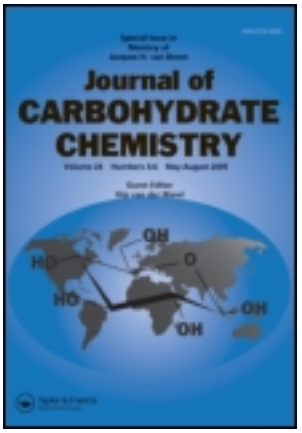

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Synthesis, Characterization, and Antimicrobial Activity of Carboxymethyl Chitosan-Graft-Poly(N-acryloyl,N′-cyanoacetohydrazide) Copolymers

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Synthesis, Characterization, and Antimicrobial Activity of Carboxymethyl Chitosan-Graft-Poly(N-acryloyl,N′-cyanoacetohydrazide) Copolymers

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Carboxymethyl chitosan was grafted with N-acryloyl,N′-cyanoacetohydrazide in homogenous aqueous phase using potassium persulfate initiator. The maximum grafting yield achieved was 448% at 0.03 mol/L potassium persulfate, 0.75 mol/L N-acryloyl,N′-cyanoacetohydrazide, and 60°C within 2 h. The grafted copolymers showed better thermal stability than that of carboxymethyl chitosan. The samples with percent grafting values up to 98% were soluble in water, but a higher grafting extent resulted in insoluble copolymers. The grafted copolymers are nontoxic materials and showed an inhibition effect on both *Escherichia coli* and *Staphylococcus aureus* bacteria and *Aspergillus flavus* and *Candida albicans* fungi better than those of chitosan and carboxymethyl chitosan themselves.

Keywords Carboxymethyl chitosan; N-acryloyl,N′-cyanoacetohydrazide; Graft copolymerization; Characterization; Antimicrobial activity

INTRODUCTION

Chitosan is a biocompatible and biodegradable polymer[1] and its degradation products are nontoxic, nonimmunogenic, and noncarcinogenic.[2] It has found many industrial, pharmaceutical, and medical applications.[3] Chitosan films, fibers, microparticles, and nanoparticles have been reported for tissue engineering and drug, vaccine, and DNA delivery applications.[4] However,

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it is only soluble in a few dilute acids, which limits its applications. Recently, considerable attention has been directed to chemical modification of chitosan to improve its water solubility and widen its applications. Chemical modification of chitosan by its graft copolymerization with various vinyl monomers can produce multifunctional materials of desired chemical and physical properties and enlarge the fields of their potential applications such as flocculent agents by grafting chitosan with acrylamide using gamma radiation,[5] for dye absorption where chitosan is grafted with a series of methacrylate monomers,[6] as a metal chelating agent by grafting chitosan with N-acryloyl,N′-cyanoacetohydrazide,[7] for wastewater purification especially removal of heavy metals such as mercury,[8] for enhanced antibacterial activity via grafting chitosan with N-vinylimidazole,[9] and for antifungal activity via grafting chitosan with vinyl acetate[10] and N-acryloyl,N′-cyanoacetohydrazide.[11] However, the properties of grafted chitosan have been improved only to a limited extent because of its regular structure and the strong intermolecular hydrogen bonds. Recent researchers showed that grafting onto premodified chitosan is quite significant in view of preparing polysaccharide-based advanced materials with multifunctions. However, there are limited reports about the graft copolymerization of premodified chitosan derivatives.[12–15]

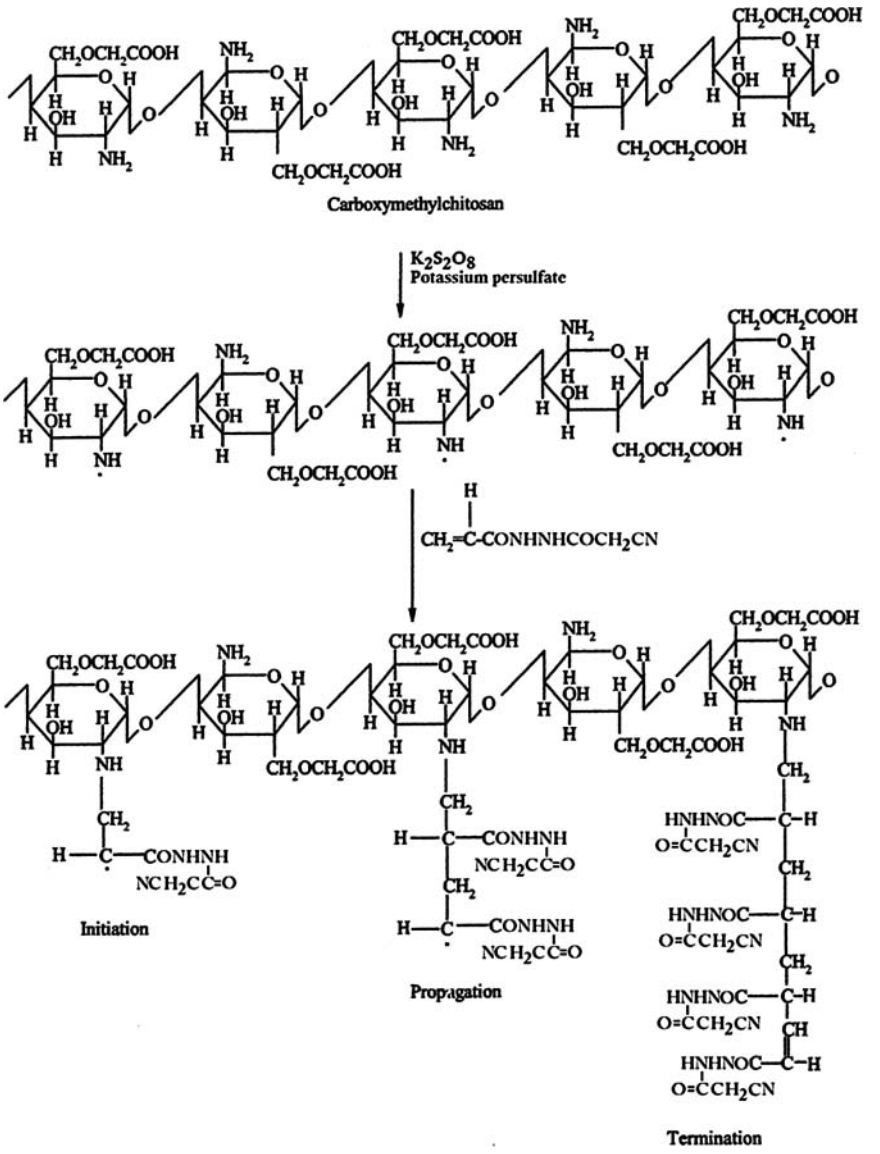
Multiple-derivatized chitosan, carboxymethyl chitosan-graft-poly(N-acr yloyl,N′-cyanoacetohydrazide) copolymers, will be prepared for the first time in this work by carboxymethylation of chitosan with monochloroacetic acid followed by graft copolymerization with N-acryloyl,N′-cyanoaceto hydrazide, which contains two active moieties, a hydrazide and a cyano group. The effects of reaction conditions such as concentration of potassium persulfate initiator, concentration of monomer, reaction temperature, and reaction time on the graft copolymerization will be studied. Carboxymethylation and grafting processes will be elucidated by FTIR spectroscopy, scanning electron microscopy observations, and x-ray diffraction. Characterization of the grafted copolymers for their properties such as thermal behavior, solubility, and antimicrobial activity will also be reported.

