



New Chitosan/Ag/Carbacylamidophosphate nanocomposites: Preparation and antibacterial study

Nasrin Oroujzadeh

Department of Chemical Technologies, Iranian Research Organization for Science and Technology (IROST), Tehran, Iran

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ABSTRACT

Two new Chitosan-based nanocomposite films were prepared: Chitosan /7% Ag nanoparticles (NPs) (NC1) and Chitosan/7% Ag NPs/5%Carbacylamidophosphate(NC2), in which the carbacylamidophosphate derivative is N-Nicotinyl-N',N"- bis(hexamethylenyl) phosphorictriamide (NHE) with the formula: $C_5H_4NC(O)NHP(O)(NC_6H_{12})_2$. X-ray Powder Diffraction (XRD), Field Emission Scanning Electron Microscopy (FE-SEM) and Energy Dispersive X-ray Spectroscopy (EDS) methods were used to characterize and confirm the prepared frameworks. XRD graph of the two nanocomposites showed all the characteristic peaks of NHE, Ag NPs, and chitosan, indicating the fact that the preparing process has not made any changes in the phases of the nanocomposites components. All the SEM micrographs and EDS analysis results also confirmed the desired structures. To study the effect of the additive NHE on the antibacterial activity of the films, *in vitro* antibacterial tests were done on the prepared nanocomposites against two Gram-positive (*Staphylococcus aureus*, *Bacillus cereus*) and two Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) in Brain-Heart Infusion(BHI) medium. Results showed that the antibacterial effects of the nanocomposite containing NHE on each of the four bacteria is stronger than those for the nanocomposite without NHE.

1. Introduction

Our environment is contaminated with various microorganisms, many of which are harmful to human health. Microbiological contamination of water has long been a concern to the public. Water-borne pathogen contamination in water resources and related diseases are a major water quality concern throughout the world. From the 1920's-1960's, the bacillus which causes typhoid fever was considered a major problem in the water supply [1]. There are also dangerous soil-sourced microorganisms that, beside water- born harmful microbes, can cause many diseases through the food. *Staphylococcus aureus* (*S. aureus*), *Bacillus cereus* (*B. cereus*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli* (*E. coli*) are examples of common bacterial food pathogens. *Staphylococcus aureus* is a gram-positive, round-shaped bacterium that is one of the most common causes of

bacteremia and infective endocarditis. Additionally, it can cause various skin and soft tissue infections [2,3]. *Escherichia coli* is a gram-negative, facultatively anaerobic, rod-shaped, coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms (endotherms) [4]. *Escherichia coli* O157:H7 enterohemorrhagic (EHEC) can cause hemolytic-uremic syndrome [5]. *Bacillus cereus* is a Gram-positive bacterium commonly found in soil and food. It is the cause of "fried rice syndrome". Some *Bacillus cereus* strains are very harmful to humans and cause foodborne illness [6-8]. *Pseudomonas aeruginosa* is a common Gram-negative, rod-shaped bacterium that can cause disease in plants and animals, including humans. An opportunistic, nosocomial pathogen of immunocompromised individuals, *P. aeruginosa* typically infects the airway, urinary tract, burns, and wounds, and also causes other blood infections [9]. Increasing interest in controlling environmental pathogens

