The Effect of Nanoclay Type on the Mechanical Properties and Antibacterial Activity of Chitosan/PVA Nanocomposite Films

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Abstract: Nanoclays are a class of nanomaterials extensively used to prepare polymer nanocomposites. In this study, four types of common nanoclays were selected to prepare chitosan–polyvinyl alcohol (CP) nanocomposite films. Montmorillonite cloisite Na+ (MMT), organically modified montmorillonite (OMMT), and bentonite (BNT), as layered aluminosilicates, and halloysite nanotubes (HNT), as a tubular nanoclay, were blended with CP films at concentrations of 1.5, 3 and 4.5%. The nanocomposite films were characterized by FTIR, XRD, SEM/EDX, AFM, tensile strength, and antibacterial tests. SEM/EDX results showed a more uniform distribution of the OMMT and HNT nanoclays in the polymer matrix. AFM images showed a rougher surface for nanocomposite films compared to CP film. Increasing the nanoclay concentration in the films from 1.5 to 4.5% resulted in higher tensile strength for HNT and MMT while the trend was reversed for OMMT and BNT. Among the samples, nanocomposite films composed of OMMT and BNT showed the highest tensile strength at the lowest concentrations (CP-OMMT1.5 99 ± 3.7 MPa, CP-B1.5 81 ± 1.5 MPa). The nanocomposite films prepared from OMMT showed the highest antibacterial activity against E. coli and S. aureus with an inhibition zone of 15 and 19 mm, respectively. The results of this study showed that BNT and OMMT are promising nanoclays for enhancing the mechanical properties and antibacterial activity of hydrophilic polymers. The results of this research can provide new insights into selecting suitable nanoclays for different applications.

Keywords: nanocomposite; montmorillonite; bentonite; halloysite; chitosan; organically modified nanoclay

1. Introduction

Nanoclays are natural layered aluminosilicate nanomaterials used to enhance the mechanical, thermal, or electrical properties of polymers. Nanoclays can be classified as planar or tubular according to the geometry of their molecular structure. Montmorillonite (MMT), bentonite (BNT), kaolinite, hectorite, laponite, . . . can be mentioned as planar layered nanosilicates, and halloysite (HNT) is an example of a tubular nanoclay. MMT is a common type of planar nanoclay with a 2:1 structure that is widely used to prepare polymer nanocomposites [1]. Aluminosilicate layers in natural MMT are tightly bonded by Na+ ions (Na+-MMT) with a very small interlayer distance. MMT usually forms agglomerates...
due to the strong interactions between the layers. The hydrophilic nature of natural MMTs limits their dispersion in hydrophobic matrix polymers. Accordingly, MMTs are modified with different chemicals, such as quaternary ammonium salts, to increase the interlayer distance and improve their dispersion into the polymer matrix. BNT is another type of mineral nanoclay which is composed of MMT as the major phase and some other minor phases such as quartz, illite, bassinite, feldspar, halite, and crytoballite [2]. BNT has a number of valuable properties, such as high ion exchange capacity and protein binding capability [3,4]. HNT is a type of dominantly tubular aluminosilicate with a 1:1 dioctahedral structure, an inner diameter of 10–30 nm, an outer diameter of 40–70 nm, and a length of 0.2–1.5 µm [5]. HNT is widely used in medical applications such as bionanocomposites [6], drug delivery [7], and tissue engineering [8].

Chitosan (CS) is a cationic polysaccharide derived from chitin through the deacetylation reaction. The degree of deacetylation is never complete. CS is a biocompatible, biodegradable, and non-toxic biopolymer approved for internal use [9]. CS is one of the most attractive polymers due to its unique properties, such as non-toxicity, biodegradability, and antimicrobial and anti-fungal properties [10,11]. Furthermore, different forms of CS have been used for many other applications such as wound dressing [12,13], clarifying agents [14,15] and drug delivery [16,17]. However, its wide applications as a food packaging material have been limited due to inadequate mechanical and antimicrobial properties [18,19]. Poor mechanical properties and water permeability make the CS films an almost inappropriate choice, especially for moisture-sensitive packaging applications. Thus, much research in this area has focused on the reinforcement of CS films and hydrogels [20–22]. In a previous study, a CS biopolymer was used for the intercalation of MMT, and the bio-modified nanoclay was incorporated into the polymerization reaction of poly(AMPS) to prepare super-absorbent nanocomposite hydrogels. The results of this research have shown that bio-modified MMT improved the properties of hydrogels, such as gel strength, gel content, and thermal stability, without any toxicity to fibroblast cells. It has also been shown that the modification of MMT with CS has the ability to exfoliate the silicate nanolayers [23].

CS is insoluble in most organic solvents, but it dissolves in acidic solvents such as acetic acid and formic acid. The amine group (NH$_2$) present in the structure of CS transforms into a cationic group (NH$_3^+$) by receiving a hydrogen atom from the acidic medium. Although CS is a cationic polysaccharide, when dissolved in acidic solvents, because of its cationic nature, CS possesses antimicrobial activity against a wide range of microorganisms, including fungi, algae, and some bacteria [24]. These properties have led to the development of CS applications in biomedical materials and devices [25].

