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Article

Synthesis and Characterization of Carboxymethyl Chitosan Nanogels for Swelling Studies and Antimicrobial Activity

Reem K. Farag [1] and Riham R. Mohamed [2,*]

[1] Egyptian Petroleum Research Institute (EPRI), Nasr City, Cairo, 11727, Egypt

[2] Department of Chemistry, Faculty of Science, Cairo University, Giza, 12613, Egypt

* Author to whom correspondence should be addressed; E-Mail: rihamrashad@hotmail.com

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Abstract: Nanogels of a binary system of carboxymethyl chitosan (CMCh) and poly-(vinyl alcohol) PVA, were successfully synthesized by a novel *in situ* process. They were also characterized by various analytical tools like Fourier transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM) and X-ray diffraction (XRD). They were studied for their unique swelling properties in water and different pH solutions. They were also investigated for their great ability to capture or isolate bacteria and fungi from aquatic environments.

Keywords: carboxymethyl chitosan; poly(vinyl alcohol); nanogels; antimicrobial activity; swelling ability

1. Introduction

Hydrogels—generally—have the ability to swell in water, retaining a significant fraction of water within their structure without dissolving [1]. They have been studied for various applications in different fields: medicine, pharmacy, biotechnology and controlled drug release [2–4]. New technologies of tissue engineering, drug delivery and regenerative medicine highlight the need for new biomaterials which are biocompatible, processable and biodegradable.

Thus, the development of biomaterials using natural polymers is an important and promising channel of research. Chitosan is an inexpensive and renewable material with many applications in cosmetics, pharmaceuticals, food science and biotechnology [5,6].

Due to the poor solubility of chitosan, *O*-carboxymethyl chitosan (CMCh), which is a water-soluble chitosan derivative, has attracted much attention as it widened its application fields. Carboxymethyl chitosan not only has a good solubility in water, but also has unique chemical, physical and biological properties such as high viscosity, large hydrodynamic volume, low toxicity, biocompatibility and good ability to form films, fibers and hydrogels [7,8].

For this reason, it has been extensively used in many biomedical fields such as a moisture-retention agent, a bactericide, wound dressing agent, artificial bone and skin, blood anticoagulant and as a component in different drug delivery matrices [9–11].

Carboxymethyl chitosan is prepared by means of carboxymethylation, as some of the –OH groups of chitosan were substituted by $–CH_2COOH$ groups. Therefore, the reactive ligands such as –COOH and $–NH_2$ groups are still amenable to chemical modifications to improve its physical properties for metal chelation and dye binding.

Poly(vinyl alcohol) (PVA) is a water-soluble polyhydroxyl polymer, used in practical applications because of its easy preparation, excellent chemical resistance and physical properties; it is completely biodegradable and cheap [12]. Blended hydrogels based on chitosan and poly(vinyl alcohol) (PVA) chemically crosslinked by glutaraldehyde were studied for their swelling in simulated body fluid. It was found that by increasing the chitosan to PVA ratio, simulated body fluid uptake of the material was significantly altered. All the tested hydrogels have clearly presented adequate cell viability, non-toxicity, and suitable properties which can be tailored for prospective use in skin tissue engineering [13]. A hybrid polymeric network was synthesized and modified by chemical crosslinking using genipin for potential use in a variety of biomedical applications [14]. Blended hydrogels are widely applied in medical fields, carboxymethyl chitosan (CMCS) and poly(vinyl alcohol) (PVA) blended hydrogels were prepared by Li *et al*. [15] using freezing and thawing techniques. The results showed that addition of CMCS can improve the physical properties of pure PVA hydrogels and could be applied as oral delivery systems for protein drugs. Wang *et al*. [16] prepared PVA/CMCS blend films by a mechanical blending method. He *et al*. showed that physically crosslinked poly(vinyl alcohol)/chitosan (CS) composite hydrogels were prepared by cyclic freezing/thawing techniques, and the microstructure and swelling behavior of the hydrogels in the simulated gastric (pH 1.0) and intestinal (pH 7.4) media were investigated [17].

Blend films exhibited different trends for bovine serum albumin (BSA) and bovine fibrinogen (BFG) adsorption when the pH of the medium changed. A novel chitosan derivative blend film of (HTCC)/PVA was prepared by chemical-crosslinking with glutaraldehyde, swelling equilibrium of blend films were improved with the increase of HTCC content. The results showed that the crosslinked (HTCC)/PVA blend film has good potential applications in biomedical fields, such as drug delivery and wound-dressing [18]. Biomedical applications of nanomaterials have become one of the most significant trends in the nanotechnology area. The nanometer size of polymeric nanoparticles, which is much smaller than that of blood cells, allows them to readily move in biological environments. Encapsulation of drugs and imaging agents into polymeric nanoparticles through physical or chemical conjugation was found to have a great potential in drug delivery and diagnostic applications. Polymer-based nanoparticles are the most extensively studied nano-sized drug carriers. The nanoparticles used for drug delivery generally have a size ranging from a few nm to 1,000 nm, with versatile structures and morphologies. The advantage of nanogels is based mainly on their small

particle size which provides large surface area with good surface properties. The small size of nanogels helps in increasing drug/protein stability and confers useful controlled release properties. Nanoparticles could cross the biological barriers to reach the target sites inside the cells due to their small size, thus achieving an improved effect as reported in the literature [19,20].