*Corresponding author. Telephone: +989123337857

E-mail address: n_roujzadeh@irost.ir

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evidenced by a large number of recent publications [10] clearly corroborates to the need for studies that synthesize knowledge from multiple fields covering comparative aspects of pathogen contamination. Currently, there has been growing interest in antibacterial material research area. Among polymer-based potential antimicrobial agents Chitosan-based materials, especially those contain silver in their structure, have shown excellent antibacterial properties. The antibacterial behavior of various nanocomposites based on Chitosan/Ag NPs have been studied by many researchers [11–14]. Chitosan, because of its biodegradable, biocompatible and nontoxic properties has been applied in several biomedical areas such as wound healing [15], tissue engineering [16,17], drug delivery and gene delivery [18–20]. In the last few years, carbacylamidophosphates, as an important biologically active class of organophosphorus compounds, has become very attractive [21–30]. Due to the unique physicochemical properties and existence of a peptide like group -C (O) NHP (O) - in their skeleton, these materials have valuable biological activities and critical roles in catalytic and metabolism processes [31–34]. Some carbacylamidophosphates have potential applications as antimicrobial agents [35], anti-tumor drugs [36] and also inhibitors for cholinesterase and butyrylcholinesterase enzymes [37,38]. Here, two new nanocomposite films with the composition: Chitosan/7%Ag NPs (NC1) and Chitosan/7%Ag/5%Carbacylamidophosphate (NC2), in which the carbacylamidophosphate derivative is N-Nicotinyl-N', N''-bis(hexamethylenyl) phosphoric triamide(NHE), were prepared and characterized by X-ray Powder Diffraction (XRD), Field Emission Scanning Electron Microscopy (FE-SEM) and Energy Dispersive X-ray Spectroscopy(EDS) analysis methods. In vitro antibacterial activities of these nanocomposites were also evaluated against two Gram-positive bacteria: *S. aureus*, *B. cereus* and two Gram-negative bacteria: *E. coli*, *P. aeruginosa* in Brain-Heart Infusion (BHI) medium.

2. Materials and methods

2.1. Materials

Silver nitrate, Sodium citrate, PCl_5 , Hexamethyleneimine, CCl_4 , Acetonitrile, Ethanol, Acetic acid, Nicotinamide and distilled water obtained from Merck Co. High molecular weight Chitosan was purchased from Loba Chemie Pvt. Ltd. and Glutar aldehyde (25 wt% in water) obtained from Daejong Co. All compounds were used without any more purification.

2.2. Synthesis

2.2.1. N-Nicotinyl-N', N''-bis (hexamethylenyl) phosphoric triamide(NHE)

This compound was synthesized according to our previously reported method [39]. Briefly, to a mixture of N-

Nicotinyl phosphoramidic dichloride (1 mmol), Hexamethyleneimine (4 mmol) and acetonitrile was added at 0 °C. After 8 h, the precipitate was filtered and washed with distilled water and dried at 25 °C to obtain the final product.

2.2.2. Synthesis of Ag nanoparticles

The Ag nanoparticles were synthesized by citrate reduction method [40]. Briefly, an aqueous solution 2 mmol of silver nitrate was added to 1 mmol aqueous sodium citrate solution, under atmospheric conditions. In order to avoid light-induced reduction of Ag, the reaction flask was heated in a dark environment, to about 140 °C for 5 h. Then, it was cooled to the room temperature and the Ag nanoparticles were filtered and dried after washing with distilled water, for several times.

2.2.3. Preparation of Chitosan/ Ag NPs/ NHE nanocomposite films

For preparing the nanocomposite, at first, 0.5 g of chitosan powder was dispersed in 5 ml of distilled water and 3 ml acetic acid was added to the mixture. Next, the mixture was placed in an ultrasonic bath for 2 h to yield a homogenous brown viscose gel. Then, the carbacylamidophosphate (NHE) powder (0 and 5% w/w of chitosan, for the films NC1 and NC2 respectively) was dissolved in ethanol, added to the chitosan and placed into the ultrasonic bath for 30 min. After that, Ag NPs (7% w/w of chitosan) was added to the chitosan/ NHE flask and placed in the ultrasonic bath for 30 min. The homogenous mixtures were then poured into proper plates and were allowed to be dried. Finally, the prepared films were put in a desiccator containing glutar aldehyde steam with the pressure of 0.5 atm for 24 hours, for cross linking of chitosan and obtaining the desired nanocomposite films.

2.3. Instrumentations

The phase analysis of fabricated framework was done by an X-ray Diffraction (XRD) diffractometer, model: Equinox 3000, Intel Co. The micrographs and elemental analysis were obtained from Field Emission Scanning Electron Microscopy (FE-SEM) model: Mira II, Tescan Co. and Energy Dispersive Spectroscopy (EDS) model: Mira II, Tescan Co. instruments. Ultrasonic bath, model: S 4000, Misonix Co. with output power: 600W was used in the preparation process.