Polymer–clay nanocomposites have been widely studied in the literature [26,27]. In our previous work, we prepared MMT and HNT-reinforced CS/poly(vinyl alcohol) (PVA) nanocomposite nanofibers for biomedical applications [8,28]. It was shown that the MMT layers were exfoliated, the mechanical properties of the CS/PVA/MMT and CS/PVA/HNT nanofibers were highly increased, and the nanocomposites were biocompatible. In another study, CS nanocomposites were prepared with delaminated MMT particles [13]. They found that the biocompatibility of the nanocomposites was increased with incorporation of the delaminated MMT particles, which enhanced antimicrobial activity and led to a faster biodegradation rate. It was also reported that the presence of nanoclays increased the antimicrobial activity of the nanocomposites. We have also reported similar results for intelligent CS/PVA nanocomposites reinforced with BNT as intelligent food packaging. Nanoclay-reinforced polymer nanocomposites are widely applied in active food packaging applications due to their enhanced mechanical and thermal properties as well as the permeability of polymer films [29–31]. In a study, rosemary essential oil was incorporated into CS/MMT films to prepare bio-nanocomposites for active food packaging [32]. It was shown that small amounts of nanoclay in CS films improves their physical and mechanical properties significantly. Crosslinked CS/PVA films containing modified HNT nanoclay and phytic acid showed effective flame retardancy and blocked ultraviolet light as well
as improved tensile strength and thermal stability [33]. In another study, CS/PVA films were reinforced with sepiolite, a fibrous needle-shaped phyllosilicate, and loaded with the cefazolin drug. It was found that nanocomposite films released more drugs compared to CS/PVA film and showed greater antibacterial activity against Gram-positive and Gram-negative bacteria [34]. Nanoclay-reinforced nanocomposites were also studied for cell or enzyme immobilization [35–37], wound healing [38–40], tissue engineering [41–43], dye absorption [44], drug delivery [7,45–47], the controlled release of chemical and protective agents [48,49], environmental [50], and optoelectronic applications [51].

As mentioned above, CS/PVA nanocomposite films have been widely used in many applications. The type of nanoclay used has an important role in the mechanical, thermal, and biological properties of nanocomposite films. According to our knowledge, until now, a comprehensive study of the effects of planar and tubular nanoclays in CS/PVA films has not been reported in the literature. Therefore, in this study, four common types of nanoclays were selected and used for the preparation of CS/PVA nanocomposites to study the effect of nanoclay type on the mechanical properties and antibacterial activity of the films. The planar nanoclays selected include BNT, Na-MMT, and MMT which were organically modified with quaternary ammonium salt (O-MMT) and are the most commonly prepared nanocomposites. HNT was also used as a tubular nanoclay. The nanocomposite films reinforced by different amounts of nanoclays were prepared by solution intercalation. The nanocomposites films were characterized by X-ray diffraction (XRD), scanning electron microscopy/energy dispersive X-ray scattering (SEM/EDX), Atomic force microscopy (AFM), and antimicrobial activity tests.

2. Experimental Section

2.1. Materials

CS (low viscosity, degree of deacetylation ~88%) was purchased from Sigma (Livonia, MI, USA); PVA (molecular weight ~72,000 g/mol, degree of hydrolysis ~99%) and glacial acetic acid were supplied from Merck (Darmstadt, Germany). BNT was supplied from mines in eastern Iran (Khorasan Province). Unmodified MMT (cloisite Na+) was purchased from Southern clay products Inc. (Grand Prairie, TX, USA). Dimethyl dialkyl amine-modified montmorillonite (OMMT) was purchased from Grafen Chemical Industries Co., (Ankara, Turkey). HNT was purchased from NaturalNano Inc. (Rochester, NY, USA). Double-distilled water was used for preparation of solutions.

2.2. Nanocomposite Film Preparation

O-MMT nanoclay was dispersed in ethanol (96% v/v) at a concentration of 1% and stirred at 60 °C for 3 days. The other nanoclays, i.e., BNT, HNT, and Na-MMT were dispersed in double-distilled water at a similar concentration and stirred for 3 days at 60 °C. A PVA aqueous solution (10% w/v) was prepared by dissolving 10 g PVA in 90 mL of water by heating to 90 °C and vigorous stirring. After complete dissolution of the PVA flakes, the heating was stopped, and the solution was stirred overnight. The homogenized solution was sealed and kept in ambient temperature for further use. Then, 1.5 g CS was dissolved in 98.5 mL acetic acid to prepare a 1.5% w/v CS solution and stirred until a homogenized solution was achieved. The prepared CS and PVA solutions were mixed at the same volume ratio. For the sample without nanoclay (CP), CS/PVA solution was cast and allowed to dry under normal lab temperature and humidity conditions. For the nanocomposite samples, a CS/PVA solution was prepared and mixed with nanoclay dispersion prepared in advance. The formulations prepared are listed in Table 1. For each nanocomposite sample, a volume of the relevant nanoclay dispersion was added into CS/PVA solution to reach the nanocomposite compositions based on the total weight of polymers while dry (17.5 cc, 35 cc and 52.5 cc to reach nanocomposite films containing 1.5, 3 and 4.5 wt% nanoclay). The final mixtures were cast into Petri dishes and allowed to dry at room temperature for at least 48 h. After drying, the films were peeled off the Petri dishes and kept in cool dry place for further characterizations. Sample code and composition of the films are presented in Table 1.
Table 1. Codes and compositions of the prepared nanocomposite films (dry basis).