This work attempts to synthesize new nanogels by a novel method (surfactant free method) and characterization of their properties via various analyses. Different applications were studied for these nanogels, namely swelling ability at different pH values and their antimicrobial activity.

2. Results and Discussion

A novel (surfactant free) method for nanogel synthesis was used to give nanogels based on CMCh and PVA taken in different ratios. This method depends on preparing complex nanogels in the absence of any surfactant. Thermosensitive polymer chains can collapse to form stable dispersed nanospheres above their lower critical solution temperature (LCST) in aqueous media. Characterization was done on the prepared nanogels via various analysis tools.

2.1. IR Analyses

FTIR spectra of chitosan—Figure 1a—shows two bands at 3445 and 3422 cm^{-1} corresponding to the $–NH_2$ group, while the spectra of CMCh (Figure 1b) shows a strong peak at 1412 cm^{-1} which could be assigned to the symmetrical COO^- group stretching vibration. The asymmetrical stretching vibration of the COO^- group near 1550 cm^{-1} is overlapped with the deforming NH_2vibration at 1600 cm^{-1} to give a very strong peak. The C–O absorption peak of the secondary hydroxyl group became stronger and was shifted to 1074 cm^{-1}. The results indicated that the carboxymethylation process had occurred at the C_6 position of chitosan. The spectrum of CMCh shows also a peak at 1480 cm^{-1} which refers to the $–CH_2–COOH$ group. The broad peak in CMCh at 3400–3200 cm^{-1} is caused by both O–H and N–H stretching vibrations and the peak at 2900 cm^{-1} is due to the C–H stretching vibrations.

Figure 1. (**a**) FTIR for non-modified Chitosan; (**b**) FTIR for (A) CMCh, (B) PVA, (C) CMCh/PVA.

(**a**)

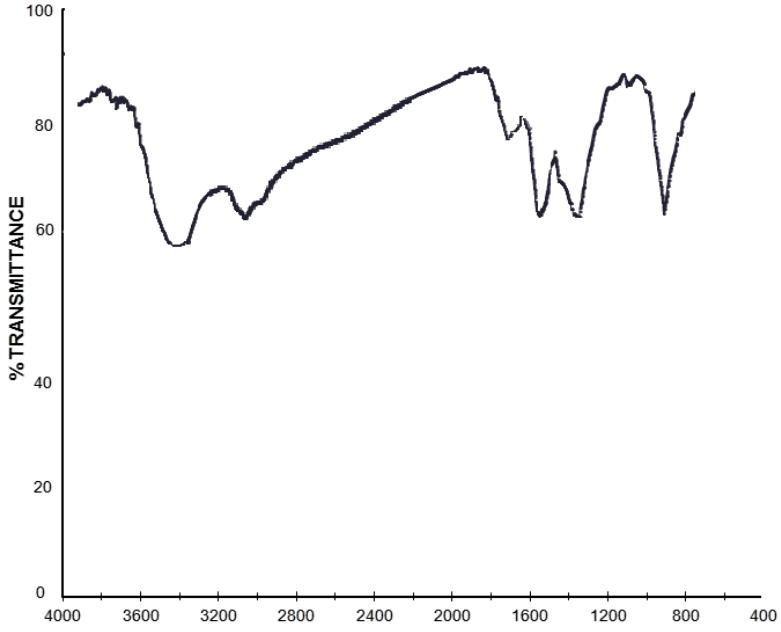
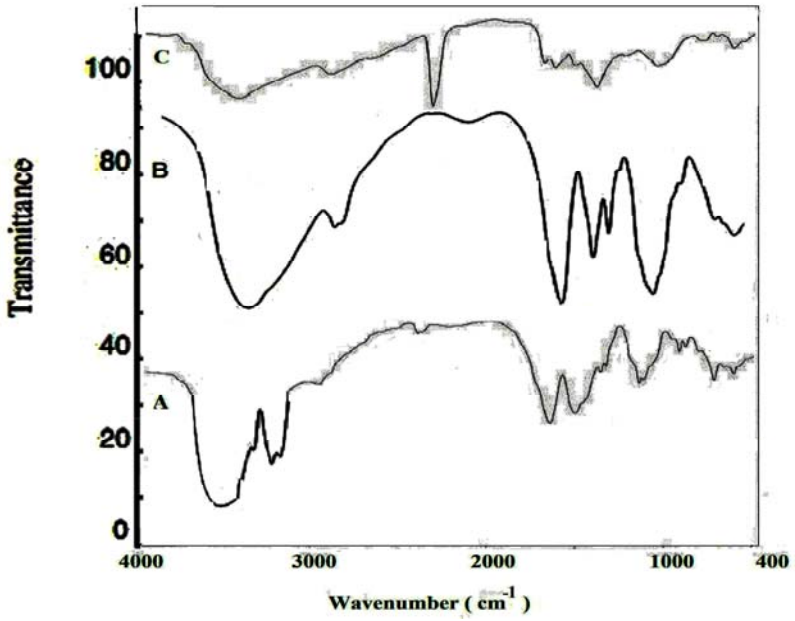