2.4. In vitro antibacterial test

The in vitro antibacterial activities of the films were evaluated by the filter paper disk method [41], with the disk diameter of 6.5 mm. The bacteria were cultured in BHI medium. About 0.005 g of each film was used in each test. The thickness of the BHI medium was kept equal in all Petri dishes. Four bacteria including two Gram-positive (*S. aureus*, *B. cereus*) and two Gram-negative bacteria

(*E. coli*, *P. aeruginosa*) were examined. The disks were incubated at 37 °C for 24 h. The inhibition zone of growth, which specifies the amount of inhibitory effect of the compounds on the growth of the bacteria, was measured and the average of three diameters was calculated for each sample (Table1).

3. Results and discussion

3.1. X-Ray Diffraction Analysis

The X-Ray Diffraction method was used to investigate the crystallinity and the phases of the material. XRD pattern of Ag NPs is given in Figure 1. Comparing XRD patterns of NHE, Chitosan and the two fabricated nanocomposite films, NC1 and NC2, are also shown in Figure 2. As it can be seen in the figures, owing to their high crystallinity, Ag NPs and carbacylamidophosphate (NHE) represent sharp peaks; but the chitosan film shows a broad pattern, which refers to its amorphous polymeric structure. XRD of Ag nanoparticles (Figure 1) shows a strong diffraction pattern,

relating to the hkl values of (1 1 1), (2 0 0), (2 2 0), (3 1 1) and (2 2 2) planes, that matches with the standard JCPDS no. [00-087-0719]. As NHE is a newly synthesized compound, no standard card can be found for its XRD pattern. This phosphoric triamide shows 12 peaks at $2\theta \approx 9, 10, 13.5, 15.5, 16.5, 18, 18.5, 20.5, 22, 23, 25$ and 35 . The very intense and sharp peak at $2\theta \approx 10$ and the smaller one at $2\theta \approx 25$ could be considered as its characteristic peaks in the nanocomposite XRD pattern. The XRD graph of chitosan, include all the characteristics peak of chitosan, reported in literature [42,43]. In XRD graph of the nanocomposites, NC1 and NC2, (Figure 2), the characteristic peaks of NHE, Ag NPs, and chitosan are observed which confirms that the mixing process has not made any changes in the phases of the nanocomposites components. The intensity of some of components peaks have been changed, which is due to the using of ultrasonic waves in the mixing process.

