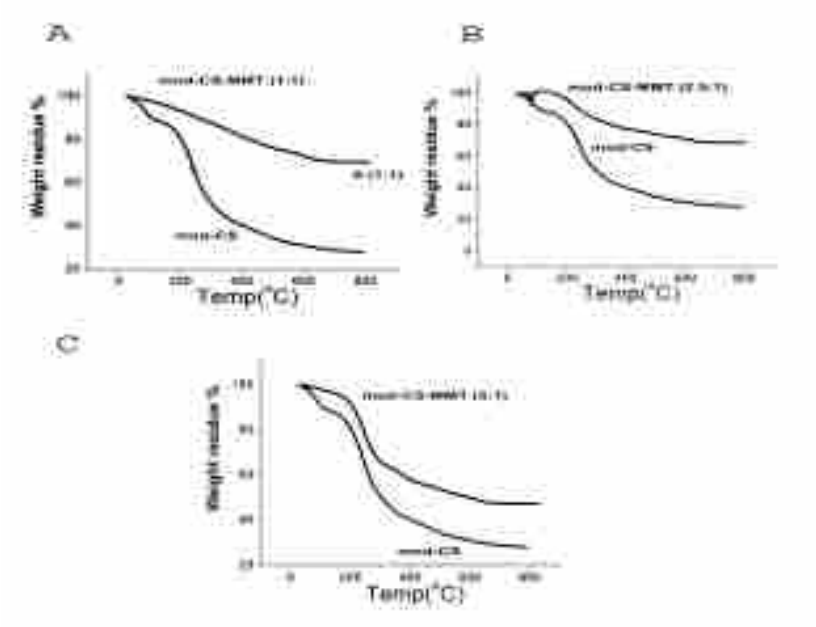

Fig. 4. TGA of mod-CS, mod-CS/MMT using different ratios of mod-CS

3.5. Antimicrobial assays:

As shown in Table 1, Na-MMT cannot inhibit the growth of microbes however; a mixture of Na-MMT/ chitosan show small inhibitory effect and mod-CS/MMT a higher inhibitory effect. The inhibitory actions are observed against a wide variety of microorganisms, including Gram-positive bacteria, Gram-negative bacteria and Fungi. Diameters of inhibition zones were ranged between 13-22 mm on Fungi, 15 mm on Gram-negative bacteria and 20 mm on Gram-positive bacteria growth after incubation for 48 h Fig. 5. The same result was observed in chitosan/organic clay nanocomposites that showed the good inhibitory for Gram positive bacteria growth, but little effect on Gram –negative bacteria.

Table1. Diameters of inhibition zones produced by CS, Na-MMT, mod-CS, mod-CS/MMT (1:1), mod-CS, mod-CS/MMT(2.5:1) and mod-CS, mod-CS/MMT(5:1) against different species of microorganisms.

Microorganism		**Diameter of inhibition zone (mm)**								
		CS		**Mod-CS/MMT(5:1)**		**Mod-CS/MMT(2.5:1)**		**Mod-CS/MMT(1:1)**		**Na-MMT**
		5 mg	**20 mg**	**5 mg**	**20 mg**	**5 mg**	**20 mg**	**5 mg**	**20 mg**	**5 mg**
Bacteria	***E.coli***	**3**	**9**	**14**	**14**	**10**	**10**	**3**	**3**	**- ve**
	B.subtillus	**2**	**5**	**9.5**	**- ve**	**5**	**6**	**-ve**	**-ve**	**- ve**
	Serratia sp.	**- ve**	**- ve**	**20**	**- ve**	**16**	**-ve**	**- ve**	**- ve**	**- ve**
	Staphylococcus aureus	**- ve**	**- ve**	**10**	**30**	**4**	**15**	**- ve**	**- ve**	**- ve**
	Pernela sp.	**-ve**	**- ve**	**15**	**- ve**	**10**	**-ve**	**-ve**	**- ve**	**- ve**
	Salmonela	**- ve**	**- ve**	**15**	**12**	**5**	**9**	**- ve**	**- ve**	**- ve**
	Klebsiella	**- ve**	**- ve**	**15**	**-ve**	**7**	**-ve**	**- ve**	**- ve**	**- ve**
	Pseudomonas	**- ve**	**- ve**	**15**	**- ve**	**9**	**-ve**	**- ve**	**- ve**	**- ve**
Fungi	***C.albicans***	**2**	**4.5**	**8.6**	**19**	**5**	**20**	**5**	**10**	**- ve**
	C. neoformans	**1**	**5.6**	**7.5**	**16**	**5**	**12**	**2**	**7**	**- ve**
	A.flavas	**-ve**	**3**	**20**	**45**	**10**	**15**	**1**	**3**	**- ve**
	Fusarium	**-ve**	**2.5**	**15**	**30**	**10**	**20**	**5**	**5**	**- ve**

It is well known that the structure of cell wall of Gram-positive different from Gram- negative bacteria. In Gram-positive bacteria, there are networks with plenty of pores which allow foreign molecules to come into the cell without difficulty. On the other hand, Gram-negative bacteria the outer membrane (peptidoglycan layer) is a

potential barrier against foreign molecules with high molecular weight. The changes in peptedology can lead to change in microbial forms.