Figure 1. *Cont.*

(**b**)



FTIR spectra of the formed nanogels were studied as represented in Figure 1b, FTIR spectra of both pure CMCh and PVA are given for comparison. In trace B, the FTIR spectrum of PVA, the following peaks are recorded: 2800–3000 cm^{-1} which corresponds to the stretching of –CH groups in alkanes, 1100 cm^{-1}, characteristic for C–OH stretching, and a wide-strong absorption peak at 3500–3350 cm^{-1} is associated with the stretching of O–H from the intermolecular and intramolecular hydrogen bonds [21]. Peaks between 1750 and 1735 cm^{-1} are due to the stretching of C=O and C–O from acetate groups remaining from PVA [22].

As for the prepared nanogels, they contain a strong absorption peak at 3400 cm^{-1} coming from both the stretching of O–H of PVA and also the O–H and N–H stretching vibrations of CMCh chains. These nanogels have a strong peak at 1100 cm^{-1} characteristic for C–OH stretching coming from PVA. These nanogels have a strong peak at 1587–1650 cm^{-1} corresponding to the –COOH groups in CMCh.

2.2. Transmission Electron Microscopy

The morphology of PVA in water and water/acetone was investigated by TEM. Figure 2 shows some changes from its initial coil or aggregate caddice-like unattached particulates. Also the micrograph shows a rope-like configuration with aggregates of about 200 nm in diameter and over 2 mm in length. TEM micrograph for PVA dissolved in (water/acetone, 15:85, v/v) reveals that PVA nanoparticles could be formed in a mixed solvent of water/acetone. Images of CMCh/PVA nanoparticles that formed via the polymerization of CMCh in 1% PVA water/acetone solution are represented in Figure 3a–c according to the different applied concentrations of CMCh, namely; 1:2, 1:1, and 2:1 g/100 mL. respectively. The morphology of the nanoparticles was greatly affected by the initial CMCh concentration in solution. In Figure 3a,b, the polymerized particles (0.5 g and 1 g/100 mL PVA) show sphere-shaped particles with an average diameter of 15 and 20 nm. Figure 3c with a higher concentration of CMCh (2 g/100 mL) resulted in nanogels with diameters of 10 nm. This shows that

the increase in CMCh concentration decreases CMCh/PVA nanogels particle size up to 2 g/100 mL concentration of CMCh. Similar results were obtained by Mrkic and Saunders [23].

Figure 2. TEM micrographs of PVA in (**a**) water, (**b**) water/acetone solution.

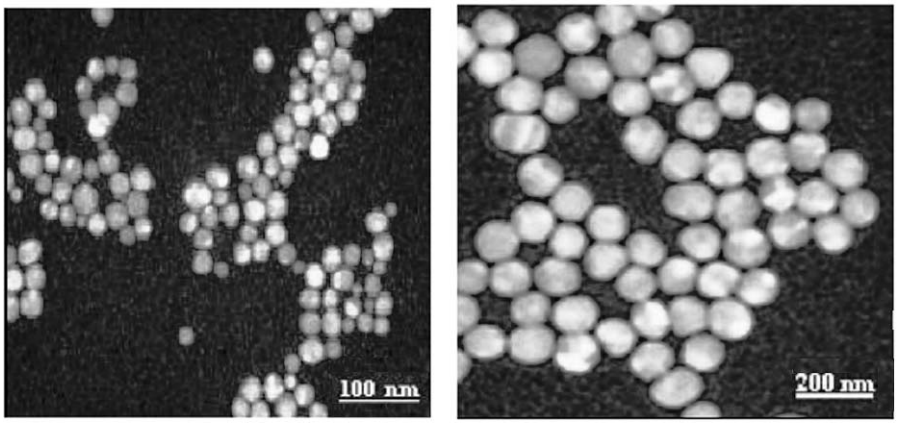
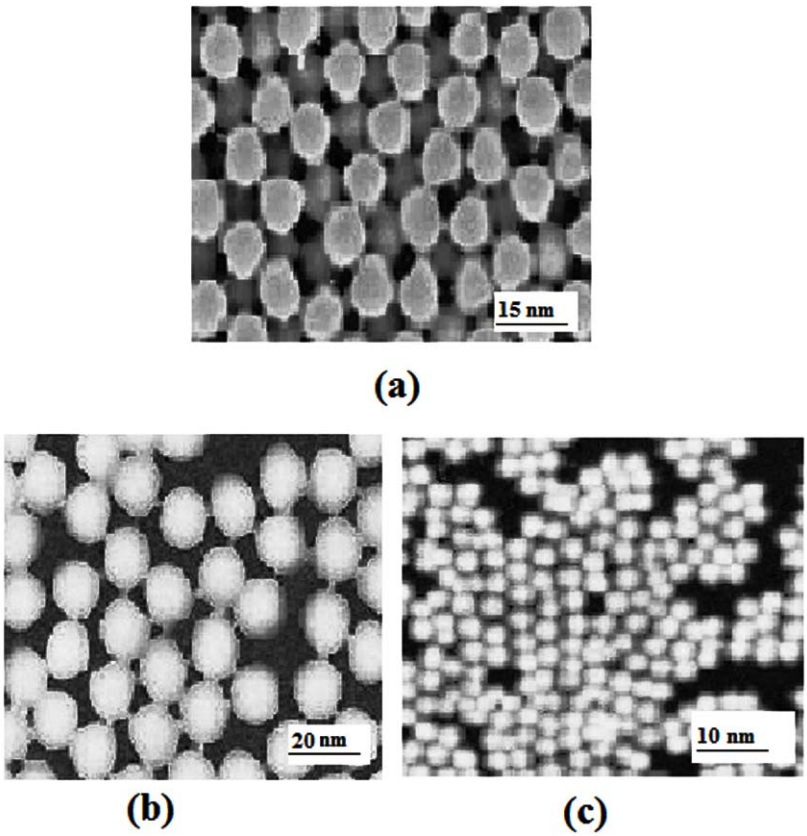