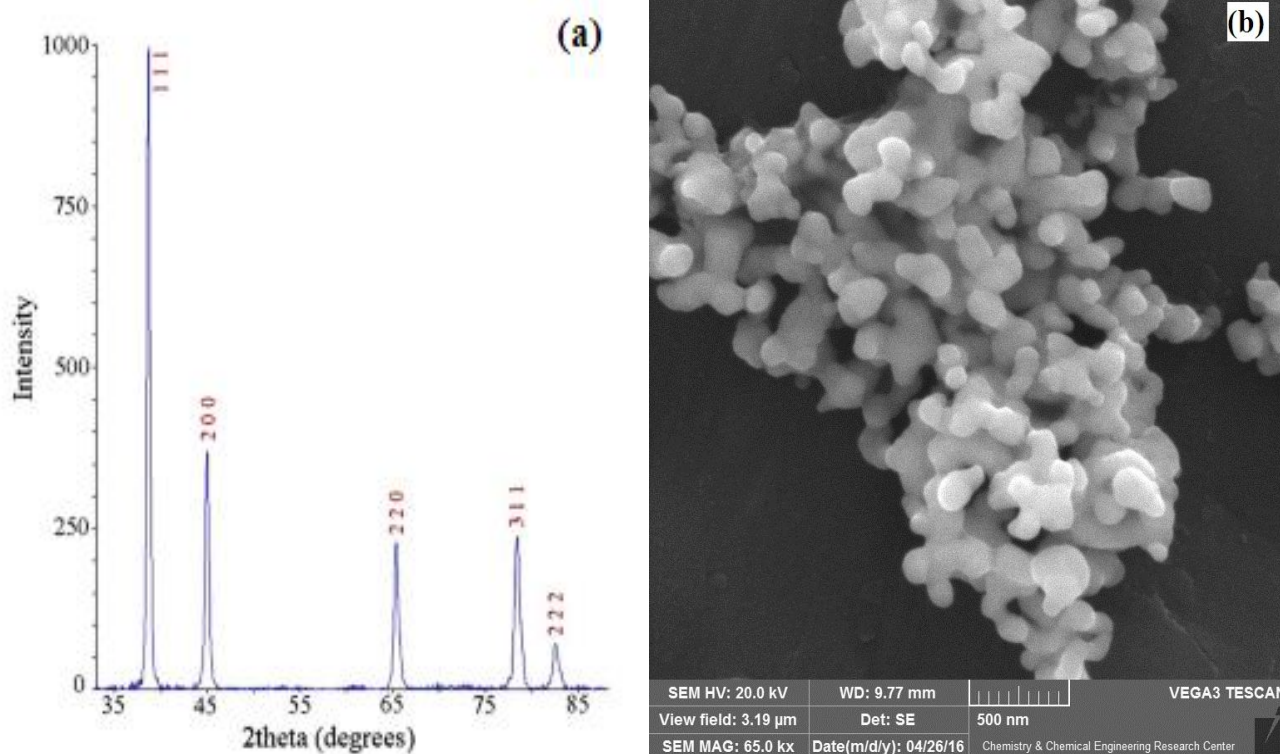


Fig. 1. The XRD pattern (a) and the FE-SEM (b) micrograph of Ag nanoparticles

