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Synthesis and characterization of photo-crosslinkable hydrogel membranes based on modified chitosan

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ABSTRACT

In order to develop a non toxic and biocompatible hydrogel system with potential ability in biotechnology, modified photo-crosslinkable hydrogel membranes based on chitosan were prepared. Using an EDC/NHS conjugation method, chitosan was chemically modified to incorporate a photosensitive α-cyano-4-hydroxycinnamic acid moiety with various degrees of substitutions. Fourier transform infrared spectra (FTIR), proton nuclear magnetic resonance (^{1}H NMR) and ultraviolet–visible light spectra (UV–vis) were used for structural characterization of modified chitosan, The obtained membranes were crosslinked by irradiation in the ultraviolet region, where the photosensitive monomers showed maximum sensitivity. The prepared photo-crosslinked hydrogel membranes were investigated by thermal gravimetric analysis (TGA) and wide angle X-ray diffraction (WAXD). The swelling behaviors were investigated in terms of pH, time of swelling, and degree of substitution. Also, mechanical properties of the different photo-crosslinked hydrogel membranes were studied in both dry and wet conditions.

1. Introduction

Chitosan membranes have been explored in many uses, such as in water ethanol pervaporation [1–3], enzyme immobilization and cationic specimen transportation [4,5], protein separation and concentration [6,7], controlled ingredient-release [8–10], environmental applications [11–14], among others. The preparation of chitosan structures explores the polycationic nature of this biopolymer. Chitin and chitosan present amino and hydroxyl groups highly reactive and resistant to organic solvents. Most of membrane architectures made with these natural polymers are usually obtained by casting and coagulation of their acidic solutions. Chitosan is a well-known filmogenic material. Further treatments are usually done in order to turn chitosan more bacteriostatic, and to improve chemical and mechanical resistance. Previous work explored a very interesting route to modify chitosan by crosslinking using glutaraldehyde [14,15]. The use of a bifunctional agent can block amino groups and turn chitosan structures more inert and resistant to acidic media. Chitosan can also be modified easily to form porous hydrogels for existence of reactive amino group [16]. For this reason, photo-polymerizated injectable chitosan have received great attention [17]. For chitosan hydrogels by photo-polymerization, the temperature and pH can be similar with body environment on the polymerization process. Additionally, control of polymerization reaction can be accomplished though adjusting the exposure area and the time of light incidence. Several methods have been used to modify chitosan and the properties of modified polymers have been investigated [18–22].

In an effort to devise a crosslinking strategy that is efficient, achieves precise control over the nature and the degree of crosslinking, and facilitates spatial and temporal control over the reaction process, we describe herein a versatile strategy, which includes the loading of α-cyano-4-hydroxycinnamic acid onto the chitosan backbone through the amide bond formation using carbodiimide and *N*-hydroxysuccinimide (EDC/NHS) via initial carboxyl group activation followed by reaction with chitosan bearing amino groups. The known photo-sensitivity of cinnamate groups is primarily based on the π electron density of the photoactive chromophore (–CH=CH–), which can be dimerized as a result of [2π + 2π] electron cycloaddition reaction [23,24]. Significantly, this reaction does not require the addition of a light sensitive initiator, which is typically required for crosslinking reactions based on photosensitive acrylate, acrylamide or azide moieties [25–27]. As a consequence, unanticipated side reactions due to the presence of free radical initiators are minimized. Although cinnamate chemistry has been widely applied in photo-lithography and has potential applications in liquid crystal display technology, its application in the

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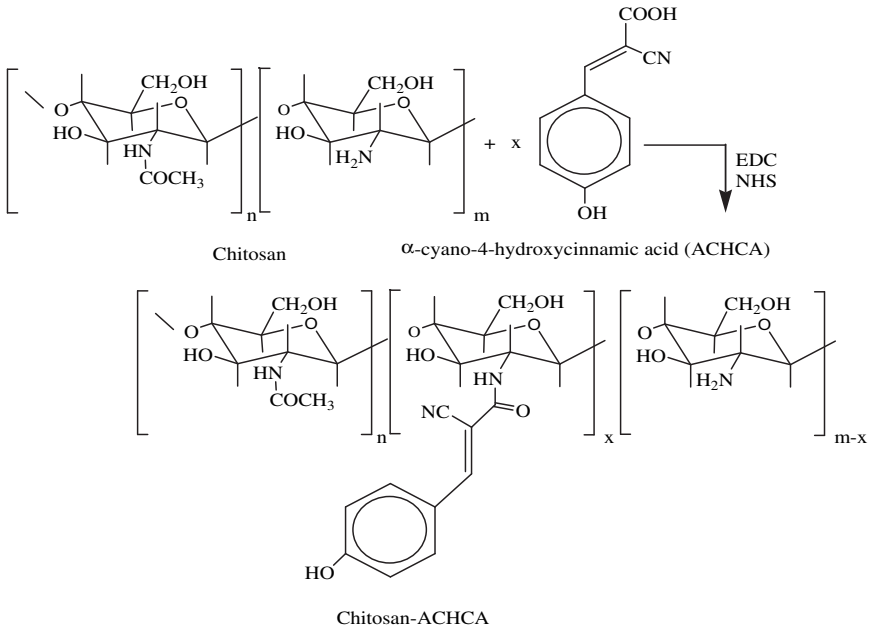