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Nanoclay Type</th>
<th>Nanoclay (wt%)</th>
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<tbody>
<tr>
<td>CP</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>CP/HNT1.5</td>
<td>HNT</td>
<td>1.5</td>
</tr>
<tr>
<td>CP/HNT3</td>
<td>HNT</td>
<td>3</td>
</tr>
<tr>
<td>CP/HNT4.5</td>
<td>HNT</td>
<td>4.5</td>
</tr>
<tr>
<td>CP/BNT1.5</td>
<td>BNT</td>
<td>1.5</td>
</tr>
<tr>
<td>CP/BNT3</td>
<td>BNT</td>
<td>3</td>
</tr>
<tr>
<td>CP/BNT4.5</td>
<td>BNT</td>
<td>4.5</td>
</tr>
<tr>
<td>CP/MMT1.5</td>
<td>MMT</td>
<td>1.5</td>
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<tr>
<td>CP/MMT3</td>
<td>MMT</td>
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<tr>
<td>CP/MMT4.5</td>
<td>MMT</td>
<td>4.5</td>
</tr>
<tr>
<td>CP/OMMT1.5</td>
<td>O-MMT</td>
<td>1.5</td>
</tr>
<tr>
<td>CP/OMMT3</td>
<td>O-MMT</td>
<td>3</td>
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<tr>
<td>CP/OMMT4.5</td>
<td>O-MMT</td>
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2.3. Characterization

2.3.1. SEM

SEM images were obtained using a VEGA TESCAN Co. (Brno, Czech Republic) working at room temperature with a voltage of 5–30 kV and a vacuum of $10^{-5}$ Pa. The images were obtained from the surface and cross-section of the films. For the cross-section images, the films were cryogenically fractured in liquid nitrogen before the test. All of the samples were sputter coated before imaging using a sputter coater device (K450X, Emitech, Chelmsford, UK) working under a vacuum of $10^{-1}$ Pa with an electric current of 5–20 mA.

2.3.2. AFM

Topography of the surface of the films was observed with a scanning probe and optical microscope (Dual scope C-26, DME 95-50-E, Semilab, DME nanotechnologies, GmbH, Lower Saxony, Germany) in AFM mode in air ambient. Surface roughness of the plain CP film and nanoclay embedded CP nanocomposite films were assessed using the dynamic tapping mode of operation. An area of $10 \mu m \times 10 \mu m$ was scanned and root mean-square ($R_{\text{RMS}}$) roughness was obtained to compare the samples. In the present study, DME-SPM software (ScanTool™, 2016) was used to analyze the results of AFM test.

2.3.3. XRD

The X-ray patterns of samples were obtained using an X-ray diffractometer (PW1800, Philips, The Netherlands) equipped with Cu-Kα radiation source. The wavelength of radiation was 0.15406 nm, the scanning range was 4–60° with a rate of 1°/min.

2.3.4. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of the pure clay nanoparticles and nanocomposite films was obtained to characterize the interactions between the matrix and filler. A piece of film was hand milled and made into powder. Film and clay powders were mixed with KBr and pressed into a translucent discs and used for the test. FTIR spectra were collected using a Bruker Tensor 27 device (Hardtstraße, Karlsruhe, Germany) with a resolution of 4 cm$^{-1}$ and 40 scans per sample.

2.3.5. Tensile Strength (TS)

TS of the films was measured according to the standard test method ASTM D882 using a CRE tensile strength measuring machine (Gotech, Zuhai, China). The samples were prepared by cutting the films into rectangular shapes of dimensions 10 mm $\times$ 50 mm and stretched with a strain rate of 10 mm/min.
2.4. Antibacterial Activity

The antibacterial activity of the nanocomposite films was evaluated by the agar disk diffusion method [52]. Three pathogenic bacteria were used as organisms for testing the samples in which two of them were Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and one Gram-positive (*Staphylococcus aureus*). Each bacterium was cultured at a concentration of $1.5 \times 10^6$ in culture plates prefilled with Muller–Hinton agar medium. The films were punched into circular shapes and sterilized by ethanol 70% for 10 min followed by UV irradiation for 30 min. The samples were placed in the culture plates and incubated for 24 h at 37 °C. The size of the inhibition zone was measured and reported for each sample.

2.5. Statistical Analysis

The data obtained from the tensile strength test were statistically analyzed for the significance of means between the samples with a confidence level of 95 using Minitab 19 software. The analysis of variance was performed followed by Tukey’s comparison and $p$-value < 0.05 was considered as significantly different.

3. Results

3.1. XRD

The nanoscale structure of the clays used in this study have been approved before. In our previous research, we have confirmed the nanostructure of HNT [7,53], MMT [28] and BNT [3]. The nanostructure of OMMT has also been approved in a previous work [54,55]. XRD results for the pure clays are shown in Figure 1A. HNT diffractions appeared at $2\theta = 11.9, 19.9, 24.5, 35$, and 38° which have been confirmed with data from the literature [56]. A small peak at around 26.5° may be due to the presence of small amounts of a quartz phase. HNT is a tubular nanoclay and the peaks observed here reveal its excellent purity. The (001) reflection appeared at $2\theta = 11.9$ which corresponds to a d-spacing of 0.74 nm. BNT showed different peaks at $2\theta = 12.5, 17.9, 25, 26.8, 30, 35$, and 45.6° due to the presence of different crystalline structures such as montmorillonite as major phase and kaolinite, quartz, illite, bassinite, feldspar, halite, and crytoballite as minor phases [3,57]. The (001) reflection peak appeared at $2\theta = 12.5$ which corresponds to a d-spacing of 0.71 nm. MMT showed peaks at $2\theta = 6.5, 17.5, 19.8, 26.6$, and 35°. The (001) reflection peak appeared at $2\theta = 6.5$ which corresponds to a d-spacing of 1.36 nm. OMMT showed diffractions at $2\theta = 5.1, 7.5, 19.9, 26.6$, and 35°. The (001) reflection peak which appeared at $2\theta = 5.1$ corresponds to a d-spacing of 1.73 nm. The diffraction of OMMT at 5.1° is lower than unmodified MMT and BNT and HNT. This is because of the modification of OMMT structure by alkyl ammonium compounds which result in higher spatial distance between the layered silicates. Accordingly, OMMT shows the highest d-spacing value among the nanoclays used in this work.