Figure 3. TEM micrographs of CMCh/PVA nanogels; (**a**) CMCh/PVA (2:1); (**b**) CMCh/PVA (1:1); (**c**) CMCh/PVA (1:2).

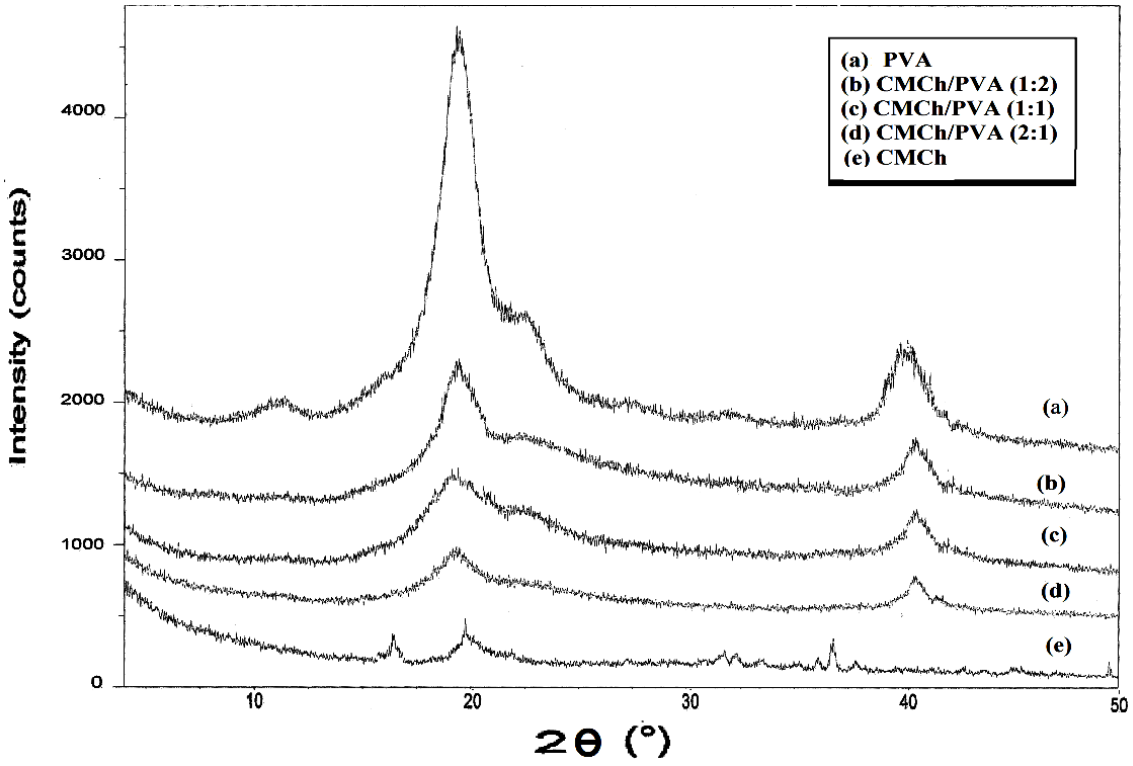


2.3. X-ray Diffraction

The high crystalline structure of PVA is confirmed by the observed intense and strong peaks in Figure 4. CMCh and CMCh/PVA nanogels are also shown for comparison. The diffraction peaks of carboxymethyl chitosan are located around 16.5°, 20° and 36° and they are very weak, indicating low

crystallinity. This is in a good agreement with literature [24]. However diffraction peaks of pure PVA are located at 2θ~20° and 40° and they are strong and intense indicating the high crystalline structure of PVA. On the other hand, the X-ray investigations showed that CMCh/PVA nanogels contained two diffraction peaks occuring at 2θ~20° and 40°. Their intensities decreased with increasing CMCh content. Similar results of XRD analysis were reported in literature for PVA/chitosan hydrogels [20], PVA/carboxymethyl cellulose [25] and similar XRD chart was obtained in case of PVA/carboxymethyl chitosan blend hydrogels by the same author in this research [26]. Therefore, addition of CMCh suppresses the capacity of PVA to crystallize into nanogels. In PVA blends, this may imply an interaction between the components [26].

Figure 4. X-ray diffraction patterns for PVA, CMCh and CMCh/PVA nanogels [26].



