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Abstract

Crosslinked carboxymethyl Kappa Carrageenan (CMKc)/ poly(ethylene glycol) (PEG) nanocomposites with various ratios of CMKc: PEG (1:1), (1:2) and (2:1) were synthesized using calcium chloride as a crosslinker, in presence of montmorillonite (MMT) in different weight ratios. Characterization of nanocomposites was carried out using various analysis techniques like; FTIR, SEM, TEM, TGA and XRD. Swelling behavior was studied in different buffer solutions. Data revealed that the swelling ability of cross-linked CMKc/PEG nanocomposites increased in the neutral medium. Metal ions adsorption was also examined to show that cross-linked CMKc/PEG nanocomposites adsorbed different metal ions more than the cross-linked CMKc itself. Antimicrobial activity was examined to show that CMKc almost has no activity, whereas the introduction of PEG into nanocomposites improved their antimicrobial activity. Biodegradation in soil was examined to reveal that cross-linked CMKc/PEG in presence of MMT had the highest ability to degrade in soil with time.

Keywords: Carboxymethyl Kappa Carrageenan; Poly (Ethylene Glycol); Nanocomposites; Swelling; Metal Uptake; Antimicrobial Activity

Introduction

Nowadays, plenty of biopolymers are used in different fields such as pharmaceutical, biomedical and food industries [1-3]. Hydrogels are three-dimensional cross-linked polymers that have high swelling properties. Consequently, they are super absorbent hydrophilic polymers [4-7]. Hydrogels are classified into bio hydrogels or synthetic hydrogels depending on the nature of their precursors. Synesthetic hydrogels that are formed from synthetic polymers like poly (ethylene glycol) (PEG), poly (vinyl alcohol) (PVA) and poly (N-isopropyl acrylamide) (PNIPAAM) are prevalently used because of their applications in the medical field [8-11].

Carrageenans are hydrophilic polysaccharides that are cable of forming bio hydrogels that are extracted from natural materials like marine red algae [12-17]. These linear polysaccharides consist of D-galactose and 3,6-anhydrogalactose units with different numbers of sulfate groups [18]. In which Carrageenans exist in three different forms: kappa carrageenan, iota carrageenan and lambda carrageenan with one, two and three sulfate groups per disaccharide unit respectively [19-20]. Kappa-Carrageenan is considered as the most commercial type of carrageenans [21] and is vastly used in biomedical, biosensors, cosmetics, paints and in food industries [22,23]. Also, due to its high alkaline nature, it can be used in wound dressing and tissue engineering in order to expand the applicability of k-carrageenan,

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Carboxymethyl kappa carrageenan was synthesized owing to its wide industrial and scientific applications. Carboxymethyl Kappa Carrageenan is enhanced and developed for metal absorption, and encapsulation in drug delivery [27].

Poly (ethylene glycol) PEG is a hydrophilic, non-volatile and non-hazardous polymer that has common properties with oils, it undergoes the production of many medical aims such as cream manufacturing, dispersing agents and solvents [28]. PEG is a high biocompatible polymer that has an important role in macromere chemistry, as a result, it has encouraged the improvement of smartly-designed hydrogel systems for regenerative medicine applications [29-33].

Recently, Clay Minerals have been used in a wide range in the field of nanocomposites because it has excellent intercalation properties. Clays consist of layers of aluminosilicate with (-OH) groups located on the surface. As a result of the interaction between the clay powder and the polymer matrices super absorbent hydrogels are produced [34]. Montmorillonite (MMT) clay is the most abundant clay mineral that is frequently used in many industries, it is used as an absorbent in removing dyes and organic pigments [34,35] owing to their large surface area and their high exchange capacity of cations. The synthesis of polymer/clay nanocomposites had attracted huge attention owing to their low production cost [36].

In this study, crosslinked CMKc/PEG nanocomposites were synthesized in presence of MMT nanofillers in different weight ratios using CaCl₂ as a cross-linker, then their swelling properties, heavy metal ions uptake, thermal stability, antimicrobial activity towards different Gram-positive, Gram-negative bacteria and fungi and their biodegradation in soil were investigated.