RESULTS AND DISCUSSION

Grafting Copolymerization Reaction

Identification of the grafted copolymers

The designed grafting copolymerization of N-acryloyl,N′-cyano acetohydrazide with carboxymethyl chitosan is illustrated in Scheme 1. It was expected that nitrogen radicals would be formed on carboxymethyl chitosan after treating with potassium persulfate, which would be followed



Scheme 1: Grafting of N-acryloyl,N′-cyanoacetohydrazide onto carboxymethyl chitosan.

by radical addition to N-acryloyl,N′-cyanoacetohydrazide and subsequent polymerization to generate various copolymers.

The existence of the grafting was evidenced by the weight increase as reflected by grafting percentage (%G) and grafting efficiency (%GE) (Figs. 1 through 4), and by observing the changes in the FTIR spectra of the pure chitosan, carboxymethyl chitosan, and grafted copolymers as shown in Figure 5. The FTIR spectrum of chitosan showed four strong peaks at 1155, 1073, 1030,

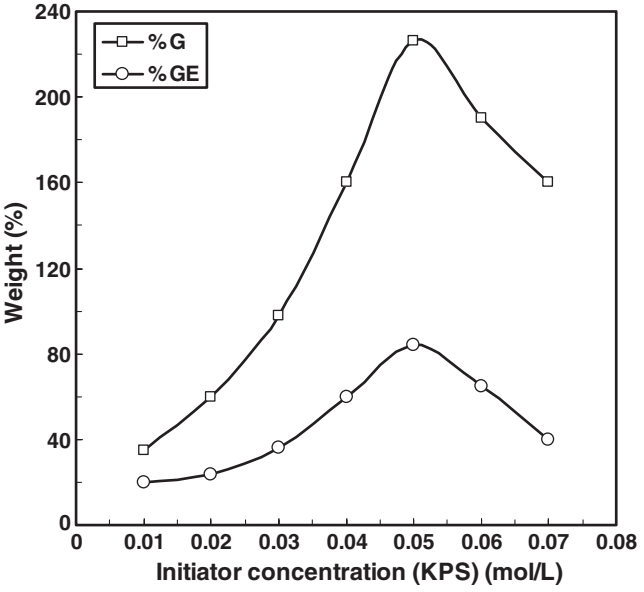


Figure 1: Effect of initiator ($K_2S_2O_8$) concentration on grafting percentage (%G) and grafting efficiency (%GE). Reaction conditions: 0.5 g carboxymethyl chitosan, 0.35 mol/L N-acryloyl,N′-cyanoacetohydrazide, 25 mL water, 60°C; 120 min.

and 895 cm^{-1}, which were characteristic peaks of the saccharide structure. The very strong broad peak around 3600 to 3200 cm^{-1} should be assigned to the stretching vibration of O—H, the extension vibration of N—H, and the intermolecular hydrogen bonds of the polysaccharide. Primary amines have two peaks in this region. There were weak absorption peaks at 1653 and 1567 cm^{-1} corresponded to amide I and amide II, respectively, which indicated that chitosan had a high deacetylation degree. The IR spectrum of carboxymethyl chitosan showed, in addition to the above peaks, a strong peak at 1454 cm^{-1}, which could be assigned to the symmetrical stretching vibration of the COO^- group. The asymmetrical stretching vibration of the COO^- group (around 1550 cm^{-1}) is overlapped with the deforming vibration of NH_2 at 1604 cm^{-1} to obtain a very strong peak. The C—O absorption peak of the hydroxyl group became stronger and moved to 1097 cm^{-1}. The results, which are in accordance with the work of Xie et al., [16] indicated that substitution occurred at the C_6 position. On comparing the FTIR spectrum of carboxymethyl chitosan and the grafted copolymers, an additional peak at 2265 cm^{-1} was observed in the spectrum of the copolymer, which is attributed to the presence of the CN group of the monomer; its intensity increases with increasing the grafting extent. Also, CH_2 bending vibration of the monomer appears at 1392 cm^{-1}. Further, the characteristic peak of the C=O of the hydrazide group appeared around 1660 cm^{-1}. From the IR data, it is clear that the grafted copolymers had both

characteristic peaks of poly(N-acryloyl,N′-cyanoacetohydrazide) and the saccharide of chitosan and carboxymethyl chitosan, which could be effective evidence of grafting. Moreover, Figure 5 has proved that the initiation step for grafting was done on the amino group at C_2. This is well illustrated by the disappearance of the doublet peak at 3382 and 3170 cm^{-1} corresponding to the $-NH_2$ group and the appearance of a singlet peak at 3434 cm^{-1} for the $-NH$ group, which indicates the abstraction of hydrogen atom by the $KSO_4^{\cdot}$ radical derived from decomposition of the potassium persulfate initiator[12]:

$$\text{carboxymethyl chitosan } -NH_2 + KSO_4^{\cdot} \rightarrow \text{carboxymethyl chitosan } -NH^{\cdot} + KHSO_4$$

^{1}H NMR spectroscopy was also used to confirm the grafting process as shown in Figure 6, which represents the spectrum of 98%G copolymer. Elemental analyses could be used as further evidence for the occurrence of grafting through the noticeable increase in the nitrogen content with grafting (the gravimetrically 98%G sample was found to have nitrogen of 20.7%, which corresponds to a %G of 101.7%).

Impact of initiator concentration on the graft copolymerization

The graft copolymerization was conducted at different concentrations of initiator, which was varied from 1 to 7 $\times$ 10^{-2} mol/L by keeping the monomer concentration constant at 0.35 mol/L, the temperature at 60°C, and the reaction time at 2 h. As shown in Figure 1, the %G and the %GE increase with increases in the initiator concentration and reach their maximum values at 5 $\times$ 10^{-2} mol/L. The increase of %G may be ascribed to the increase of macroradicals. With the increase in initiator concentration, more initiator attacked the characteristic NH_2 group of the saccharide unit of carboxymethyl chitosan, more carboxymethyl chitosan macroradicals were generated, and thus more active sites of carboxymethyl chitosan could initiate and propagate the graft copolymerization process with monomer. A further increase of the initiator concentration (>5 $\times$ 10^{-2} mol/L) resulted in a decrease of the %G and %GE. This could be due to several known reasons such as the competition between initiation and termination reactions through chain transfer to the initiator or the coupling between initiator radicals.