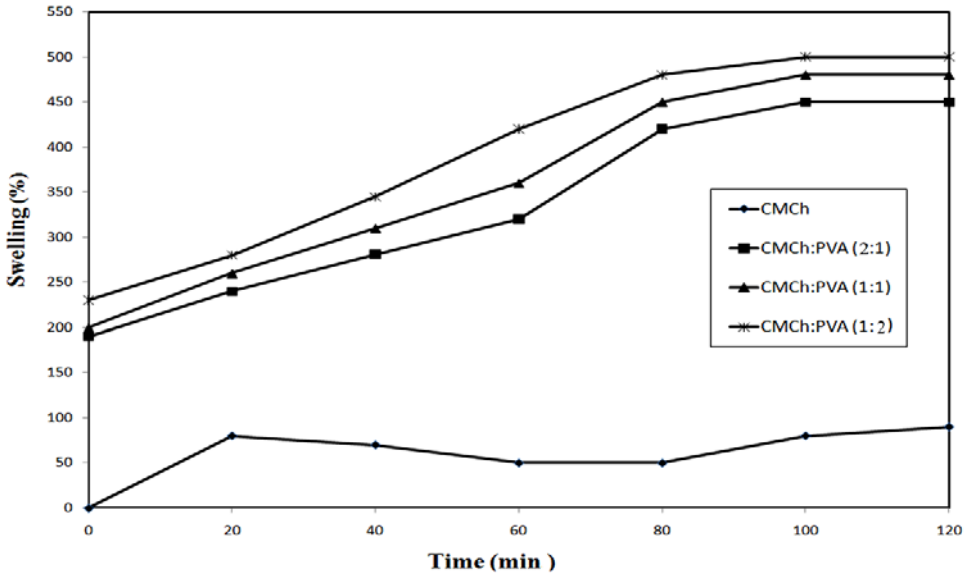
2.4. Swelling Behavior

The affinity of polymeric hydrogels for solvents can be easily determined by their sorption ability. The swelling process of polymers involves the diffusion of the liquid phase into the hydrogel bulk, which is possible by the mobility of the polymeric chains and the free-volume among chains. The molecules of the liquid remain entrapped in these free spaces up to an equilibrium point, as determined by both polymer solvent interactions and the elasticity of the hydrogel. Figure 5a shows the water swelling kinetics of CMCh, CMCh/PVA (2:1), CMCh/PVA (1:1) and CMCh/PVA (1:2) at room temperature. The increase in water sorption is related to the increase in the amount of PVA dispersed in the hydrogel, which enhances the polarity of the nanogels. Similar results to that were described for a system based on chitosan, in which the blending with PVA increased the water uptake of the resulting material up to 150 wt% [27]. The water uptake rate sharply increases and then begins to level off. The equilibrium swelling was achieved after 100 min. The swelling corresponds to the

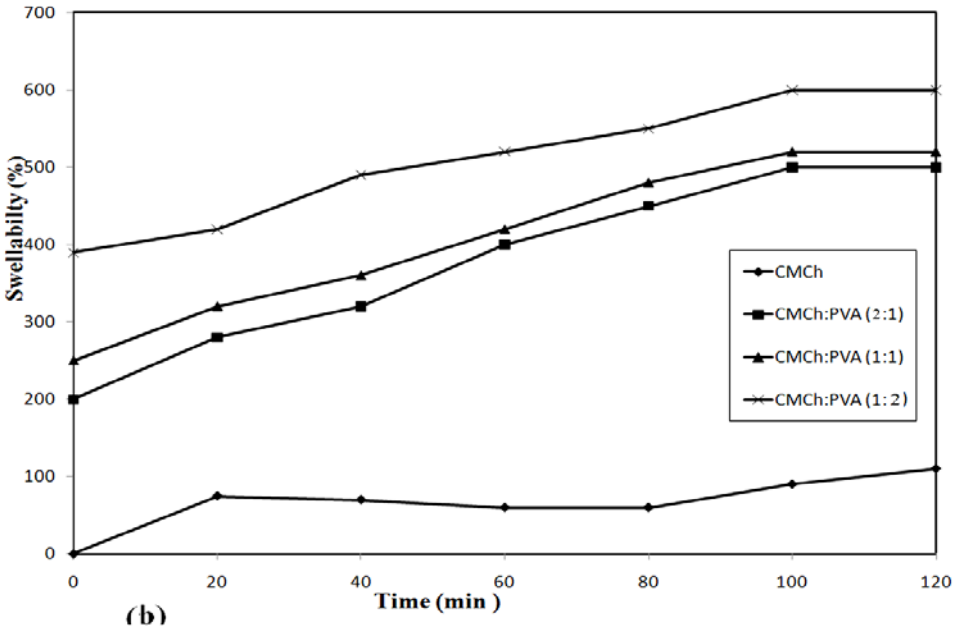
crosslinking behavior. In this study, the prepared nanogels made from CMCh/PVA show maximum water-swelling % of 500.