Experimental Study

Materials

Kappa Carrageenan (WG-2000) was purchased from Titan biotech Ltd, Poly (ethylene glycol) (Average Molecular weight = 6000 g mol⁻¹) was obtained from Oxford London, UK. Mono-chloroacetic acid was obtained from Loba chemi -PVT. Ltd, Bombai, India.

Ni (II), Copper (II), Cobalt (II) chlorides and Lead acetate salts were purchased from Aldrich Germany. Nanoclay "Nanomer" 1.31PS, montmorillonite clay surface modified with 15 - 35 wt% octadecylamine and 0.5 - 5 wt% aminopropyl triethoxysilane, was purchased from Merck Germany, CaCl₂ was purchased from Chem-Lab NV Belgium and Different buffer solutions were purchased from Loba chemie.

Synthesis of carboxymethyl kappa carrageenan CMKc

Carboxymethyl Kappa carrageenan CMKc was synthesized as reported in literature [37]. 80g of Kappa-carrageenan was suspended in 720 mL of 80% (w/v) 2-propanol. NaOH 93.5 mL of 40% (w/v) was added drop wisely with stirring at room temperature. In order to activate the alkaline property of the mixture, stirring was continued for another 1h at 40°C. Then 49.1g of monochloroacetic acid (MCA) were added to the mixture. Stirring was continued for 3h at 40°C. Subsequently, the mixture was filtered, the product was collected and washed three times with 80% (w/v) 2-propanol then washed with pure 2-propanol and finally allowed to dry in an oven at 40°C [37].



Scheme 1: Carboxymethylation of Kappa-carrageenan.

Synthesis of cross-linked carboxymethyl Kappa carrageenan/PEG/Clay nanocomposites

CMKc solution was prepared by dissolving 1.5g dry powder CMKc in 30 ml of distilled water. PEG solution was prepared by dissolving 1.5g in 15 ml of distilled water. CMKc and PEG solution were mixed and stirred together for 15 minutes. MMT clay suspension (1 or 3 or 5%) of CMKc weight was added to CMKc/PEG mixture with stirring at room temperature. CaCl₂ solution 2.0N was added drop wisely to carboxymethyl kappa carrageenan/PEG/MMT nanocomposites with continues stirring to form hydrogels.



Scheme 2: Synthesis of crosslinked carboxymethyl Kappa Carrageenan/PEG.

Sample	CMKc (g) (mmol)	PEG (g)	Clay% (gm)	
Cross-linked CMKc	1	-	-	
Cross-linked CMKc 1% MMT	1	-	0.01	
CMKc/PEG (2:1)	2	1	-	
1 % MMT	2	1	0.02	
3% MMT	2	1	0.06	
5% MMT	2	1	0.1	
CMKc/PEG (1:1)	1.5	1.5	-	
1% MMT	1.5	1.5	0.015	
3% MMT	1.5	1.5	0.045	
5% MMT	1.5	1.5	0.075	
CMKc/PEG (1:2)	1	2	-	
1% MMT	1	2	0.01	
3% MMT	1	2	0.03	
5 % MMT	1	2	0.05	

Table 1: Composition of the prepared cross-linked CMKc/PEG/MMT nanocomposites.

Characterization of synthesized hydrogels

KBr discs are used to prepare samples in order to measure FTIR spectra by Testcan Shimadzu IR-Spectrometer (FTIR model IR Affinity-1) at room temperature within the wave number range of 4000-400 cm⁻¹, it was done in Cairo University (CU).

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Measurements of XRD were carried out by Bruker D₈ x-ray diffractometer. The dry sample was coated with gold and measured by Zeiss Scanning electron microscope (SEM). It was done in American University in Cairo (AUC).

Atomic absorption was done on AAnalyst 100 win lab Perkin Elmer to determine the amount of heavy metal ions remaining in the hydrogel liquor was done in National research center (NRC).

Thermal analysis was done on TGA-50 from TA instrument. Samples were heated from 0 to 500°C in a platinum pan with a heating rate 10°C min⁻¹. It was done in American University in Cairo (AUC).

TEM analysis was done by JEOL JEM-1400 electron microscope at Faculty of Agriculture - Cairo University. Antimicrobial tests were done at Cairo University (CU) labs.