Impact of monomer concentration on the graft copolymerization

Figure 2 illustrates the effect of monomer concentration on the grafting parameters. The monomer concentration was studied within the range of 0.25 to 1.25 mol/L, while the initiator concentration was kept constant at 3 $\times$ 10^{-2} mol/L, the temperature at 60°C, and the reaction time at 2 h. With an increase in concentration of monomer, %G and %GE increased continuously, reached

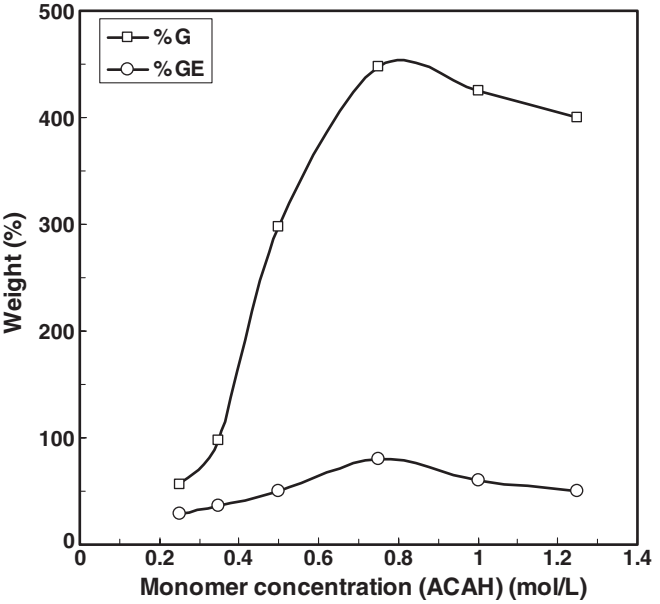


Figure 2: Effect of monomer (N-acryloyl,N′-cyanoacetohydrazide) concentration on %G and %GE. Reaction conditions: 0.5 g carboxymethyl chitosan, 0.03 mol/L potassium persulfate, 25 mL water, 60°C; 120 min.

the maximum values when the concentration of monomer was 0.75 mol/L, and then decreased, showing that higher monomer concentrations do not promote further grafting. This behavior could be explained by the fact that an increase of monomer concentration leads to the accumulation of monomer molecules in close proximity to the carboxymethyl chitosan backbone. The decrease of %G after saturation could be associated with depletion in the available monomer concentration as well as a reduction in the active sites on the carboxymethyl chitosan backbone as graft copolymerization proceeds. It can also be noted that once the graft copolymer radical has formed, the excess monomer will shield the graft copolymer, which may inhibit the rate of graft copolymerization. Further, the observed behavior could be attributed to a substantial amount of polymer grafted onto carboxymethyl chitosan backbone, which creates steric hindrance for further grafting. It is worth mentioning that the high value of %G can be attributed to the fact that the presence of bulky groups such as $-CH_2$-COOH in the carboxymethyl chitosan may open up its structure, thereby increasing the diffusion of the initiator and monomer into carboxymethyl chitosan.

Impact of reaction temperature on the graft copolymerization

The effect of reaction temperature on %G and %GE was investigated by changing the reaction temperature from 45°C to 70°C and keeping the

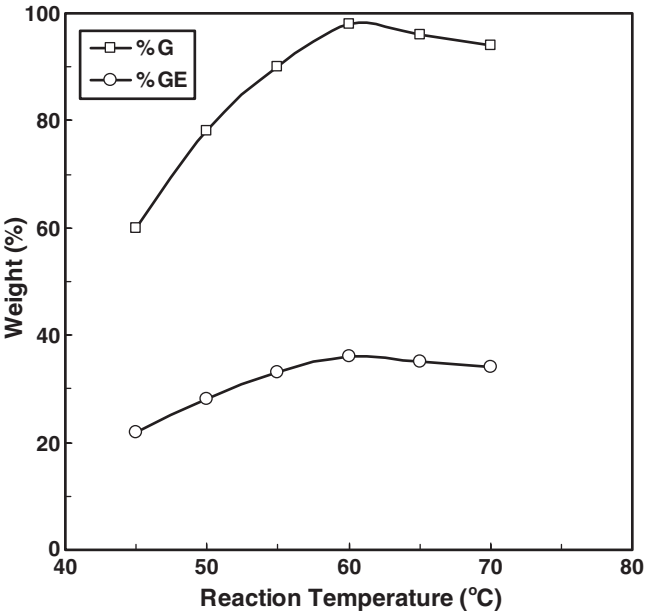


Figure 3: Effect of reaction temperature on %G and %GE. Reaction conditions: 0.5 g carboxymethyl chitosan, 0.35 mol/L N-acryloyl,N′-cyanoacetohydrazide, 0.03 mol/L potassium persulfate, 25 mL water, 120 min.

initiator concentration constant at 3×10^{-2} mol/L, the monomer concentration at 0.35 mol/L, and the reaction time at 2 h. As shown in Figure 3, the %G and %GE increase with rise in temperature from 45°C to 60°C, but decrease with further rise in temperature to 70°C. At low temperature, a redox reaction between the initiator and NH_2 group of carboxymethyl chitosan was slow, the amount of radicals generated was small, and thus %G was low. With the increase in temperature, the collision chance of carboxymethyl chitosan and initiator increased and resulted in the increase of carboxymethyl chitosan macroradicals, and thus enhanced the graft copolymerization. At higher temperatures chain transfer reactions and accelerated termination reactions are favored, decreasing the extent of grafting. Also, inefficient activity of initiator at elevated temperatures would give rise to less efficient initiation, causing a decrease in grafting. Consequently, the combined factors of less efficient initiation and increased rate of termination accounts for the decreasing amount of grafted copolymer.

Impact of reaction time on the graft copolymerization

The influence of reaction time on grafting is shown in Figure 4. The reaction time was changed from 0.5 to 4 h, keeping the reaction temperature constant at 60°C, the initiator concentration at 3×10^{-2} mol/L, and the monomer

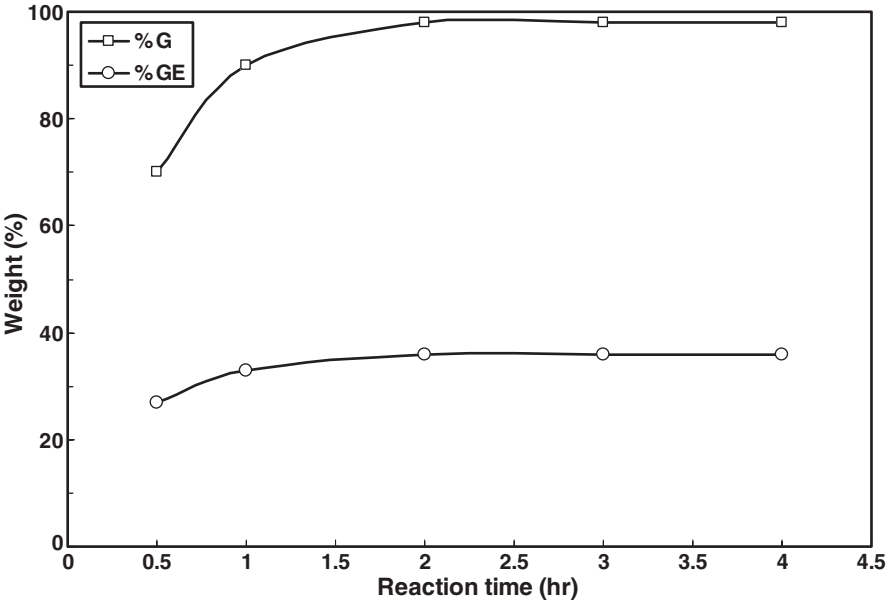


Figure 4: Effect of reaction time on %G and %GE. Reaction conditions: 0.5 g carboxymethyl chitosan, 0.35 mol/L N-acryloyl,N′-cyanoacetohydrazide, 0.03 mol/L potassium persulfate, 25 mL water, 60°C.