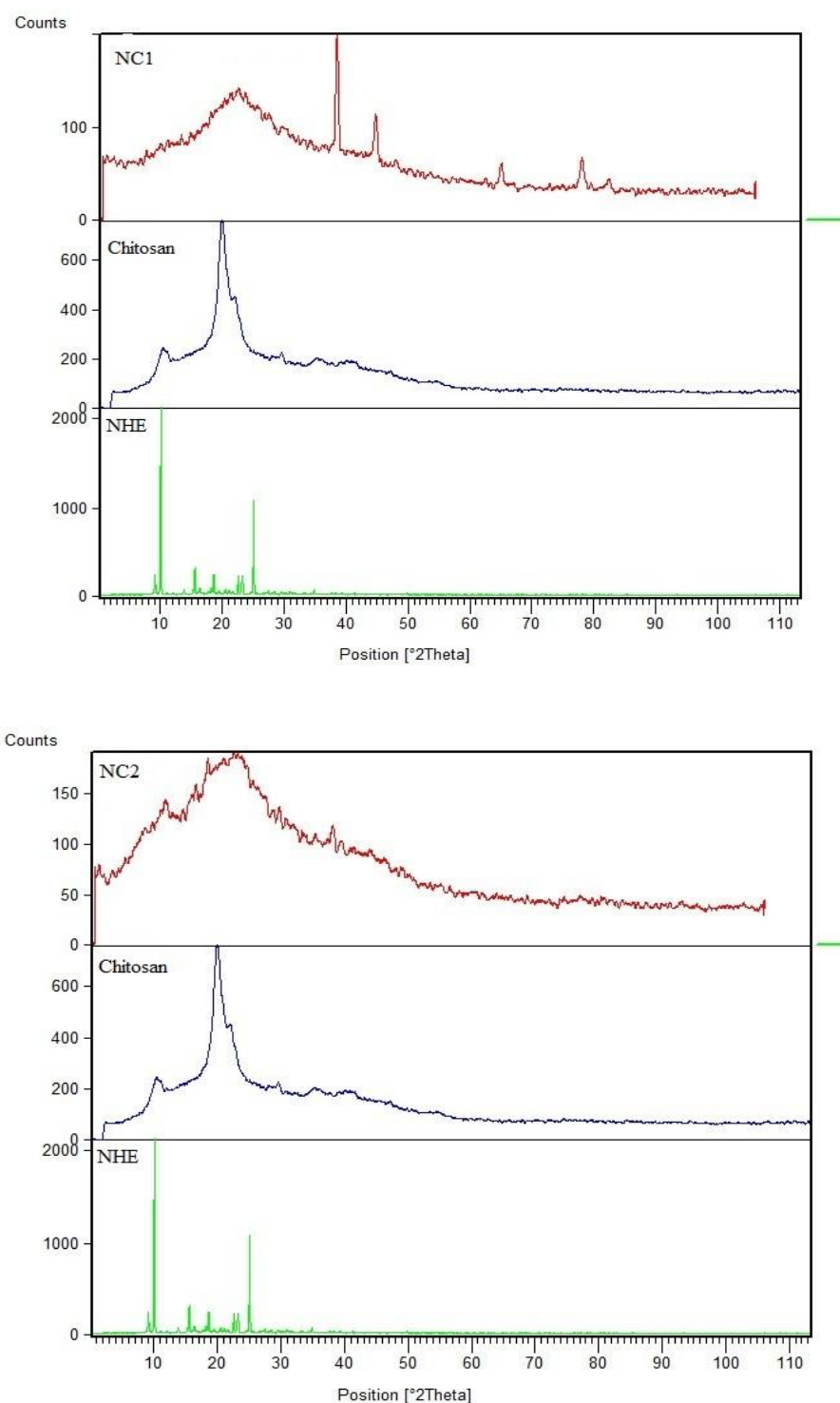


Fig. 2. The XRD pattern of nanocomposites NC1(0% NHE) and NC2 (5% NHE) comparing to their components