Antimicrobial measurements

Antimicrobial activity of the tested samples was determined using a modified Kirby-Bauer disc diffusion method [38]. Concisely, 100 µl of the test bacteria/fungi were grown in 10 ml of fresh media until they reached a count of approximately 108 cells/ml for bacteria or 105 cells/ml for fungi [39]. 100 µl of microbial suspension was spread onto agar plates equivalent to the broth in which they were maintained. Isolated colonies of each organism that might be playing a pathogenic role should be selected from primary agar plates and tested for susceptibility by disc diffusion method [40]. Plates inoculated with filamentous fungi as *Aspergillus flavus* at 25°C for 48 hours; *Gram (+)* bacteria as *Staphylococcus aureus, Bacillus subtilis; Gram (-)* bacteria as *Escherichia coli, Pseudomonas aeruginosa*. They were incubated at 35 - 37°C for 24 - 48 hours and yeast as *Candida albicans* incubated at 30°C for 24 - 48 hours, then the diameters of the inhibition zones were measured in millimeters [38].

Standard discs of Ampicillin (Antibacterial agent), Amphotericin B (Antifungal agent) served as positive controls for antimicrobial activity but filter discs impregnated with 10 μ l of solvent (distilled water, chloroform, DMSO) were used as a negative control. Blank paper disks (Schleicher and Schuell, Spain) with a diameter of 8.0 mm were impregnated 10 μ of tested concentration of the stock solutions. When a filter paper disc impregnated with a tested chemical is placed on agar, the chemical will diffuse from the disc into the agar. This diffusion will place the chemical in the agar only around the disc. The solubility of the chemical and its molecular size will determine the size of the area of chemical infiltration around the disc. If an organism is placed on the agar, it will not grow in the area around the disc if it is susceptible to the chemical.

This area of no growth around the disc is known as a "Zone of inhibition" or" Clear zone". For the disc diffusion, the zone diameters were measured with slipping callipers of the National Committee for Clinical Laboratory Standards. Agar-based methods such as Etest and disk diffusion can be good alternatives because they are simpler and faster than broth-based methods [41,42].

Applications done on CMKc/PEG/clay nanocomposites

Swelling studies

Water uptake of nanocomposites was studied at room Temperature in buffered solutions of various pH 4, 7 and 9. Using the wellknown Tea-bag method, around 0.1g of each sample was packed in a tea-bag, each packed tea bag was balanced and its weight was recorded. In a beaker, 25 ml of each buffer was poured and the tea-bag of each sample was submerged in the buffer then was left for 2h. The tea bags were hanged and left for 15 min to get rid of the excess buffer solution. Finally, the teabags were balanced to calculate the water uptake% [43].

The swelling % was calculated using the below equation:

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Water uptake %= $[(W_s - W_s)/W_s] \times 100$

W_s = Weight of wet tea-bag, W_o = Weight of dry tea-bag.

The average of three experimental trials was done on each sample in different buffer solutions.

Adsorption of heavy metals ions

 $CuCl_2$, $CoCl_2$ and $NiCl_2$ were used to prepare solutions of 150 ppm concentration were prepared. 0.1g of each sample was soaked for 24h in 10 ml of each of the above mentioned solutions at room temperature, samples were soaked in the metal solutions overnight, then they were removed. The remained solution was analyzed to measure the concentration of the metal remained in it using atomic absorption technique.

The amount of remaining heavy metal ions will be estimated using the following equation:

 $Q (mgg^{-1} wet weight) = V (C_0 - C_A) / 1000 W$

Where V (l) is the volume of the salt solutions, W (g) is the weight of the nanocomposite, C_0 (mg l⁻¹) is the initial metal ions concentration and C_A (mg l⁻¹) is the metal ion concentration at definite time.

Biodegradation in the soil

0.1 g of samples were buried in organic soil for 20 days in room temperature. The degradation percentage was calculated using the below equation:

Weight Loss % = 100
$$\frac{wi - wf}{wi}$$

Where, is the initial weight before burying is final weight after burying.

Results and Discussion

FTIR spectroscopy analysis

FTIR spectra charts for KC, CMKc and cross-linked CMKc are presented in figure 1a, Kc has a band at 928 cm⁻¹ representing C-O-C vibration of 3,6-anhydro-D-glactose, the peaks appeared at 1265 cm⁻¹ and 848 cm⁻¹ were assigned to 0=S=0 symmetric vibration and $-0-SO_3$ stretching vibration at C-4 of β -D-galactose residue [44].