concentration at 0.35 mol/L. %G and %GE increased gradually with increase in the reaction time and leveled off after 2 h, reaching a saturation grafting value. This may be attributed to the depletion of the monomer, initiator, and available grafting sites as the reaction proceeds. The remarkable feature of this copolymerization system is that no homopolymer was formed under all these investigated conditions.

Characterization of the Grafted Copolymers

Scanning electron microscopy observations of the grafted copolymers

The scanning electron micrographs of chitosan, carboxymethyl chitosan, and two samples of grafted copolymers (56%G and 448%G) are shown in Figure 7. Carboxymethylation and graft copolymerization affected the surface morphology and also the physical and chemical characteristics of chitosan. It is clearly noted that the flaky fibrous nature of chitosan was modified in the carboxymethylation process as there appear more lumps on the smooth surface of chitosan due to the formation of bulky $-CH_2$-COOH groups. Carboxymethyl chitosan was totally modified in the graft copolymer, wherein distinct morphological differences were discernible in their surface topography. The grafted

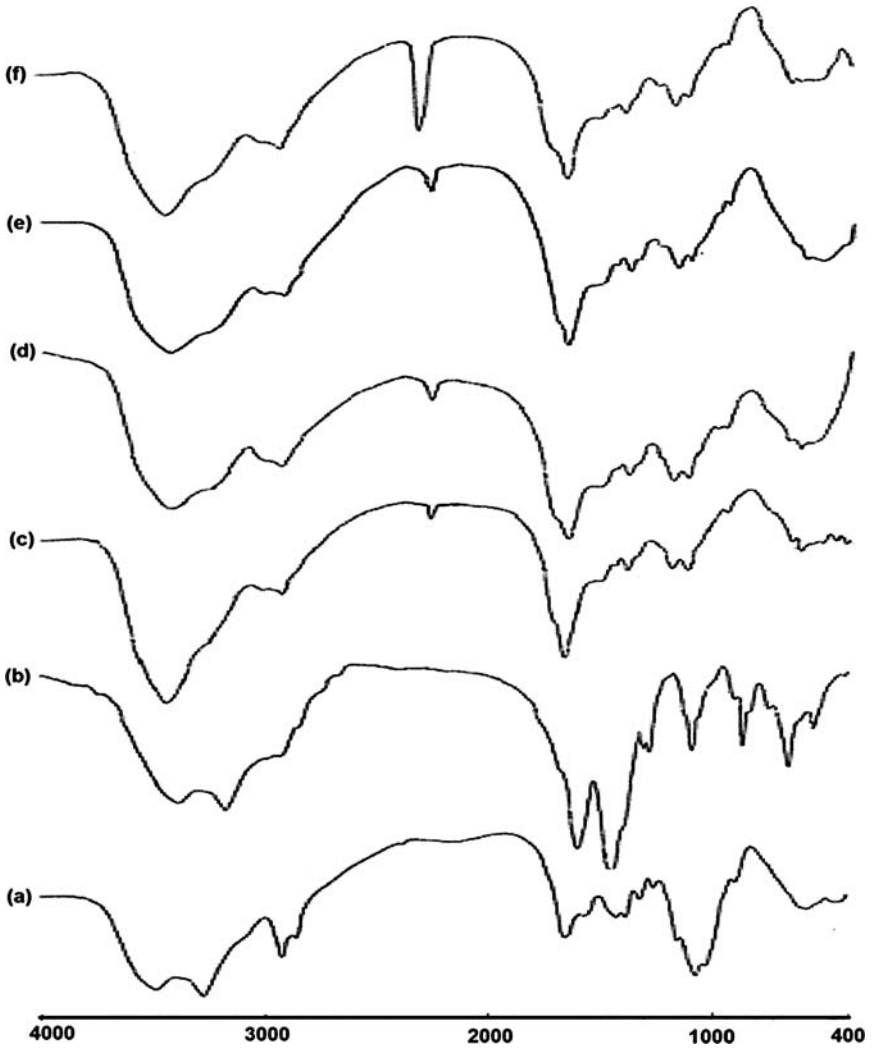


Figure 5: FTIR spectra of (a) chitosan, (b) carboxymethyl chitosan, (c) grafted copolymer (56%G), (d) grafted copolymer (98%G), (e) grafted copolymer (298%G), and (f) grafted copolymer (448%G).

copolymers showed the clustered irregular structure, which increases with increasing the percentage of grafting.

X-ray diffraction of the grafted copolymers

Figure 8(a–d) shows the powder x-ray diffractograms of chitosan, carboxymethyl chitosan, and two samples of grafted copolymers (56%G and 448%G), respectively. From Figure 8(a), the two peaks showing the maximum intensity were obtained at $2\theta = 31.6^{\circ}$ and $2\theta = 45.4^{\circ}$, indicating that chitosan is highly crystalline in nature. Also, carboxymethyl chitosan exhibits