XRD results for the nanocomposite films containing 3% of each nanoclay are shown in Figure 1B. The prepared CP film showed sharp peaks at 14.1, 17, 18.7, 19.4, and 25.6° due to the crystalline structures of CS and PVA. These peaks indicate the interactions between CS and PVA in forming different crystalline regions. In our previous research, we prepared CS/PVA nanofibers using the electrospinning method [58]. It was found that the structure of nanofibers is mainly amorphous because of the fast solvent evaporation in the electrospinning process. The CP films in this research were prepared by the solvent casting method. In this process, the solution is dried within a few days in normal temperature conditions which provides enough time for polymer chains to interact and form crystalline regions.
Figure 1. Cont.
Figure 1. XRD patterns for (A) pure nanoclays and (B) CP film and its nanocomposites.

For the nanocomposite films, XRD patterns showed peaks similar to the CP films with slight changes in the diffraction angle, and the new peaks were related to presence of...
nanoclays. The intensity and position of the nanoclay peak observed in the XRD pattern of the nanocomposite films can provide valuable information about the distribution and dispersion of nanoclays in the polymer matrix. There are two distinct structures for nanoclay–polymer nanocomposites: intercalated and exfoliated [59]. Intercalation is the process of polymer chains penetrating the intergalleries distance of the clays, represented by a shift in $2\theta$ to lower values [60]. Exfoliation is the complete separation and dispersion of individual silicate layers into the polymer matrix, represented by the absence of the nanoclay related $2\theta$. Based on these criteria, the XRD patterns for the nanocomposite films were analyzed. CP-HNT3% showed peaks at $2\theta = 14.7, 17.6, 19.4,$ and $26.2^\circ$. This result shows that HNT is completely exfoliated and well-dispersed in the CP matrix, since the peak for pristine HNT at $2\theta = 11.9$ did not appear for the nanocomposite film. For CP-BNT3% several peaks were observed at $2\theta = 9.2, 14.1, 17.1, 20.2, 27.4^\circ$. The peak at 9.2 reveals that the crystalline structures of BNT were intercalated in CP matrix which is in accordance with our previous results [3]. CP-MMT3% showed diffraction patterns at $2\theta = 7.0, 14.2, 17.0, 19.5, 25.6^\circ$. The small peak at 7.0° indicates that MMT nanoclays are intercalated in the matrix. In the case of CP-OMMT, peaks at $2\theta = 4.8, 13.8, 16.7, 19.5,$ and $25.3^\circ$ were observed. The peak at 4.8° also indicates the intercalated structure for OMMT as the interlayer distance was increased compared to the pure OMMT (5.2°). This result shows that, compared to MMT, OMMT has a better dispersion in the CP matrix. This is attributed to the alkyl amine modification of OMMT and the increased interlayer distance which allows polymer chains to better diffuse into the galleries. From the results of the XRD, it can be concluded that HNT has an exfoliated and MMT, OMMT, and BNT have an intercalated structure in the CP matrix.

3.2. FTIR Results

FTIR spectra of the pure nanoclays are presented in Figure 2A. HNT is mainly composed of aluminosilicate nanolayers formed into a tubular structure with hydroxyl groups on its outer surface. The two intense peaks at 3623 and 3698 cm$^{-1}$ correspond to vibrations in the OH groups. The peak at 1655 cm$^{-1}$ is related to Si-O vibrations in amorphous SiO$_2$. Peaks at 911 and 1038 cm$^{-1}$ are related to Al-OH and Si-O group vibrations, and Al-O-Si vibrations are detected at 535 cm$^{-1}$. The FTIR spectrum observed for HNT agrees well with the literature [61]. BNT showed peaks at 3629 and 3431 cm$^{-1}$ which are also related to the vibration of the OH groups. The presence of the absorption bands at 525, 670, 1026, and 1119 cm$^{-1}$ is related to the presence of a quartz phase containing Si-O-Si bonds, and the peak at 1652 is related to amorphous Si-O stretching vibrations [62]. Similar peaks with slight changes in the wavenumbers were observed for MMT and OMMT, except for the peaks at 2925, 2847, and 1486 cm$^{-1}$ which were only observed for OMMT. These peaks are related to the vibrations in the CH$_2$ group of alkyl amine compound used for the modification of the nano-clay [63].