The CMCh/PVA nanogels swelled much more than CMCh in acidic buffer solution, as shown in Figure 5b. This swelling ability of the nanogels increases with the increase of the PVA content due to its hydrophilicity. The equilibrium swelling was achieved also after 100 min and the CMCh/PVA nanogels show the maximum water-swelling % of 600. On the other hand, the swelling ability of CMCh/PVA nanogels is much less than that of CMCh in basic buffer solution due to its strong acidic character. This swelling ability decreases as the PVA content increases, as shown in Figure 5c.

Figure 5. (**a**) Water swelling kinetics of CMCh/PVA nanogels at room temperature; (**b**) Swelling kinetics of CMCh and CMCh/PVA nanogels in pH 4 at room temperature; (**c**) Swelling kinetics of CMCh and CMCh/PVA nanogels in pH 9 at room temperature.

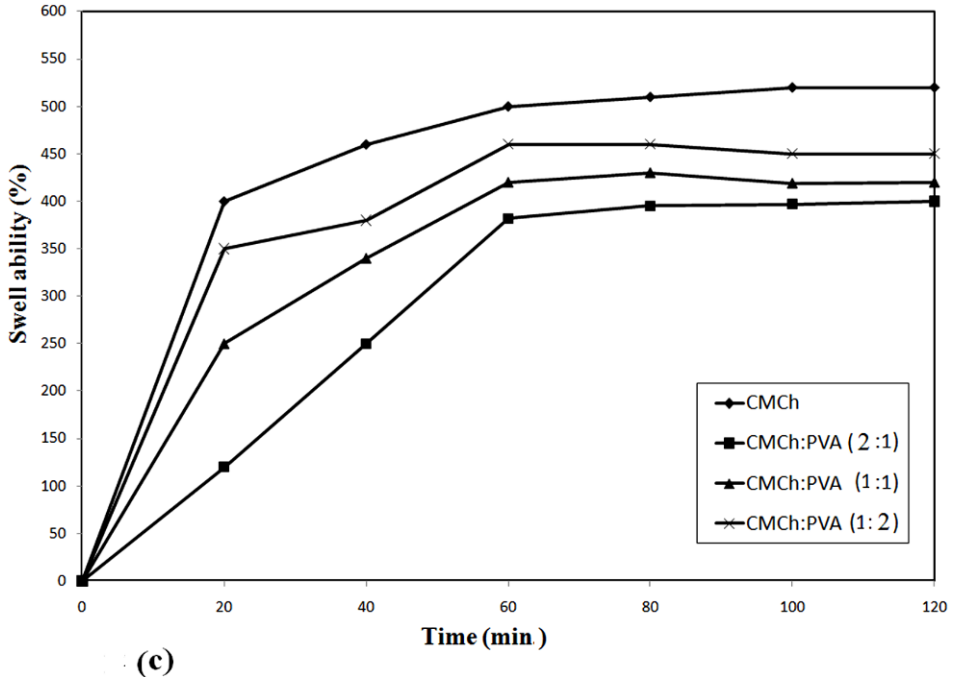


(a)



(b)

Figure 5. *Cont.*



2.5. Antimicrobial Activity for Investigated Nanogels

Table 1 shows the effect of exposure of the Gram positive *S. aureus* and the Gram negative *E. coli* to CMCh, which caused a decrease in viable cell counts of both bacterial spp. It was observed that CMCh was effective in decreasing the viable cell count of *S. aureus* (inhibition zone diameter 25 mm/sample), and CMCh was more potent (inhibition zone diameter 30 mm/sample) in case of *E. coli.* Similar behavior was noticed with Sabaa *et al.* [28]. Also, CMCh/PVA (1:1) had higher inhibitory activity on *E. coli* (inhibition zone diameter 38 mm/sample) than CMCh/PVA (2:1) with an inhibition zone diameter 35 mm/sample. It was obvious that increasing the percentage of PVA caused higher inhibition of viable cell counts of both *S. aureus* and *E. coli*, while pure PVA has no antibacterial activity [18]. Generally, the antibacterial activities of the tested nanogels were stronger against *E. coli* than *S. aureus*. Results also reveal the antifungal activities of CMCh and CMCh/PVA nanogels when tested on the pathogenic fungus *Aspergillus flavus* and *Candida albicans*. CMCh showed an inhibitory effect on the growth of both *Aspergillus flavus* (inhibition zone diameter 18 mm/sample) and *Candida albicans* (inhibition zone diameter 15 mm/sample). It was obvious that addition of PVA onto CMCh was also successful in inhibiting fungal growth, CMCh/PVA (1:2) exhibited a higher effect (inhibition zone diameter 35 mm/sample for *Aspergillus flavus* growth and 36 mm/sample for *Candida albicans* growth) than CMCh/PVA (1:1) which exhibits (inhibition zone diameter 30 mm/sample for *Aspergillus flavus* growth and 32 mm/sample for *Candida albicans* growth). The high antimicrobial activity of these nanogels suggested good potential for applications in biomedical fields.

Table 1. Antimicrobial activity for the CMCh/PVA nanogels.

Sample		Inhibition zone diameter (mm/sample)			
		Esherichia coli ***(G^-)***	***Staphylococcus aureus*** ***(G^+)***	***Aspergillus flavus*** ***(fungus)***	***Candida albicans*** ***(fungus)***
Tetracycline antibacterial agent	**Standard**	32	27	--	--
Amphotericin B antifungal agent		--	--	20	18
CMCh		30	25	18	15
CMCh /PVA (2:1)		35	31	25	22
CMCh/ PVA (1:1)		38	36	30	32
CMCh/ PVA (1:2)		40	39	35	36

Error % = + or − 3%.