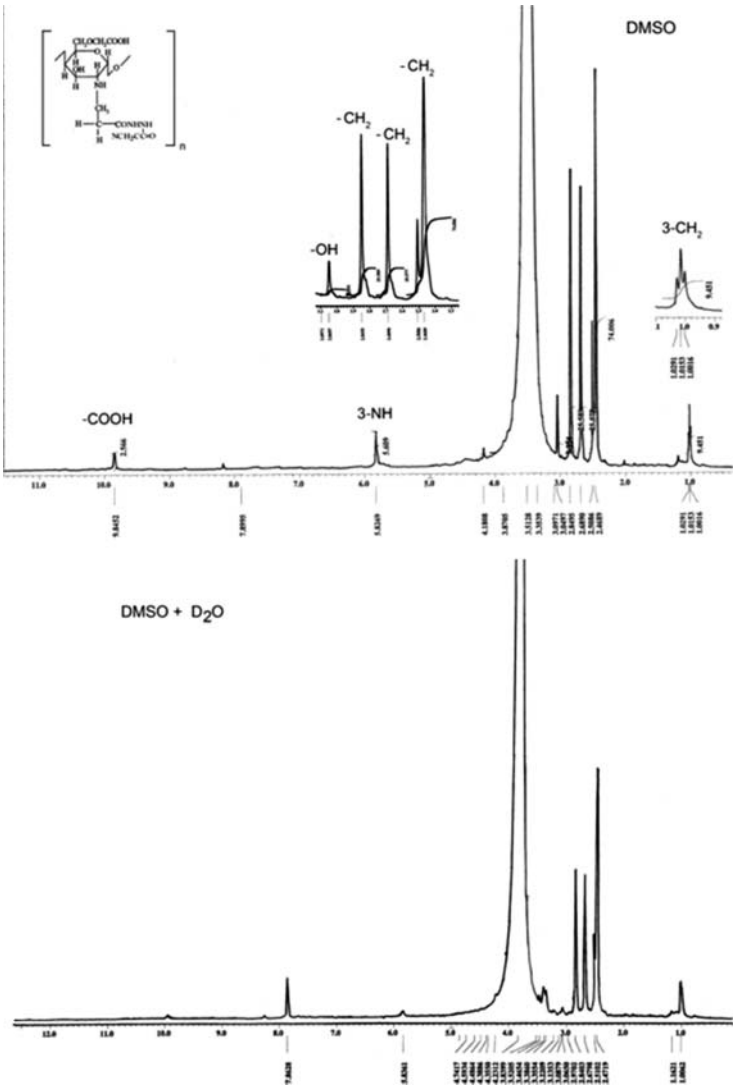


Figure 6: H NMR spectrum of 98% grafted polymer.

crystallinity as shown in Figure 8(b). The grafting of poly(N-acryloyl,N′-cyanoacetohydrazide) into carboxymethyl chitosan severely decreases the intensity of both the peaks; that is, almost no peak is obtained, which is clearly visible in Figure 8(c, d). The graft copolymerized samples showed an almost amorphous nature. The grafting of N-acryloyl,N′-cyanoacetohydrazide takes place randomly on the carboxymethyl chitosan chains, giving rise to a random copolymer, which efficiently destroys the regularity of the packing of the original carboxymethyl chitosan chains and results in the formation of amorphous copolymer.

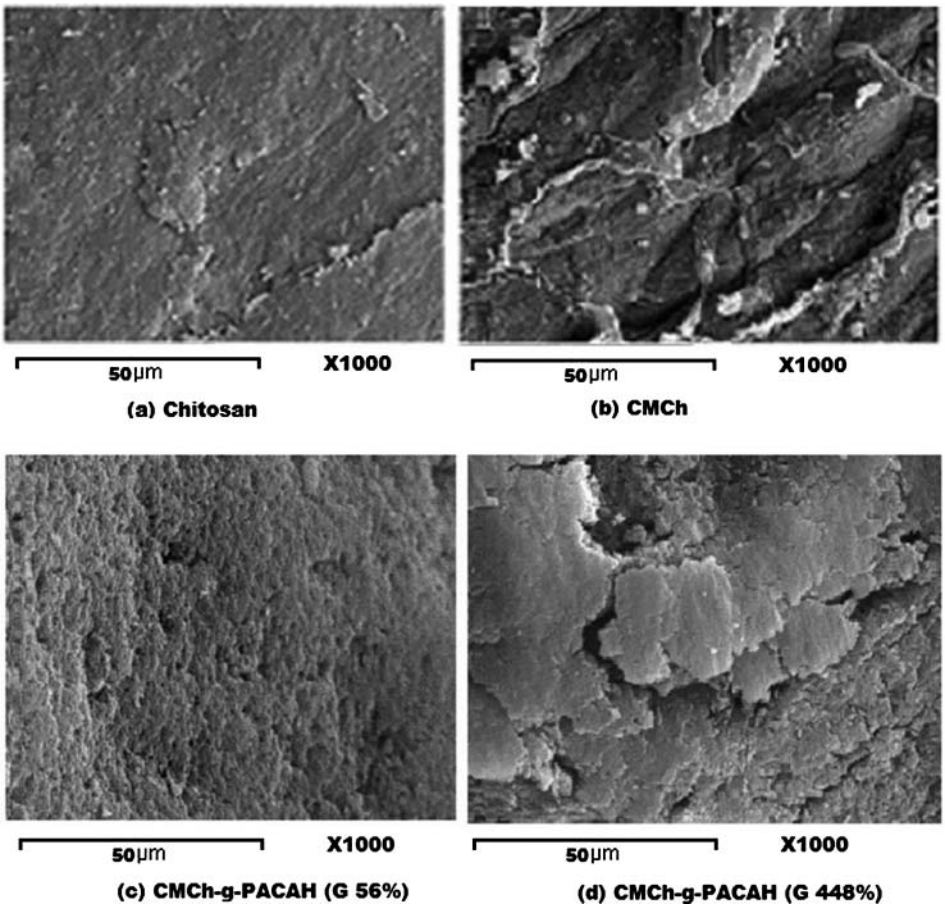


Figure 7: Scanning electron micrographs of (a) chitosan, (b) carboxymethyl chitosan, (c) grafted copolymer (G 56%), and (d) grafted copolymer (G 448%).

Thermogravimetric analyses of the grafted copolymers

Thermogravimetric analyses of carboxymethyl chitosan and the grafted copolymers (98%G and 226%G) together with that of poly(N-acryloyl,N′-cyanoacetohydrazide) homopolymer are illustrated in Figure 9. The results clearly reveal the higher initial decomposition temperature of poly(N-acryloyl,N′-cyanoacetohydrazide) (265°C) as compared with that of carboxymethyl chitosan (237°C). It can also be noted that the rate of thermal degradation of poly(N-acryloyl,N′-cyanoacetohydrazide) is better than that of carboxymethyl chitosan. Modification of carboxymethyl chitosan by grafting with poly(N-acryloyl,N′-cyanoacetohydrazide) was found to improve its thermal stability and that the initial decomposition temperature value was 250°C and 260°C for the 98%G and 226%G. The relatively low initial decomposition temperature of the parent carboxymethyl chitosan is mainly