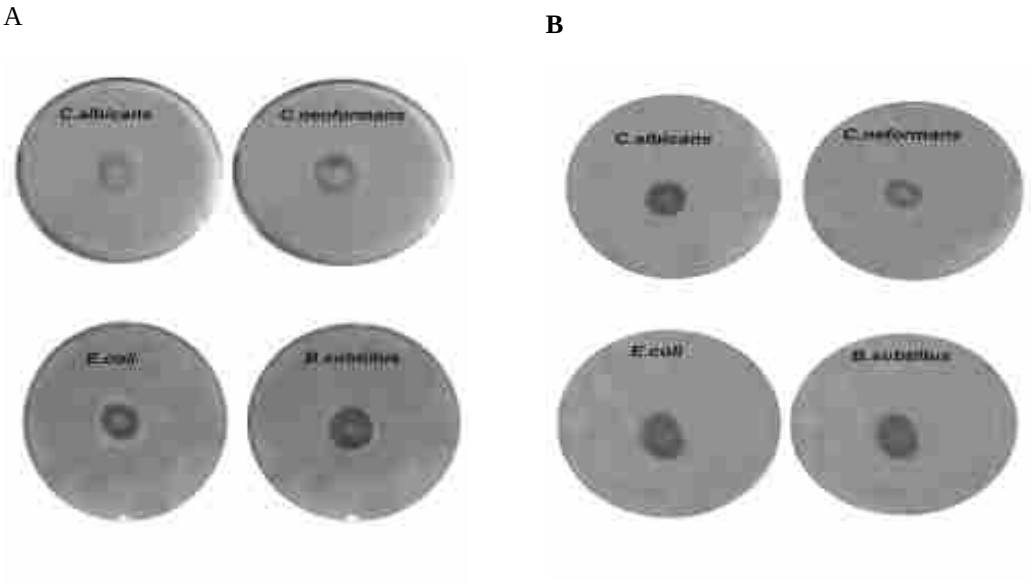

Fig.5. Inhibition zone of *(Escherichia coli* and *B. subtillus*) and fungi (*C. albicans* and *Cryptococcus neoformans* of microorganisms against A) mod-CS/MMT (2.5:1) and B) mod-CS/MMT (5:1).

3.6. Minimal inhibition concentrations (MICs).

The growth inhibiting effect was quantitatively determined by the ratio (CFU/ml) of the surviving cell number as shown in Table 2. The inhibition percentage of mod-CS/MMT was assayed with different concentrations (0, 2.5, 5, 10 and 20 mg/ml). The 20 mg/ml concentration performed zero surviving ratios on cells. At sub MICs dose (10 mg/ml) the effect of mod-CS/MMT on gram positive bacteria more than gram negative bacteria and the survival percentage of *B.subtillus* and *E.coli* were 4 % and 12 % respectively. For fungi, the survival percentage of *C. neoformans* and *C.albicans* were 36 % and 15 % respectively.

Table 2. MICs produced by Na-MMT and different ratio of mod-CS/MMT nanocomposites against different species of microorganisms.*

nanocomposite		**Microorganisms (CFU/ml)**			
Code	Conc. mg/ml	Bacteria		Fungi	
		B.subtillus	*E.coli*	*C. neoformans*	*C.albicans*
Mod-CS/MMT(1:1)	0	100	100	100	100
	2.5	25	32	71	18
	5	12	19.2	48	16
	10	4	12	36	15
	20	0	0	0	0
Mod-CS/MMT(2.5:1)	2.5	13	52	21	14.4
	5	7.4	35.2	17.5	12
	10	5	24	16	3.3
	20	0	0	0	0
Mod-CS/MMT(5:1)	2.5	12	19.2	48	16
	5	7	11	24	5
	10	0	0	0	0
	20	0	0	0	0

3.7. Effect of chitosan/MMT nanocomposite on microbial cells.

Fig. 6 Show the images of *E. coli* and *C. albicans* before and after treatment with CS/MMT and mod-CS/MMT nanocomposite respectively. It is obvious from the observation that bacteria cells were swelled and turn to irregular elongated shape. However, other microorganisms have no morphological change after treatment.