In carboxymethyl kappa-carrageenan and cross-linked carboxymethyl kappa-carrageenan; two new characteristic absorption peaks have appeared at 1608 cm⁻¹ and 1420 cm⁻¹, which were attributed to the symmetrical and asymmetrical stretching vibrations of carboxylate anions (-COO⁻) [45]. The new bands confirmed the successful carboxymethylation of Kappa carrageenan.

In figure 1b CMKc: PEG (1:1) (1:2) (2:1) nanocomposites revealed new bands that have referred to PEG around 1110 cm⁻¹ which could be attributed for stretching C-O ether group. In addition, some other peaks are related to MMT that exists in various ratios in the matrices, it has exhibited several peaks. Peaks near 1030 and 570 cm⁻¹ can be attributed to Si-O vibrations [46].

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Figure 1a: IR Charts (a). Kappa-Carrageenan Kc, CMKc and cross-linked CMKc.



Figure 1b: IR Charts (b) CMKc, PEG and CMKc/PEG/MMT (1:2) nanocomposites (1%, 3% and 5% MMT).



Figure 1c: IR Charts (c) CMKc, PEG and CMKc/PEG/MMT (1:1) nanocomposites (1%, 3% and 5% MMT).



Figure 1d; IR Charts (d). CMKc, PEG and CMKc/PEG/MMT (2:1) nanocomposites (1%, 3% and 5% MMT).

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XRD analysis

In figure 2 the X-ray diffraction patterns of pure PEG, CMKc and CMKc/PEG nanocomposites are presented. CMKc has a non-crystalline pattern, while PEG has a more crystalline pattern. CMKc has four main diffraction Peaks at $2\theta = 8^{\circ}$, 31° , 33° and 45° . On the other hand, PEG has two diffraction Peaks at $2\theta = 18^{\circ}$ and 23° . Hence, the ratios of the matrices nanocomposites have collaborated peaks from both CMKc and PEG, the more ratio of the PEG, the more crystalline its peaks and vice versa. In CMKc: PEG (1:2) ratio, four peaks are showed at $2\theta = 18.5^{\circ}$, 21.5° , 31° and 45° . While in CMKc:PEG (2:1) four peaks appeared at $2\theta = 28^{\circ}$, $2\theta = 31^{\circ}$, $2\theta = 40^{\circ}$ and $2\theta = 45^{\circ}$. For CMKc: PEG (1:1) ratio all the peaks that are specific for both CMKc and PEG appeared at $2\theta = 18^{\circ}$, 23° , 28° , 32° , 40° and 45° .



Figure 2: X-ray diffraction patterns of PEG, CMKc and CMKc/PEG nanocomposites.

Thermal analysis

The thermal analysis for Kc, CMKc, PEG and cross-linked CMKc/PEG/MMT nanocomposites are shown in figure 3. The curves showed that CMKc is thermally more stable than KC. PEG was found to be thermally stable till 350°C, then at that temperature it begins to degrade, as it unexpectedly decomposed at 420°C. Cross-linked CMKc/PEG nanocomposites are clearly more thermally stable than CMKc and Kc individually. It is commonly suggested that MMT plays an important role in controlling the thermal stability of the nanocomposites. Figure 3 shows that thermal stability of the nanocomposites increased gradually by adding MMT from 1 to 5% as MMT postponed weight dissociation of CMKc/PEG matrices.



Figure 3a: TGA chart of Kc, CMKc, and CMKc/PEG/MMT (1%, 3% and 5%) nanocomposites.

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Figure 3b: TGA chart of Kc, CMKc, and CMKc/PEG nanocomposites.



Figure 3c: TGA chart of Kc, CMKc, and CMKc/PEG/MMT nanocomposites.

SEM analysis (Magnification X 36000)

As shown in figure 4a-4c CMKc surface morphology is completely different from that of Kc surface, as the former had many bulky lumps on its surface referring to carboxymethylation process. On introducing PEG to the matrix, whilte aggregates started to appear on surface. on adding MMT, the matrix morphology gradually fades away and a network structure masked the original morphology of CMKc and PEG, this network structure increased more clearly with increasing MMT%.



Figure 4a: SEM micrographs of Kc and CMKc.