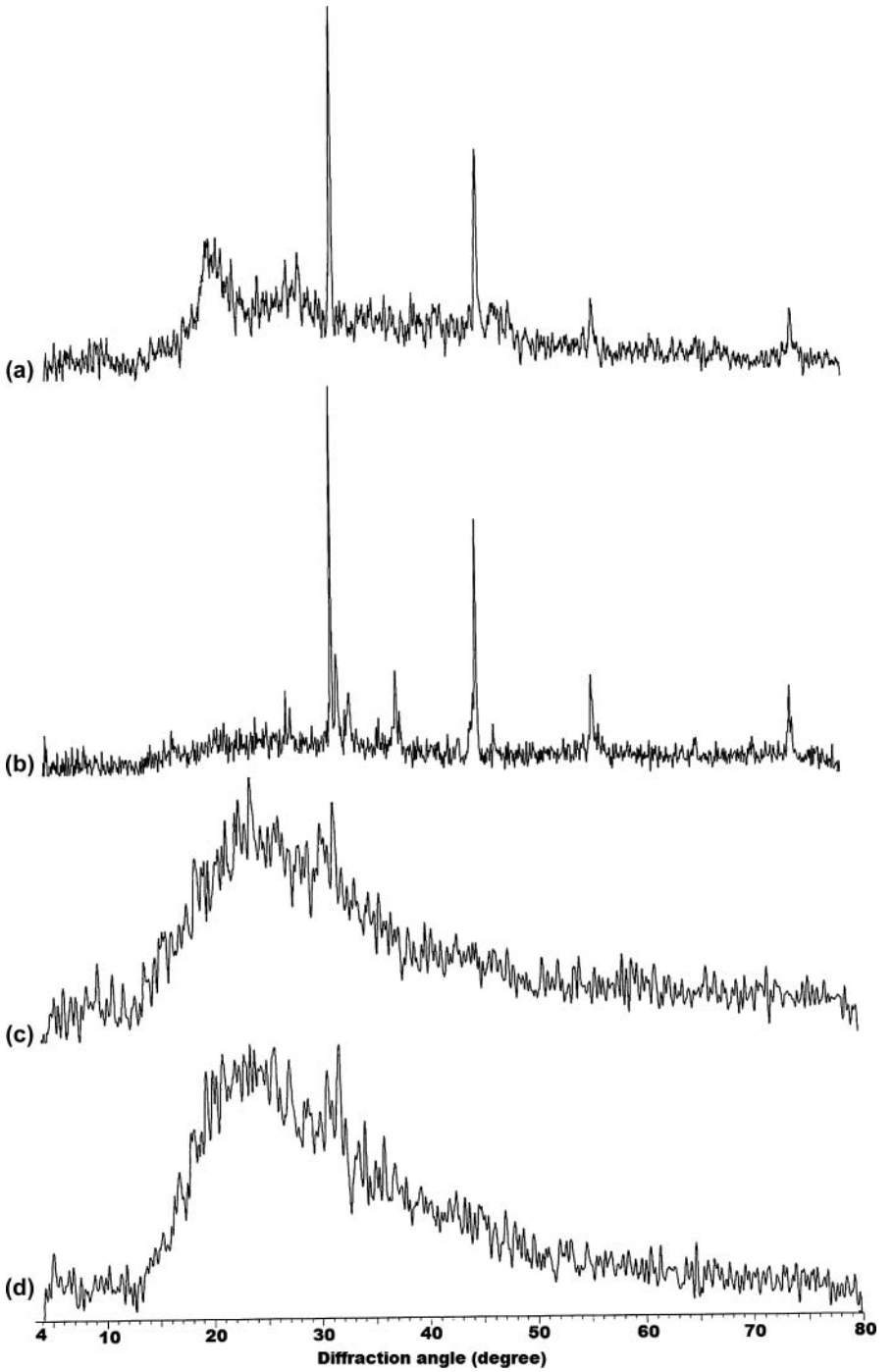


Figure 8: Powder x-ray diffraction of (a) chitosan, (b) carboxymethyl chitosan, (c) grafted copolymer (G 56%), and (d) grafted copolymer (G 448%).

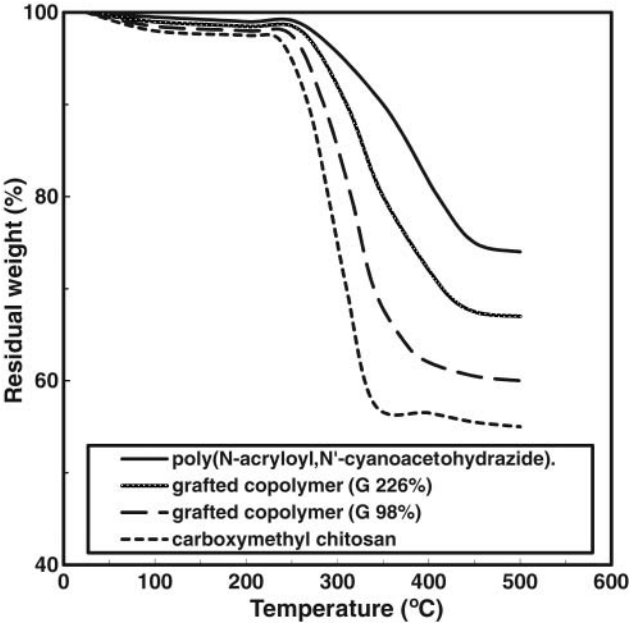


Figure 9: Thermogravimetric curves of carboxymethyl chitosan, grafted copolymers, and poly(N-acryloyl,N′-cyanoacetohydrazide). All the thermograms were recorded in nitrogen atmosphere at a heating rate of 10°C min^{-1} and under a gas flow rate of 30 mL/min^{-1}.

attributed to the presence of −COOH groups that may decompose, giving up CO_2 gas. The same behavior was reported for carboxymethyl cellulose.[17] The data also reveal that the rate of thermal degradation was found to be better for the grafted copolymers as compared with the parent carboxymethyl chitosan. This is well illustrated by comparing the recorded temperatures for the same weight loss values of different investigated samples. Moreover, the weight loss at 500°C was found to be lower for the grafted samples relative to the parent carboxymethyl chitosan. The improvement in the thermal stability of carboxymethyl chitosan grafted copolymers is mainly attributed to the cyclization reactions of poly(N-acryloyl,N′-cyanoacetohydrazide), which may occur due to the reaction of the long pendant groups attached to the poly(N-acryloyl,N′-cyanoacetohydrazide) backbone according to the thermal degradation mechanism of poly(N-acryloyl,N′-cyanoacetohydrazide).[18] The cyclization reaction takes place via addition of the amido hydrogen atoms on the nitrile groups to form stable aromatic pyrazole moieties, which were then detached from the polymeric backbone, and sequences of stable fused γ-pyran rings were produced.[18] This will, no doubt, lead to a delay in the degradation process of grafted copolymers. The thermal stability of grafted copolymers increases with increasing the graft extent.

Table 1: Solubility of the grafted copolymers

Solvents	56%G	98%G	298%G	448%G
Distilled water	sol.	sol.	swelled	swelled
DMSO	sol.	sol.	sol.	sol.
1% Acetic acid	sol.	sol.	swelled	swelled
Methanol	insol.	insol.	insol.	insol.
1% Acetic acid- methanol (1:1)	cloudy	cloudy	cloudy	cloudy
DMF	insol.	insol.	insol.	insol.
THF	insol.	insol.	insol.	insol.