The FTIR spectrum for CP film showed peaks confirming the chemical structure of CS and PVA and their interactions (Figure 2B). The NH$_2$ in CS and OH in PVA showed a single broad peak at around 3361 cm$^{-1}$. As only one peak is observed for both groups, it reveals the favorable hydrogen bonding interactions between the two polymers. CS is a natural polysaccharide with a rod-like macromolecular structure in its charged state. PVA is a water-soluble polymer with a coil configuration. When CS and PVA solutions are blended, OH groups present in both chains form hydrogen bonding interactions and ensure the miscibility of both polymers, at least at the micro-scale. This is also approved by the smooth surface of the films observed by SEM (Figure 3A). The peak at 2940 cm$^{-1}$ is related to alkyl CH stretching vibrations and the peak at 1733 is due to the carbonyl groups in the remaining unhydrolyzed acetate groups of PVA and acetyl groups in CS. Other peaks relevant to the structure of CS and PVA were also observed.
Figure 2. FTIR spectra of (A) HNT, BNT, MMT, and OMMT pure nanoclays, CP films (B), and their nanocomposite films with HNT (B), BNT (C), MMT (D), and OMMT (E).
The FTIR spectrum for CP film showed peaks confirming the chemical structure of CS and PVA and their interactions (Figure 2B). The NH2 in CS and OH in PVA showed a single broad peak at around 3361 cm\(^{-1}\). As only one peak is observed for both groups, it reveals the favorable hydrogen bonding interactions between the two polymers. CS is a natural polysaccharide with a rod-like macromolecular structure in its charged state. PVA is a water-soluble polymer with a coil configuration. When CS and PVA solutions are blended, OH groups present in both chains form hydrogen bonding interactions and ensure the miscibility of both polymers, at least at the micro-scale. This is also approved by the smooth surface of the films observed by SEM (Figure 3A). The peak at 2940 cm\(^{-1}\) is related to alkyl CH stretching vibrations and the peak at 1733 is due to the carbonyl groups in the remaining unhydrolyzed acetate groups of PVA and acetyl groups in CS. Other peaks relevant to the structure of CS and PVA were also observed.

Figure 3. SEM images of the nanocomposite films: (A) CP, (B) CP-HNT3%, (C) CP-BNT3%, (D) CP-MMT3%, and (E) CP-OMMT3%.

After the addition of HNT into the films, some changes were observed in the spectrum of the nanocomposite films compared to the CP film. The strong peak at 1655 cm\(^{-1}\) is absent in the CP film and confirms the presence of HNT in the nanocomposite films, which was further investigated by EDX (Figure 4A). The peak at 3350–3360 cm\(^{-1}\) appeared to be broader compared to the CP film. This result is due to the presence of OH groups on the surface of HNT and its hydrogen bonding interactions with the OH and NH\(_2\) groups of PVA and CS, respectively. By increasing the HNT content in the nanocomposite films, the shape and intensity of the peaks in the spectrum did not change significantly.
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Figure 4. SEM/EDX mapping images for Si atom in the cross section of the nanocomposite films: (A) CP-HNT3%, (B) CP-BNT3%, (C) CP-MMT3%, and (D) CP-OMMT3%.
In the case of BNT (Figure 2C), a peak at around 1666 cm\(^{-1}\) is observed, which is related to Si-O vibrations and proves the presence of BNT in the nanocomposite films. The immense interaction of BNT with CS and PVA chains resulted in a broad peak in the range 3300–3500 cm\(^{-1}\). This result may be due to the high cation exchange capacity of BNT. Similar results were observed for MMT (Figure 2D); however, the peak for CP-MMT 1.5% and CP-MMT 3% is sharper compared to CP-MMT 4.5%. Also, for OMMT nanocomposites (Figure 2E), the shape of the peak in this range is broad. This result confirms strong interactions between the CS and PVA chains and OMMT. As OMMT is a modified clay, the distance between the layers is higher and polymer chains can diffuse between the layers more easily and result in higher interaction.

3.3. SEM/EDX

In order to observe the dispersion of nanoclays in the CP matrix, SEM images were obtained from the surface and cross section of the films. The CP film shows a smooth surface without any aggregation or phase separation which will also be demonstrated in AFM images in the next sections (Figure 5A). The smooth surface indicates that CS and PVA exist in a single phase and the hydroxyl groups of PVA have formed hydrogen bonds with the hydroxyl and amine groups of CS. CS is a polysaccharide and has a rod-like chain structure while PVA has a random coil chain. Thus, the two polymers are not physically compatible; however, they can be mixed without phase separation due to the formation of hydrogen bonding interactions. For CP-HNT3% (Figure 3B), the surface is also smooth and, in accordance with XRD data, exhibits the well dispersed, exfoliated structure of HNT in CP matrix. For CP-BNT% (Figure 3C), the aggregates of BNT are observed in the surface of the nanocomposite film. This result is in accordance with the XRD data which showed that intercalated crystalline clay structures are present in the matrix. In the case of CP-MMT3% (Figure 3D), some aggregates of clay nanolayers are observed while CP-OMMMT3% (Figure 3E) has a more uniform dispersion which is also in agreement with XRD graphs. As mentioned in the XRD results, OMMT is a type of modified nanoclay with a higher spatial distance between the aluminosilicate layers. Thus, it shows a more uniform dispersion, as confirmed by the XRD and SEM data in this study.