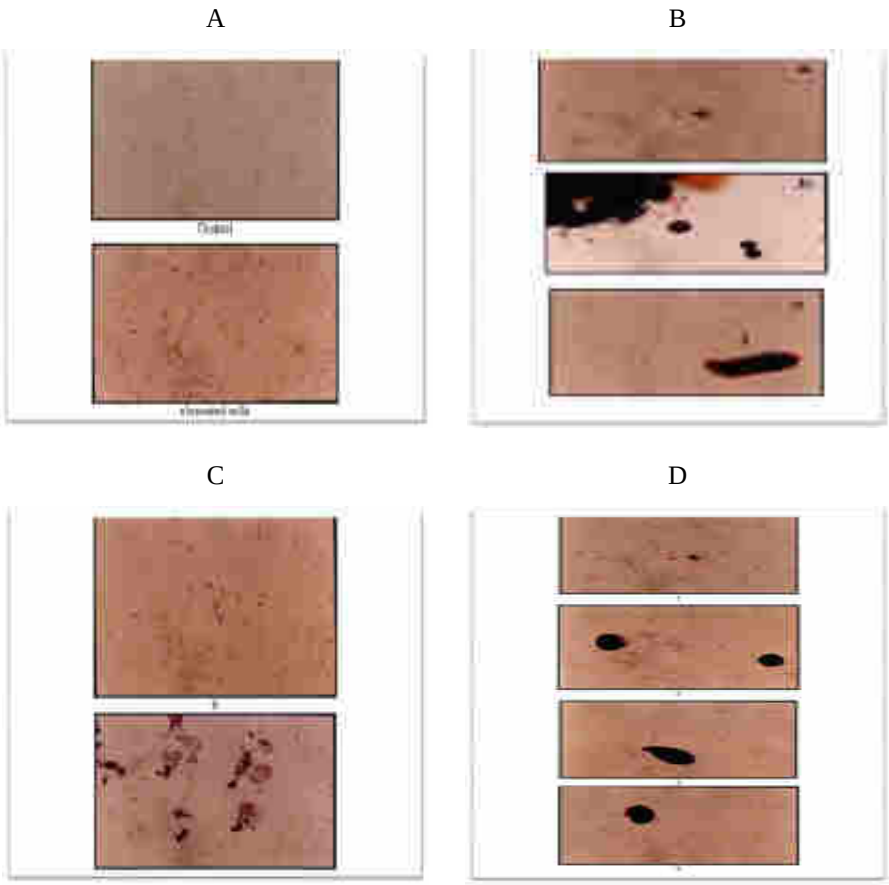

Fig 6. The morphology of (A) *E-coli* (a) before treatment; (b,c,d) After treatment (B) *B.subtillus* (C) *C.albicans* (D) *C. neoformans* after treatment with mod-CS/MMT (5:1)

It is well known that adsorption on to the negatively charged bacterial cell surfaces are expected to be enhanced with increasing charge density of the antimicrobial polymers. Therefore, adsorption onto the bacterial cell surface is much more enhanced for polymers, compared with that for model compounds (monomers) (Fan et al., 2004). The disruption of the membrane was expected to be as a result of the interaction of the bound polymers with the membrane that facilitated with increasing amounts and molecular weight of the bound polymers. It was reported that layered silicate can adhere to bacteria selectively (Xia et al., 2005). The negatively charged bacteria will not be significantly adsorbed onto these clays. However, clay mineral will be positively charged after modification by cationic polymers. In this study, MMT was modified by CS with different ratio producing nanocomposites with positive charge and various degrees of hydrophobicity. The positive charges of these nanocomposites enhance their ability to adsorb bacteria through electrostatic interactions.

Consequently, It is now generally accepted that the mechanism of action involves: Disruption of the transmembrane proton motive force leading to an uncoupling of oxidative phosphorylation and inhibition of active transport across the membrane; inhibition of respiration or catabolic anabolic reaction; disruption of replication; loss of membrane integrity resulting in leakage of essential intracellular constituents such as potassium cations and coagulation of intra cellular material.

Conclusion

The intercalation of CS and mod-CS into Na-MMT through a cationic exchange process provides nanocomposites with both functional and structural properties. Triphenyl phosphine reacted with the free amine in the polymer forming phosphonium salt nanocomposites. The techniques employed in the characterization of the mod-CSMMT nanocomposites, XRD, TEM, TGA, IR, confirm the intercalation of the mod- polymer between the clay layers through an electrostatic interaction. The antimicrobial activity against microorganisms is

observed with the highest concentration mod-CS/MMT (5:1). The antimicrobial action is related to nanostructure and the length of the polymer backbone. The nanocomposite could permeate into the cell membrane, damage the cell wall, disturb the natural processes of the cell and finally result in the fast death of microorganism.

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