Solubility characteristics of the grafted copolymers

Solubility of the grafted copolymers is summarized in Table 1. The graft copolymers with percent grafting values up to 98% are found to be soluble in water (clear solutions, as observed by the eye, are obtained), while higher grafting extent results in insoluble but highly swollen copolymers. The latter behavior may be attributed to slight cross-linking at higher grafting yields. The water-soluble copolymers are of practical value as the lack of solubility of chitosan in water limits its useful applications. A similar behavior was observed when 1% acetic acid was examined for solubility of the copolymers. Further, the grafted copolymers were found to be freely dissolved in DMSO, but the solubility starts to decrease in 1% acetic acid:methanol mixture (1:1) and then becomes totally insoluble in methanol, DMF, and THF solvents.

Cytotoxicity and Antimicrobial Activities of the Grafted Copolymers

Cell toxicity was monitored on normal Madin-Darby bovine kidney epithelial cells by determining the effect of the grafted copolymers (56%G and 98%G) on cell morphology and cell viability. Different concentrations of the grafted copolymers (0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50 μg/mL) were investigated. The results clearly revealed no change on both the cell viability and cell morphology, indicating that there were no cytotoxic effects of the grafted copolymers on the investigated normal cell line.

The antimicrobial activity of chitosan depends on several factors, such as the kind of chitosan used (deacetylation degree, molecular weight), medium pH, incubation temperature, etc. Several hypotheses elucidating the antimicrobial activity of chitosan have been postulated. The most feasible hypothesis is a change in cell permeability due to interactions between the polycationic chitosan and the electronegative charges on the cell surface. This interaction leads to the leakage of intracellular electrolytes and proteinaceous constituents.[19–23] Other mechanisms are the interaction of diffused hydrolysis

Table 2: Inhibition zone tests for the grafted copolymers

	Inhibition zone (mm) Tested microorganisms			
	Bacteria		Fungi	
Sample	*E. coli*	*S. aureus*	*A. flavus*	*C. albicans*
Chitosan	10.4 ± 0.09	11.1 ± 0.03	11.2 ± 0.08	13.4 ± 0.1
Carboxymethyl chitosan	14.1 ± 0.06	8.2 ± 0.05	10.2 ± 0.09	12.4 ± 0.2
Grafted copolymer (56%G)	21.7 ± 0.03	16.6 ± 0.03	13.2 ± 0.01	15.4 ± 0.04
Grafted copolymer (98%G)	24.5 ± 0.04	18.9 ± 0.01	15.2 ± 0.01	19.4 ± 0.04

products with microbial DNA, which leads to inhibition of mRNA and protein synthesis[24] and the chelation of metals, spore elements, and essential nutrients.[25] The water-soluble copolymers promise to be useful as antibacterial and antifungal agents. The antibacterial study of the grafted copolymers (56%G and 98%G) was carried out against *Escherichia coli* (gram-negative bacteria) and *Staphylococcus aureus* (gram-positive bacteria) using the inhibition zone method. The results are shown in Table 2. Chitosan and carboxymethyl chitosan showed antibacterial activity against both *E. coli* and *S. aureus*. It was noted that while chitosan, compared with carboxymethyl chitosan, was much effective in increasing the inhibition zone diameter of *S. aureus* (11.1 ± 0.03 mm and 8.2 ± 0.05 mm for chitosan and carboxymethyl chitosan, respectively), carboxymethyl chitosan was more potent (14.1 ± 0.06 mm inhibition zone) in the case of *E. coli* than chitosan (10.4 ± 0.09 mm inhibition zone). Grafted copolymers showed better antibacterial activity than those of carboxymethyl chitosan and chitosan themselves. It was observed that increasing the graft percentage caused a higher inhibition zone diameter of both *S. aureus* and *E. coli*. Again the antibacterial activities of the tested graft copolymers were stronger against *E. coli* than *S. aureus*. This might be attributed to their different cell walls. *E. coli* is a typical gram-negative bacterium, the cell wall of which is made up of a thin membrane of peptidoglycan and an outer membrane constituted of lipopolysaccharide, lipoprotein, and phospholipids. Chitosan and its derivatives with large molecular weight can be expected to coat the cell surface and prevent the leakage of intracellular components.[26] Helander et al.[27] have shown that chitosan disrupted the barrier properties of the outer membrane of gram-negative bacteria, as chitosan is protonated at acidic conditions and the carboxyl and phosphate groups of the bacterial surface are anionic and offer potential sites for electrostatic binding. The NH group in the poly(N-acryloyl,N′-cyanoacetohydrazide) derivatives can be protonated under acidic conditions, so it can react with the carboxyl and phosphate groups of the bacterial surface and therefore

show antibacterial activity against gram-negative bacteria. On the other hand, *S. aureus,* a typical gram-positive bacterium, has a cell wall composed mainly of peptidoglycan, which does not allow the formation of a surface layer. Table 2 also summarizes the antifungal activity of the grafted copolymers (56%G and 98%G) when tried on *Aspergillus flavus* and *Candida albicans* fungi. Chitosan and carboxymethyl chitosan showed antifungal activity against both *A. flavus* and *C. albicans*. Chitosan is slightly effective in increasing the inhibition zone diameter of *A. flavus* and *C. albicans* (11.2 ± 0.08 mm and 13.4 ± 0.1 mm, respectively) as compared with carboxymethyl chitosan (10.2 ± 0.09 mm and 12.4 ± 0.2 mm, respectively). It was observed that the grafting improved the antifungal activity of carboxymethyl chitosan. While the inhibition zone diameter for carboxymethyl chitosan ranged between 10.2 ± 0.09 mm and 12.4 ± 0.2 mm against indicated fungi, the inhibition zone increased up to 19.4 ± 0.04 mm (against C. *albicans*) by grafting. Although the difference is not significant, activity of *C. albicans* seems to be more pronounced; increase in the inhibition zone diameter is about 7 mm in *C. albicans,* whereas it is nearly 5 mm in *A. flavus*. Grafted samples showed an increasing antifungal activity as the degree of grafting increased for both the fungi; a minimum of 2 mm increase was observed consistently when the grafting percentage increased from 56% to 98%.

CONCLUSION

Carboxymethyl chitosan was grafted with N-acryloyl,N′-cyanoacetohydrazide for the first time in this work. The grafting process was conducted in a homogenous aqueous phase using potassium persulfate as an initiator. It was possible to control the graft content by changing the reaction parameters such as monomer concentration, initiator concentration, reaction temperature, and time. The grafted copolymers showed higher thermal stability relative to carboxymethyl chitosan as judged by a higher initial decomposition temperature and a lower weight loss at a particular temperature. The thermal stability of the grafted copolymers is affected by the extent of poly(N-acryloyl,N′-cyanoacetohydrazide) grafted onto carboxymethyl chitosan, being higher for copolymer with greater graft content. The crystallinity of the carboxymethyl chitosan was reduced by grafting. Grafting of N-acryloyl,N′-cyanoacetohydrazide onto carboxymethyl chitosan yields a range of copolymers from fully water-soluble to completely insoluble products. Thus, water solubility of the grafted copolymers is controlled by the extent of grafting. The grafted copolymers with lower grafting values are soluble in water, while increasing grafting yield decreases the water solubility. The grafted copolymers showed no cytotoxic effects against normal Madin-Darby bovine kidney epithelial cells. Grafted products showed a greater antibacterial activity as compared

with chitosan and carboxymethyl chitosan. The antibacterial activity of the copolymers against *E. coli* is stronger than against *S. aureus*. Grafting of N-acryloyl,N′-cyanoacetohydrazide onto carboxymethyl chitosan was more successful in inhibition of the growth of *C. albicans* fungus than *A. flavus* fungus. The increase in the grafting percentage leads to higher suppression of both bacterial and fungal growth.