3. Experimental

3.1. Materials

Chitosan (code KB-002) was purchased from Funakoshi Co. LTD, Tokyo, Japan. Deacetylation content was determined to be 88.2%. PVA with a hydrolysis degree of 99.0%–98.0% (molecular weight = 7.7×10^4 g/mol) was obtained from Loba Vhemi PVT. Ltd, Bombai, India. *N,N′*-methylene bisacrylamide (MBA) and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were all purchased from Aldrich Chemical Co. (Steinheim am Albuch, Germany); potassium peroxydisulfate (KPS), sodium hydroxide, monochloroacetic acid, acetic acid and methanol were all used without further purification.

3.2. Preparation of Carboxymethyl Chitosan

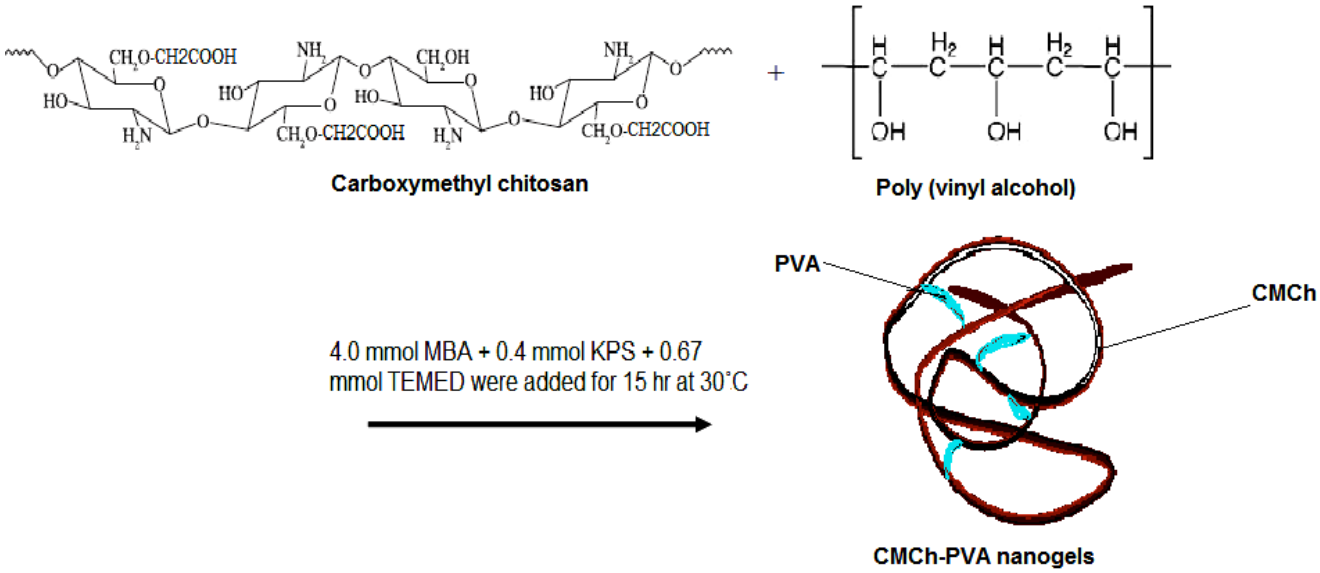
Carboxymethylation was carried out by stirring chitosan (5 g) in 20% NaOH (w/v, 100 mL) for 15 min. then monochloroacetic acid (15 g) was added dropwise to the reaction mixture and the reaction was continued for 2 h at 40 ± 2 °C with stirring. Then the reaction mixture was neutralized with 10% acetic acid, then was poured into an excess of 70% methanol. The produced carboxymethyl chitosan was filtered using a G_2 sintered funnel and was washed with methanol. The product was dried in a vacuum at 55 °C for 8 h to give 6.5 g of dried carboxymethyl chitosan. The degree of substitution of CMCh was determined to be 0.75 according to the method described in literature [29].

3.3. Synthesis of CMCh/PVA Nanogels

PVA (1 g) was dissolved in water (85 mL) at 45 °C. After the PVA-water solution was cooled to room temperature, acetone (15 mL) was added dropwise to the vigorously stirred PVA solution for 15 min to form about a 1% (w/v) PVA solution. Then the solution was kept at 5 °C for 24 h till it became light yellow, which indicated that the long chains of PVA shrank to nanoparticles. Then different amounts of CMCh (0.5, 1 and 2 wt%) were added to the solution. The solution was purged with N_2 for 30 min, then 4.0 mmol MBA, 0.4 mmol KPS, and 0.67 mmol TEMED were added to the solution to carry out polymerization for 15 h at 30 °C. The formed nanogels could be used directly or could be frozen to get

freeze dried powder, which can be easily predispersed into water forming nanoparticles dispersion [30]. The reaction could be shown with a simple schematic diagram (Figure 6).

Figure 6. Schematic diagram of nanogels formation.