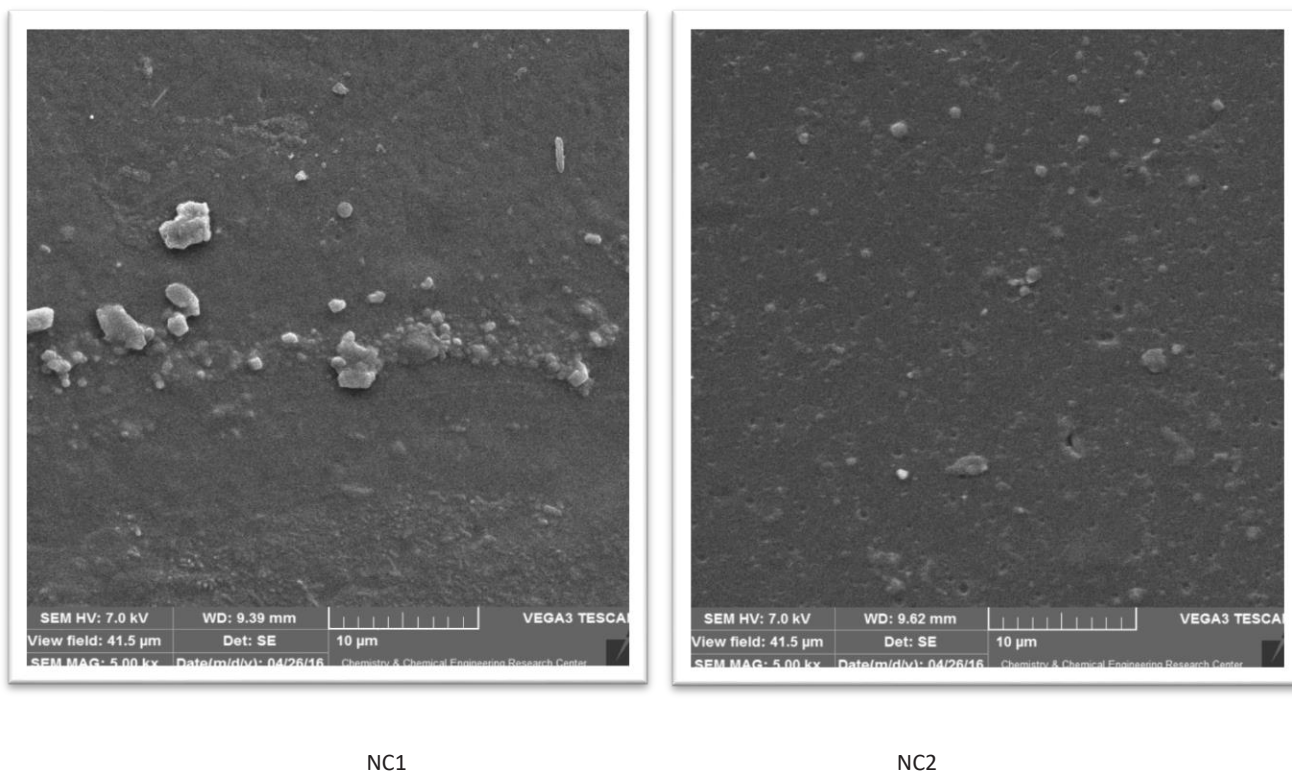
3.2. FE-SEM and EDS

To study the surface morphology of the nanocomposites and measuring the average particle size of the Ag NPs, the FE-SEM micrographs were obtained (Figures 1(b) and 3). FE-SEM micrograph of Ag NPs indicates that the average particle size of Ag NPs is about 80-90 nm and most of these

fine particles have stuck together and formed agglomerates. FE-SEM micrographs of nanocomposites NC1 and NC2, containing 0% and 5% NHE, are shown in Figure 3. The presence of Ag NPs can be obviously observed in these images.

A comparison between the micrograph of NC1 and NC2 films indicates that NC2 has a better surface with a more homogenous dispersion of smaller Ag nanoparticles. This

could be due to the more stirring time under ultrasonic waves for NC2, owing to the step of adding carbacylamidophosphate(NHE), which NC1 did not have.



NC1

NC2

Fig. 3. The FE-SEM micrographs of nanocomposites NC1 and NC2

To confirm the presence of all initial components and all their elements in the nanocomposites framework Energy Dispersive Spectroscopy (EDS) was applied. EDS analysis of NC1 and NC2, containing 0% and 5% NHE, are shown in figure 4, which verifies the existence of all the designed

parts within the nano films and confirms the desired percentages of the elements. The presence of carbacylamidophosphate (NHE) can also be obviously detected by observing the Phosphorus element (Figure4).