In order to map the dispersion of aluminosilicate layers in the CP matrix, EDX was used to detect Si atoms in the images obtained from the cross-section of the nanocomposite films (Figure 4A–D). Si atoms were selected because of their abundance in nanoclays compared to other elements such as Al, Na, Ca, etc. Si atoms were detected in all of the nanocomposite films which confirms the presence of nanoclays in these samples. The maps obtained from the distribution of Si atoms in the structure of the films are in accordance with the above explanation about SEM images. Some aggregation in the clay nanoparticles can be observed from EDX mapping (Figure 4B,C) which further confirms the conclusion obtained from SEM and XRD data.
Figure 5. AFM images, 2D (left) and 3D (right), of the surface of the films: (A) CP, (B) CP-HNT3%, (C) CP-BNT3%, (D) CP-MMT3%, and (E) CP-OMMT3%.
3.4. AFM

AFM images of the surface of nanocomposite films are presented in their 2D and 3D forms to obtain more details about surface characteristics of CP and its nanocomposite films. According to the root mean square (RMS) values mentioned on the graph (Figure 5), the CP film was shown to have a smooth surface (RMS = 43.3 nm, Figure 5A) while the nanocomposite films showed a clear increase in roughness with an increased peak-to-valley height distance (Figure 5B–E). The RMS values for HNT and OMMT were 63.4 nm and 83.4 nm, respectively which agree well with the SEM/EDX and XRD results which confirmed their better dispersion in the matrix. For BNT and MMT, the RMS values were found to be 150 and 230 nm, respectively. According to the intercalated structure of these two clays in the CP matrix, a rougher surface with higher RMS values is expected due to the formation of aggregates. In addition, nanoclays have a high surface to volume ratio. When they are dispersed in the polymeric matrices, the interactions between CP chains and the nanolayers increase the surface area of the polymer chains at the surface. Thus, the addition of nanoclays results in increased surface roughness of polymer nanocomposites which is also confirmed in previous research [64].

3.5. Tensile Strength

The tensile strength of the samples was measured to compare the effects of nano-clay type and content on the mechanical properties of the films (Figure 6). The CP film showed a tensile strength of 38 MPa in accordance with the previous reports [3]. The addition of HNT up to 3% did not change the tensile strength of the CP films significantly, while 4.5% of HNT increased the tensile strength to 48 MPa ($p$-value < 0.01). Thus, increasing the HNT concentration from 3 to 4.5% results in a higher tensile strength in CP-HNT nanocomposite films. Inversely, increasing the concentration of BNT resulted in a lower tensile strength. In this case, among the nanocomposites with 1.5, 3, and 4.5%, the highest tensile strength was obtained by 1.5% BNT and higher contents reduced the tensile strength. For CP-MMT nanocomposites, the tensile strength was significantly increased by increasing the MMT content from 1.5 to 3% but the difference between 3 and 4.5% was not significant. Interestingly, in CP-OMMMT nanocomposites, the addition of 1.5% OMMMT resulted in the highest tensile strength among all of the nanocomposite films (2.6 times higher than the CP film). However, for OMMMT nanocomposites, the changes in tensile strength with the addition of the nanofiller were reversed, and the tensile strength was reduced by the addition of OMMMT from 3 to 4.5%.

To better compare and understand the effect of the nano-clay type and content on the tensile strength of the films, the highest tensile strength for each nano-clay type and its relevant content is reported in Table 2. OMMMT and BNT showed the highest tensile strength at a low content (1.5%) while MMT and HNT showed the highest tensile strength at higher content (4.5%). Among the samples, the highest tensile strength was observed for CP-OMMMT 1.5% (99 MPa). This result is in accordance with the XRD, SEM/EDX, and AFM data which showed the uniform distribution of OMMMT in CP matrix.

Table 2. The relevant nanoclay type and content at the highest tensile strength of the nanocomposite films.

<table>
<thead>
<tr>
<th>Nano-Clay Type</th>
<th>HNT</th>
<th>BNT</th>
<th>MMT</th>
<th>OMMMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanoclay content with the highest tensile strength (%)</td>
<td>4.5</td>
<td>1.5</td>
<td>4.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Highest tensile strength (MPa)</td>
<td>$48 \pm 2.5$</td>
<td>$81 \pm 7.9$</td>
<td>$71 \pm 6.6$</td>
<td>$99 \pm 3.7$</td>
</tr>
</tbody>
</table>
Figure 6. Tensile strength of the films at room temperature at the extension rate of 10 mm/min (p-value < 0.01, groups that do not share a letter are significantly different).

3.6. Antibacterial Analysis

In order to evaluate the antibacterial activity of the prepared samples, three types of bacteria were used. The results of antibacterial tests for the prepared nanocomposites with different compositions of nanoclay are summarized in Table 3. For the control sample CP, an inhibition zone of 7 mm was observed which indicates the low antibacterial activity of this sample. PVA does not show an antibacterial effect in its pure form. CS shows an antibacterial effect mainly in acidic media. When antibiotics are loaded onto CP films, large areas of inhibition can be observed, but as there was no antibacterial drug loaded onto our films, the diameter of the inhibition zone is not large for the CP film. Due to the high molecular weight of CS, its degradation and diffusion in the bacterial culture is low and results in a small halo diameter for the CP film. The type of bacterium also did not affect this result. Larger halo diameters were observed for the nanocomposite samples, at least in one concentration from each type of nanoclay. For CP-MMT films, CP-MMT 1.5 and CP-MMT 4.5 showed larger halo diameters against *E. coli* and *S. aureus* while there was no difference between the films against *P. Aeruginosa*. OMMT showed the largest inhibition zones among the samples. The inhibition zone was increased up to 15 mm against *E. coli* and up to 19 mm against *S. aureus*. In the case of HNT, only CP-HNT4.5 nanocomposite film showed a larger inhibition zone, up to 12 mm, against *E. coli*, and CP-HNT3 showed a halo of 10 mm against *S. aureus*. Nanocomposite films containing BNT also showed great effects against the bacteria tested. CP-BNT1.5 showed a halo diameter of 14 mm against *E. coli*; however, the diameter of the inhibition zone was decreased from 14 mm to 7 mm by increasing the BNT content from 1.5 to 4.5%, respectively. Similar result was observed for *P. aeruginosa* and the halo diameter decreased from 12 to 7 mm. In the case of *S. aureus*, only CP-BNT3 showed the highest inhibition zone of 11 mm, and the halo diameter was unchanged compared to control film.