EXPERIMENTAL

Materials

Chitosan with a degree of deacetylation of 88% and molecular weight of 2.0×10^5 was purchased from Acros Organics, New Jersey, USA. Potassium persulfate was of analytical grade and was supplied from Merck Chemicals. Other reagents and solvents were of analytical grades from Aldrich and were used as received. N-acryloyl-N′-cyanoacetohydrazide (CH_2=CH−CO−NH−NH−CO−CH_2−CN) was synthesized according to the method described previously.[18] Carboxymethyl chitosan was prepared following the method reported previously,[28] where chitosan (10 g), sodium hydroxide (13.5 g), and solvent isopropanol (100 mL) were suspended in a flask to swell and alkalize at rt for 1 h. The monochloroacetic acid (15 g) was dissolved in isopropanol and added to the reaction mixture dropwise within 30 min and reacted for 4 h at 55°C. Then the reaction was stopped and isopropanol was discarded. Ethyl alcohol (80%) was added and solid product was filtered and rinsed with 80% to 90% ethyl alcohol to desalt and dewater, and vacuum dried at 50°C. The degree of substitution of carboxymethyl chitosan was determined by pH titration[29] and found to be 0.75.

Graft Copolymerization

An exact amount (0.5 g) of dry carboxymethyl chitosan was dissolved in double-distilled water in a three-necked round-bottom flask, with a 1:50 liquor ratio, followed by the addition of a predetermined amount of N-acryloyl-N′-cyanoacetohydrazide (monomer). The flask was placed in a thermostat bath at a predetermined temperature. Nitrogen gas was bubbled for 30 min under stirring to remove the dissolved oxygen. A predetermined amount of potassium persulfate (initiator) was slowly added to the flask to initiate graft copolymerization. After a suitable time, the grafted copolymers having low graft content were precipitated in cold methanol, while grafted copolymers having high graft content were originally precipitated in the grafting medium at the end of the grafting process. After that, the grafted copolymers were separated by filtration and dried under vacuum at 50°C until constant weight was reached.

Exhaustive extraction using a soxhlet for 10 h of the products with methanol allowed for the purification of the graft copolymers from the homopolymer.[11] Grafting parameters such as grafting percentage (%G) and grafting efficiency (%GE) were determined as follows: $\%G = [(W_2 - W_1)/W_1] \times 100\%GE = [(W_2 - W_1)/W_3] \times 100$, where W_1, W_2, and W_3 denote the weights of initial carboxymethyl chitosan, grafted copolymers after extraction with methanol, and monomer charged, respectively.

Identification of the Grafted Copolymers

FTIR spectra were recorded on a Testcan Shimadzu FTIR-Spectrophotometer (Model 8000) using KBr pellets within the wave number range of 4000 to 400 cm^{-1} at 25°C. ^1H NMR spectra were recorded with Jeol 270 MHz (Japan) in DMSO-d_6 as a solvent and the chemical shifts were recorded in ppm relative to (TMS) as internal standard. Elemental analyses were carried out by the microanalytical unit at the National Research Centre, Giza, Egypt.

Characterization of the Grafted Copolymers

Scanning electron microscopy observations of the investigated samples were carried out as follows: The dry samples spread on a double-sided conducting adhesive tape, pasted on a metallic stub, were coated with a gold layer of 100-μm thickness using an ion sputter coating unit (Jeol S150A) for 2 min and observed with a Jeol-JXA-840A Electron Probe Microanalyzer. All the SEM photomicrographs were obtained using an accelerating voltage of 20 kV and at a magnification of 1000×. Powder x-ray diffraction patterns of the samples were obtained using Brukur D8 Advance-Germany with a Ni monochromator. The power level was 40 kV/40 mA. The x-ray source was CuKα radiation. The samples were maintained stationary while scattering angles from 4° to 80° were scanned in the reflection mode at a scanning rate of 1°/min^{-1}. Thermogravimetric analysis curves were recorded on a TGA-50H-Shimadzu Thermogravimetric Analyzer in nitrogen under a similar heating rate of 10°C/min^{-1} and with a temperature range from rt to 500°C. The nitrogen flow rate was 30 mL/min^{-1}. Solubility of the samples was tested in water, 1% acetic acid, methanol, 1% acetic acid:methanol mixture (1:1), DMF, DMSO, and THF. In each case 1% (w/v) solution was used.

Biological Evaluation of the Grafted Copolymers

Cytotoxicity of the grafted copolymers was evaluated using viability assay at the Regional Center for Mycology and Biotechnology, Al-Azhar University. Cell toxicity was monitored on Madin-Darby bovine kidney epithelial cells by determining the effect of the grafted copolymers on cell morphology and cell viability. For cytotoxicity assay, the cells were seeded in 96-well plates at

a cell concentration of 1×10^4 cells per well in 100 μL of growth medium. Fresh medium containing different concentrations of the tested copolymer was added after 24 h of seeding. Serial twofold dilutions of the chemical compound were added in confluent cell monolayer. The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO_2 for a period of 48 h. Three wells were used for each concentration of the copolymer sample. Control cells were incubated without copolymer sample and with or without DMSO. The little percentage of DMSO present in the wells (maximal 0.1%) was found not to affect the experiment. After incubation of the cells for 24 h at 37°C, various concentrations of fungal metabolite were added, and the incubation was continued for 24 h and viable cell yield was determined by a colorimetric method. In brief, after the end of the incubation period, the crystal violet solution (1%) was added to each well for 30 min. The plates were rinsed using tap water until all excess stain is removed. Glacial acetic acid was then added to all wells and mixed thoroughly, and then the plates were read on an ELISA reader, using a test wavelength of 490 nm. Treated copolymers were compared with the control in the absence of the fungal metabolites. All experiments were carried out in triplicate. The cell cytotoxic effect of each tested copolymer was calculated.[30,31]

The antibacterial and antifungal tests were carried out by standard disc agar diffusion well method.[32] The microorganism's inoculums were uniformly spread using sterile colon swabs on a sterile Petri dish malt extract agar (for fungi) and nutrient agar (for bacteria). One hundred microliters of each copolymer was added to each well (10-mm-diameter holes cut in the agar gel, 20 mm apart from one another). The systems were incubated for 24 to 48 h at 37°C (for bacteria) and at 28°C (for fungi). After incubation, microorganism growth was observed. Inhibition zones of the bacterial and fungal growth were measured in millimeters. Tests were performed in triplicate.

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