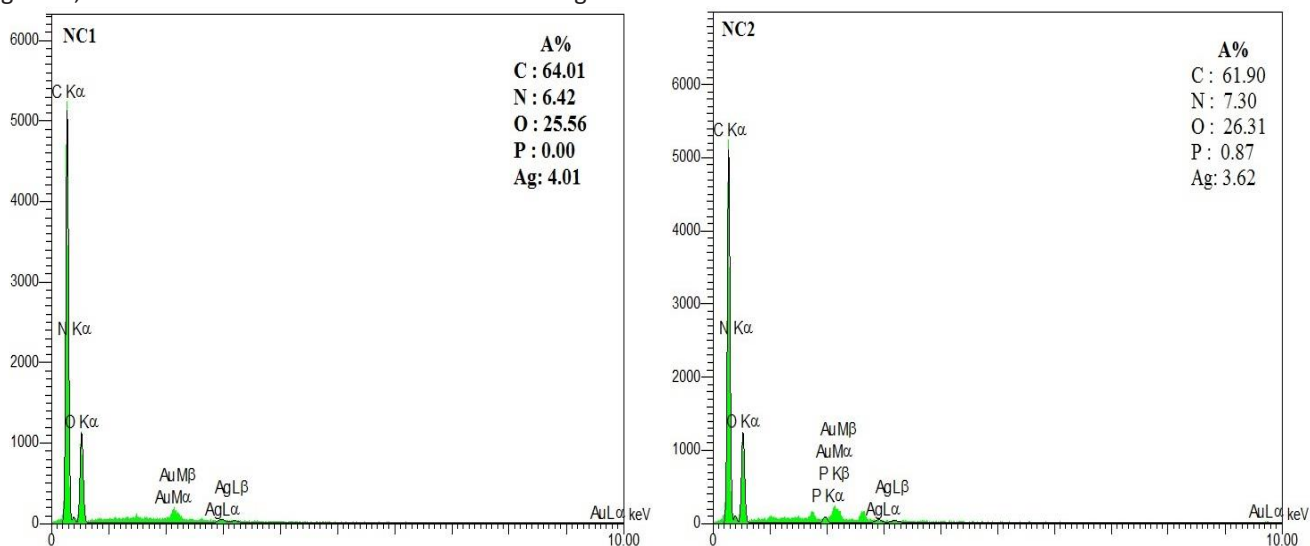


Fig. 4. The EDS of nanocomposites NC1 and NC2

3.3. Antibacterial Activity

The *in vitro* antibacterial activities of nanocomposite NC1 and NC2 were tested against four bacteria: two gram-positive (*B. cereus*, *S. aureus*), and two gram-negative (*P. aeruginosa*, *E. coli*). Each test was repeated for at least 3 times and the average of results were summarized in Table 1. As it can be seen from the table, the highest antibacterial activity of the both films is against *B. cereus* and the lowest is against *S. aureus*. Comparing the inhibition zones of the fabricated films indicates that, adding 5% of carbacylamidophosphate (NHE) to the structure of the nanocomposite results in the increasing of the antibacterial effect of the nanocomposite on all the four bacteria. This is in a good agreement with our previously reported data [41], which indicated the

antibacterial improving of a chitosan based silver-contained nanocomposite by adding 5% of an alternative carbacylamidophosphate derivative, N-Nicotinyl-N',N''-bis(tert-butyl) phosphoric triamide(NPt). Moreover, as Table1 shows, the inhibitory zone (mm) for chitosan/5% NHE/7%Ag NPs nanocomposite (NC2) is even more than those for the previously reported chitosan/10%Ag NPs nanocomposite [41]. In the other word, the effect of 5% NHE on the antibacterial activity of the nanocomposite is more than that of the 3% Ag NPs. Since silver nanoparticles are well known strong antibacterial compounds [11-14], this could confirm remarkable antibacterial activity of the additive NHE.

Table 1. The inhibition zone (mm) measured for the antibacterial activities

Bacteria Nanocomposite	Gram Negative		Gram Positive	
	<i>P. aeruginosa</i> 1074	<i>E. coli</i> 1330	<i>S. aureus</i> 1113	<i>B. cereus</i> 1254
Chitosan / 7% Ag NPs (NC1)	11.99± 0.54	8.61± 0.49	8.47± 0.58	12.02± 0.48
Chitosan /7% Ag NPs /5% NHE (NC2)	13.16± 0.42	10.45± 0.47	10.28± 0.51	13.53± 0.46
Chitosan/ 10% Ag NPs [41]	12.78± 0.47	9.33± 0.57	9.11± 0.55	12.42± 0.51

4. Conclusions

To study the effect of an additive on the antibacterial activity of a chitosan/Ag NPs two new nanocomposite films were prepared, using crosslink method: Chitosan / 7% Ag NPs(NC1) and Chitosan /7% Ag NPs /5% NHE (NC2), where the additive NHE was N-Nicotinyl-N',N''-bis(hexamethylenyl) phosphoric triamide. The desired nanoparticles and nanocomposites were characterized and confirmed by XRD, FE-SEM and EDS. The *in vitro* antibacterial test against two Gram-negative (*P. aeruginosa*, *E. coli*) and two Gram-positive (*B. cereus*, *S. aureus*) bacteria indicated that the phosphoric triamide additive (NHE) increased the antibacterial activity of the nanocomposite against all the four bacteria. This increase, which was a result of adding 5%NHE, was remarkable and more than the addition of 3%AgNps.

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