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TEM analysis

As shown in figure 5, the TEM for the crosslinked CMKc, CMKc/PEG (2:1), CMKc/PEG (2:1) nanocomposites with 1%, 3% and 5% MMT showed the distribution of MMT in the polymeric matrix. By adding MMT, the polymeric matrix started to look like a network instead of a plain bulk. Also increasing % MMT gave nanoparticles with less diameters.



Figure 5: TEM micrographs of Crosslinked CMKc, CMKc/PEG (2:1) and CMKc/PEG/MMT (2:1) in presence of 1%, 3% and 5% MMT.

Swelling behavior

Swell ability of crosslinked CMKc and CMKc/PEG/MMT with different % of MMT was studied in different pH values for 2, 10 and 24 hours.

Figure 6 showed that swell ability is optimum in pH =7 as sulfonate groups have an excessive effect on the swelling behaviour in acidic, neutral and basic media. In acidic medium (pH = 4), sulfonate groups are protonated. As a result reduction in the repulsion force between them and anionic groups occurred so, the swelling properties of the matrices in acidic medium is lowered. While in the neutral medium (pH = 7), sulfonate groups are ionized and the electrostatic repulsion forces between them and COO⁻ groups led to an increase in the swelling properties. In the basic medium (pH = 9), the excessive amount of Na⁺ in the matrices shielded the effect of sulfonate anions so that the repulsion between them and COO⁻ groups were extremely minimized leading to a decrease in the swelling behaviour for the matrices [47]. Swell ability behaviour has different manners across the matrices depending on the different ratios of PEG and MMT in polymeric matrix, as PEG % increases, the swelling behaviour decreases.

MMT enhanced the swelling ability in the matrices because it forms a loose porous matrix capable of absorbing more solvent. By increasing the clay %, the swelling behaviour started to decrease - in an opposite behaviour to the expected - owing to the accumulation of nanocomposites filling material, as reported in similar data in literature [48]. Previously mentioned reasons explains why the cross-linked CMkc with 1% MMT has the highest swelling behaviour in pH 7.

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Figure 6a: Swell ability of cross-linked CMKc CMKc/PEG/MMT nanocomposites in different pH after 2 hrs.



Figure 6b: Swell ability of cross-linked CMKc, CMKc/PEG/MMT nanocomposites in different pH after 10 hrs.

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700 600 Water uptake % 500 400 300 200 100 Caveres trable mart CHARGES 1:175% MART 0 Concres 1:1 1ºs Mart Cancers 12 10 men CHNEPEC 1:1.2% MAN Concres 123 20 mer Convertes tri 2% men Concres 12250 mer CHNECPES II:11 CANCEPES P.11 CANCEPEG 12.21 Chrepes 12:15% MM 1% MM рН 4 🛑 рН 7 🛑 РН 9



Metal ions uptake

Metal ions uptake was investigated on crosslinked CMKc and CMKc/PEG matrices in absence of MMT. Table 2 shows that the ability of polymeric matrices to uptake more metal ions increased with increasing PEG % due to the introduction of more chelating (-OH) groups into the polymeric matrices.

The metal ions adsorption efficiency is listed in the following order: $Ni^{2+} > Co^{2+} > Cu^{2+}$.

Metal ion sizes play an important role in its adsorption selectivity. The atomic radius of Ni²⁺ is 2.74 and that of Cu²⁺ is 2.06 [49].

Sample	Ni ⁺² (ppm)	Cu ²⁺ (ppm)	Co ²⁺ (ppm)	
Cross-Linked Cmkc	383	137	186	
CMKc: PEG 2:1	394	161	218	
CMKc: PEG 1:1	402	182	238	
CMKc: PEG 1:2	418	206	302	

Table 2: Metal ions uptake (ppm) for CMKc, cross-linked CMKc and CMKc/PEG matrices.

Biodegradation in soil

As shown in figure 7, CMKc/PEG/ (1:2) 5% MMT has the largest ability to degrade after 5 days and degraded completely after 10 days from the start of the burial process. In the second place, CMKc/PEG/(1:1) 5% MMT degraded completely after 15 days, and finally CMKc/PEG/(2:1) 5% MMT. While the biodegradation ability of the polymeric matrices without clay is listed as following: CMKc/PEG/(1:2) has the largest ability to degrade after 5 days then CMKc/PEG/ (1:1) and finally CMKc/PEG/(2:1). Increasing PEG% in polymeric matrix increases biodegradation rate as PEG has heteroatoms which are more subjected to biodegradation in soil. Also, it was noticed that increasing MMT % enhanced the biodegradation rate for the matrices.