The relevant nanoclay types and content with the highest antibacterial activity against each bacterium are summarized in Table 4. Our results showed that OMMT has the highest antibacterial activity against *E. coli* and *S. aureus* but its activity against *P. aeruginosa* was not high. BNT showed the highest antibacterial activity against *P. aeruginosa*. It is interesting to
point out that OMMT showed its effectiveness at a 3% concentration but BNT showed the highest halo at 1.5%. The antibacterial activity of OMMT can be related to the quaternary ammonium salts which are used as modifier for increasing the interlayer distance of the aluminosilicate layers.

Table 3. The results of the antibacterial analysis of the films (halo diameter of the inhibition zone).

<table>
<thead>
<tr>
<th>Sample</th>
<th>E. coli</th>
<th>P. aeruginosa</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (CP)</td>
<td>7 mm</td>
<td>7 mm</td>
<td>7 mm</td>
</tr>
<tr>
<td>CP-MMT1.5</td>
<td>7 mm</td>
<td>7 mm</td>
<td>10 mm</td>
</tr>
<tr>
<td>CP-MMT3</td>
<td>7 mm</td>
<td>6 mm</td>
<td>8 mm</td>
</tr>
<tr>
<td>CP-MMT4.5</td>
<td>11 mm</td>
<td>7 mm</td>
<td>7 mm</td>
</tr>
<tr>
<td>CP-OMMT1.5</td>
<td>12 mm</td>
<td>7 mm</td>
<td>11 mm</td>
</tr>
<tr>
<td>CP-OMMT3</td>
<td>15 mm</td>
<td>7 mm</td>
<td>19 mm</td>
</tr>
<tr>
<td>CP-OMMT4.5</td>
<td>12 mm</td>
<td>8 mm</td>
<td>8 mm</td>
</tr>
<tr>
<td>CP-HNT1.5</td>
<td>7 mm</td>
<td>8 mm</td>
<td>9 mm</td>
</tr>
<tr>
<td>CP-HNT3</td>
<td>8 mm</td>
<td>7 mm</td>
<td>10 mm</td>
</tr>
<tr>
<td>CP-HNT4.5</td>
<td>12 mm</td>
<td>8 mm</td>
<td>7 mm</td>
</tr>
<tr>
<td>CP-BNT1.5</td>
<td>14 mm</td>
<td>12 mm</td>
<td>7 mm</td>
</tr>
<tr>
<td>CP-BNT3</td>
<td>12 mm</td>
<td>11 mm</td>
<td>11 mm</td>
</tr>
<tr>
<td>CP-BNT4.5</td>
<td>7 mm</td>
<td>7 mm</td>
<td>7 mm</td>
</tr>
</tbody>
</table>

Table 4. The relevant nanoclay type and content with the highest antibacterial activity in the nanocomposite films.

<table>
<thead>
<tr>
<th>Nano-clay type with the highest antibacterial activity</th>
<th>E. coli</th>
<th>P. aeruginosa</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nano-clay content (%)</td>
<td>OMMT</td>
<td>BNT</td>
<td>OMMT</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.5</td>
<td>3</td>
</tr>
</tbody>
</table>

4. Discussion

Considering the results of the XRD, SEM, and AFM tests, it can be seen that the results are clearly in accordance with each other. From the results of tensile strength, it was found that, by increasing the amount of nanoclays in CP matrix, for BNT and OMMT, the tensile strength shows a decreasing trend, while for HNT and MMT the trend is reversed. This is an interesting result which can be related to the size, shape, dispersion, and interaction of nanoclays within the CP matrix.

According to SEM/EDX and XRD data, BNT showed some agglomerates in the matrix. Increasing the concentration of BNT in the matrix results in higher agglomeration and induces stress concentration in the matrix which lowers the tensile strength. However, BNT has the highest cation exchange capacity among the nanoclays used in this study. This high capacity of BNT results in a higher surface area and more filler–matrix interactions. At lower concentrations of BNT, CS and PVA chains can better diffuse between the aluminosilicate layers of BNT which results in a higher tensile strength. In case of HNT, the tubular structure results in a CP matrix with a lower surface area. The inclusion of polymer chains in the HNT structure is lower compared to BNT because of its lower ion exchange capacity. This results in HNT having a lesser effect on the tensile strength of CP-HNT nanocomposites. For MMT, the interaction of the nanoclay layers with the hydrophilic CP matrix is high which results in better dispersion and higher mechanical properties. For OMMT, the structure of nano-clay is modified with organic compounds. At low concentrations, due to higher interlayer distance, aluminosilicate nanolayers are dispersed well in the CP matrix. Due to a higher interlayer distance and better dispersion, OMMT showed the highest tensile strength among the samples at the concentration of 1.5%. However, increasing the OMMT concentration results in higher agglomeration and reduces the interaction with the CP
matrix. Similar results have been reported for CS/OMMT films containing glycerol as plasticizer. The addition of 5% OMMT resulted in the highest tensile strength compared to 10 and 15% OMM [54]. Nanoclays are composed of rigid silicate layers. If the layers are well dispersed in polymer matrix, they can drastically enhance the mechanical properties while improper dispersion results in agglomeration of the particles. Agglomerated particles act as stress concentration points and lower the mechanical properties.