3.4. Infrared Spectroscopy

FTIR spectra were recorded in KBr discs on a Shimadzu FTIR model 8000 Testcan IR-spectrometer under dry air at room temperature within the wave number range of 4,000–500 cm^{-1}.

3.5. X-ray Diffraction

X-ray Diffraction was done using a Brüker D_8 Advance instrument, at 40 KV, 40 mA using target Cu K_α with secondary monochromator (Karlsruhe, Germany).

3.6. Transmission Electron Microscopy

Micrographs of the colloidal nanogel particles were taken using a JEM-100S Transmission Electron Microscope (TEM, Jeol, Tokyo, Japan). The TEM sample was prepared by mixing one dilute drop of prepared aqueous particles dispersed in 5 mL acetone to give a slightly turbid solution on the copper grid and allowing it to dry well. The images of representative areas were captured at suitable magnifications which clarify the morphology and the size of the nanoparticles.

3.7. Swelling Behavior of Nanogels

The equilibrium swelling was performed to characterize the pH-responsive behavior of CMCh/PVA nanogels. To determine the equilibrium swelling behavior, freeze-dried CMCh/PVA (100 mg) was dispersed into distilled water (10 mL) and different buffer solutions of pH values of 4.00 and 9.00. The pH values of solutions were determined by the pH meter and the size of CMCh/PVA particle was measured using laser particle size analyzer before and after swelling. The degree of swelling was defined as the volume ratio of CMCh/PVA after and before swelling. The measurements were made in

triplicates using an analytical balance, and the error % was estimated to be 10%. The equilibrium degree of swelling of the gel was calculated as:

$$\text{Swelling} = W_e/W_d$$

where W_e is the weight of gel at the equilibrium and W_d is the weight of initial dry gel. Experiments were carried out till 120 min, and then all pieces were dried and re weighed again.

3.8. Antimicrobial Activity of the Prepared Nanogels

Antimicrobial activity of the nanogel samples was determined using a modified Kibry-Baur disc diffusion method [31,32]. Briefly, the test bacteria/fungi (100 μL) were grown in fresh media (10 mL) until they reached a count of approximately 10^8 cells/mL for bacteria or 10^5 cells/mL for fungi [33]. Microbial suspension (100 μL) was spread onto agar plates corresponding 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 [34]. Of the many media available, NCCLS recommends Mueller-Hinton agar due to its good batch-to-batch reproducibility results. Disc diffusion method for filamentous fungi was tested by using approved standard method (M38-A) developed by researchers [35] for evaluating the susceptibilities of filamentous fungi to antifungal agents. Disc diffusion method for yeasts was developed by using approved standard method (M44-P) by NCCLS [36]. Plates were inoculated with filamentous fungi as *Aspergillus flavus* at 25 °C for 48 h; *Gram* (+) bacteria as *Staphylococcus aureus*; *Gram* (−) bacteria as *Escherichia coli*. They were incubated at 35–37 °C for 24–48 h and yeast as *Candida albicans* was incubated at 30 °C for 24–48 h and then the diameters of the inhibition zones were measured in millimeters.

Standard disc of tetracycline (antibacterial agent), amphotericin B (antifungal agent) served as positive controls for antimicrobial activity but filter dics impregnated with 10 μL of solvent (distilled water, chloroform, DMSO) were used as a negative control. The agar used is Meuller-Hinton agar that is rigorously tested for composition and pH value. Further the depth of the agar in the plate is a factor to be considered in the disc diffusion method. This method is well documented and the standard zones of inhibition have been determined for susceptible and resistant values.

Blank paper disks (Schleicher & Schuell, Barcelona, Spain) with a diameter of 8 mm were impregnated with 10 μL 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 round the disc. If an organism is placed on the agar it will not grow on 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 calipers of National Committee for Clinical Laboratory Standards [36]. Agar-based methods such as Etest and disk diffusion can be good alternatives because they are simpler and faster than broth-based methods [37].

Antimicrobial measurements were done as the average of three times on four samples CMCh, CMCh/ PVA with its three ratios. PVA alone has no antibacterial activity, but in its combinations with

CMCh and when its content increases in the nanogels, it gives higher inhibition than CMCh itself till it reaches the maximum value at CMCh/PVA (1:2), a similar behavior was reported in literature with PAN [38].

4. Conclusions

A novel (surfactant free) method for nanogel synthesis based on CMCh and PVA in different ratios was reported and then characterization via various analyses was done on the prepared nanogels; the following results were obtained:

1. TEM results showed that the increase in CMCh concentration results in a decrease in CMCh/PVA nanoparticles size up to 2 g/100 mL concentration of CMCh.
2. Prepared nanogels can provide satisfying properties such as pH-sensitivity swelling, improved surface property and good antibacterial activity. The swelling study showed water absorption up to 500% after 2 h.
3. The prepared nanogels exhibited good antibacterial activities against both types of bacteria, *Eschericia-coli* (G−) and *Staphylococcus aureus* (G+). Also the two types of fungi, *Aspergillus flavus* and *Candida albicans* are affected by the prepared nanogels.
4. The antimicrobial activity of the prepared nanogels increases with the increase of PVA content.

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