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Figure 7: Biodegradation in the soil of CMKc/PEG/MMT nanocomposites.

Antimicrobial activity

As shown in table 3, Kc has no antimicrobial activity to all used strains, whereas CMKc has antimicrobial activity towards only *pseudo-monas aeruginosa*. Introducing PEG with CMKc didn't improve the antimicrobial effect but when MMT was added in different %, the antimicrobial activity was slightly improved. CMKc/PEG/MMT nanocomposites show some antimicrobial activity towards *Gram +ve* bacteria, *Gram -ve* bacteria and fungi with inhibition zone diameter reaching 11 mm. The low antimicrobial activity for crosslinked CMKc/PEG is due to presence of Ca²⁺ ions in the matrix as reported in literature [51]. Also due to the presence of huge agglomerates of nanoparticles that apparently couldn't diffuse into the cell membrane to destroy the DNA of bacteria [50].

Sample Inhibition zone diameter (mm / mg Sample)						e)	
1			Bacteri	Fungi			
1		(G⁺) (G [·])					
		Bacillus subtilis	Staphylococcus aureus	Escherichia coli	Pseudomonas aeruginosa	Aspergillus flavus	Candida albicans
ndard	Ampicillin: Antibacterial agent	26	21	25	26		
Sta	Amphotericin B Antifungal agent					17	21
0	Control : water	NA	NA	NA	NA	NA	NA
	Kc	NA	NA	NA	NA	NA	NA
	CMKc	NA	NA	NA	9	NA	NA
CM	KC: PEG (1:1) 0%	NA	NA	NA	NA	NA	NA
CMKC: PEG (1:1) 1%		10	10	9	9	NA	NA
СМ	KC: PEG (1:1) 3%	NA	9	11	9	NA	NA
CM	KC: PEG (1:1) 5%	NA	10	10	9	NA	NA
CM	KC: PEG (1:2) 0%	NA	NA	NA	NA	NA	NA
CMKC: PEG (1:2) 1%		NA	10	NA	10	NA	NA
CMKC: PEG (1:2) 3%		NA	NA	NA	NA	NA	NA
СМКС: РЕС (1:2) 5% NA		NA	NA	NA	NA	NA	NA
CMKC: PEG (2:1) 0%		NA	NA	NA	NA	NA	NA
CMKC: PEG (2:1) 1%		12	10	10	9	NA	NA
CMKC: PEG (2:1) 3%		9	9	NA	NA	NA	NA
CMKC: PEG (2:1) 5%		NA	NA	NA	11	NA	NA
Crosslinked CMKc +1%MMT		NA	9	NA	NA	NA	NA

 Table 3: Antimicrobial Activity of Kc, CMKc, CMKc/PEG/MMT in different ratios.

 NA= -ve result.

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Conclusion

Carboxymethyl Kappa carrageenan was synthesized and cross-linked in presence of PEG by using CaCl₂ as a crosslinker. MMT was added in different %. Characterization of nanocomposites was performed via different analysis tools.

Some applications on nanocomposites were performed like; metal uptake, swelling in different pH buffers, biodegradation in soil and antimicrobial activity. Following results were obtained:

- 1. Swelling behaviour was investigated in different solutions with different pH values. Neutral medium with (pH 7) is the optimum medium for the swelling behaviour. CMKc: PEG (2:1) ratios have the greatest swell ability in the presence of MMT % that enhanced swelling too.
- 2. Metal ions uptake results indicated the ability of cross-linked CMKc to adsorb metal ions increased by increasing PEG % in thematrix, the metal adsorption ability was enhanced due to chelation ability of -OH groups in PEG
- 3. Biodegradation ability in the soil is increased by increasing PEG % in the matrix, also, the addition of MMT enhanced biodegradation in the soil.
- 4. Antimicrobial activity of Kappa carrageenan is nil, whereas the activity of CMKc was weak, when PEG/MMT was added to CMKc, antimicrobial activity was improved.

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