Antibacterial activity of nanoclays loaded with metal ions such as Cu and Co, antibiotic drugs or bioactive molecules has been widely studied in the literature [65–67]. However, the effect of different nanoclays on the antibacterial activity of CS/PVA films has rarely been studied before. Considering the results of the antibacterial tests, it was found that, among the nano-clay types used in this study, OMMT and BNT provided the highest antibacterial activity. In the case of OMMT, the antibacterial effect can be related to the organic modifier which is a type of quaternary ammonium salt. Similar effects have been reported in past research [68,69]. Bacterial cell membranes are mainly composed of a peptidoglycan layer which protects the inner components of the cell such as cytoplasm, DNA, ribosomes, etc. Pure MMT does not show antibacterial effect but its strong adsorption to the surface of the cell membrane can result in the immobilization of the bacteria. In our study, MMT did not show strong antibacterial effects while OMMT produced large inhibition zones. MMT can only adsorb to the cell membrane, but it is not able to diffuse inside the membrane and disrupt it. In the case of CP-MMT films, presence of MMT can result in a higher degradation rate of CS. The release and diffusion of CS oligosaccharides in the culture medium can result in larger inhibition zones due to the electrostatic interactions of the positive charge of CS with the negative charge of the bacterial cell membrane. In the case of OMMT, when MMT is adsorbed onto the surface of the membrane, the quaternary ammonium salt can diffuse inside and result in disruption of the normal structure of the peptidoglycan layer by making complex between the slat’s positive charge and the negative charge of the peptidoglycan layer. However, the type of bacterium can also affect the antibacterial results due to the differences in the membrane structure of Gram-positive and Gram-negative bacteria. Gram-negative bacteria have a thin peptidoglycan layer in their cell wall and a lipopolysaccharide membrane and release different toxins and are more resistant to antibiotics. Gram-positive bacteria have a thicker peptidoglycan layer but they lack lipopolysaccharide outer membrane. These differences in the biological structure of the cells result in different antibacterial results. In our study, S. aureus is a Gram-positive and P. aeruginosa and E. coli are Gram-negative bacteria. In most of the samples, the inhibition zone was larger for E.coli compared to S. aureus; however, P. aeruginosa showed smaller inhibition zones. This result may be due to several reasons: P. aeruginosa is intrinsically resistant to many antibiotics, including aminoglycosides, tetracyclines, and macrolides. It can acquire additional resistance mechanisms through horizontal gene transfer, making it resistant to other antibiotics as well. P. aeruginosa can form biofilms which provide protection against antibiotics [70]. It has multiple efflux pumps that actively transport antibiotics out of the cell, reducing their intracellular concentration and making it more difficult for antibiotics to kill the bacteria [71].

5. Conclusions

The results of this work show how the type of nano-clay can affect the morphology and mechanical and antibacterial properties of CS-PVA nanocomposites. The FTIR and XRD results showed the interaction between the nanoclay and the CS/PVA chains due to hydrogen bonding interactions. HNT reached an exfoliated structure in the CP matrix while BNT, MMT, and OMMMT gained an intercalated structure in the nanocomposite films. OMMMT showed a higher d-spacing value before and after dispersion into the CP matrix compared to MMT, which was attributed to the quaternary ammonium modification. The Addition of HNT as a tubular nanoclay into CS-PVA films results in the lowest mechanical properties compared to layered nanosilicates such as BNT, MMT, and OMMMT. Among the layered nanosilicates, OMMMT was shown to have the highest mechanical properties at a
low concentration of 1.5%. Also, OMMT, as well as BNT, was shown to have the highest antibacterial activity against Gram-positive and Gram-negative bacteria. The results of this research can provide valuable insights for preparing CS/PVA/nanoclay nanocomposites with tunable mechanical properties and antibacterial activity for different applications such as food packaging, wound dressings, cosmetics, etc.

**Author Contributions:** Conceptualization, M.K.; methodology, H.F. and M.S.; software, M.K., H.F. and Y.G.; validation, T.L. and N.N.; formal analysis, H.F., M.K., Y.G.; investigation, H.F., M.S.; resources, N.N., M.A. and M.K.; data curation, M.K. and H.F.; writing—original draft preparation, H.F. and M.K.; writing—review and editing, N.N., and M.A.; supervision, N.N., M.K. and M.A.; funding acquisition, Y.G. and T.L. All authors have read and agreed to the published version of the manuscript.

**Funding:** The authors express their appreciation to the Jinan High-End Foreign Experts Recruitment Program for providing support for this research. There was no external funding provided for paying the article processing charge (APC) of this manuscript. The APC was completely paid by the author’s vouchers.

**Data Availability Statement:** The original contributions presented in this study are all included in the article in form of figures and tables, further inquiries can be directed to the corresponding author(s).

**Conflicts of Interest:** Mojtaba Koosha, Tianduo Li and Yinghua Gong were employed by the company Yi Mu E-Zhong Biotechnology (Shandong) Co., Ltd., Jinan, Shandong, China. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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