Scheme 1. Synthesis of chitosan-ACHCA [19].

crosslinking of biopolymers has been somewhat more limited. Significantly, Matsuda and colleagues [28–30] have reported the synthesis and characterization of a variety of photo-crosslinkable hydrophilic polymers, including chondroitin sulfate, hyaluronan, and gelatin, using cinnamate and other photo-dimerizable groups. However, to our knowledge this is the first report, which describes the synthesis and characterization of photo-crosslinked chitosan–cinnamate derivatives hydrogel membranes.

2. Experimental

2.1. Materials

Chitosan (molecular weight = 1.5×10^5 amu) and with degree of deacetylation of 85%, α-cyano-4-hydroxycinnamic acid (ACHCA), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, commercial grade) and *N*-hydroxysuccinimide (NHS, 98%) were purchased from Sigma Aldrich. All chemicals were used as received.

2.2. Instrumentation

Fourier transform infrared spectra (FTIR) were obtained with a Perkin–Elmer spectrophotometer with ATR accessory. The chitosan and modified chitosan were dried overnight at 60 °C under reduced pressure and pressurized with a glass slide on top of the quartz window of the ATR instrument.

Table 1
Synthesis of chitosan-ACHCA using EDC/NHS conjugation method.[a]

Formulation code	ACHCA (mol/L)	DS[b] (mol %)
CACN-1	0.0264	28%
CACN-2	0.0449	43%
CACN-3	0.0687	54%
CACN-4	0.0952	69%
CACN-5	0.1269	78%

[a] Concentration of chitosan solution = 5 g/L; EDC:NHS:ACHCA = 2:2:1; reaction time: 24 h at room temperature.

[b] Degree of ACHCA substitution (DS) determined by ^{1}H NMR, DS (mol%) = $(I_8 + I_9 + I_{10} + I_{11})/(6 \times I_2)$, where I_8, I_9, I_{10} and I_{11} denote the integrals of the peaks of the introduced ACHCA moiety, and I_2 denotes the integral of the peak of the proton H^2 in C-2 of the chitosan [19,28,29].

^{1}H NMR spectra were recorded by an Oxford NMR instrument at 500 MHz at room temperature using 1% deuterated acetic acid in D_2O as a solvent. The experimental degree of substitution (DS) of the final modified chitosan was calculated from the integration of the appropriate peaks [31].

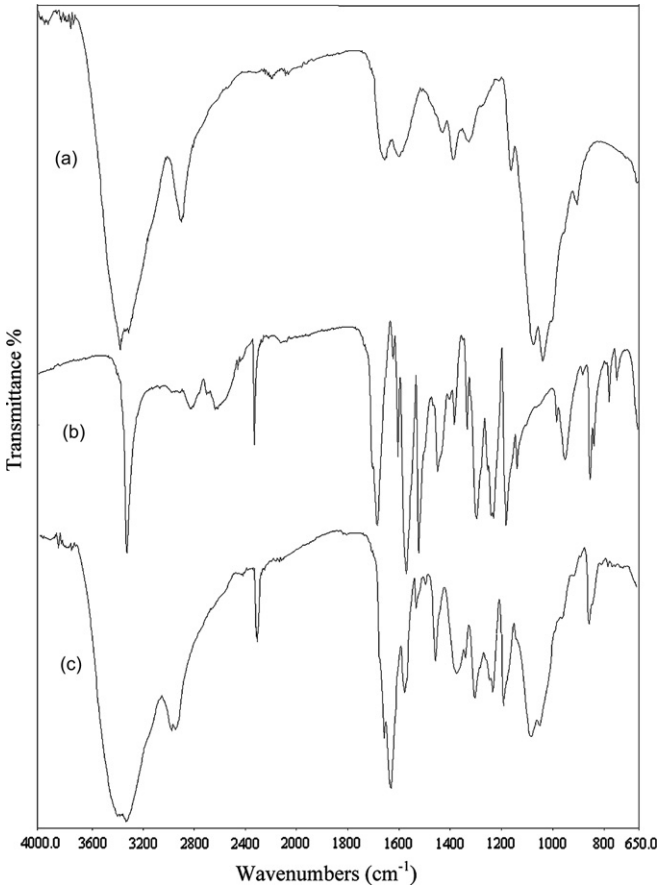


Fig. 1. FTIR spectra of (a) chitosan (b) ACHCA (c) chitosan-ACHCA (CACN-4) [19].

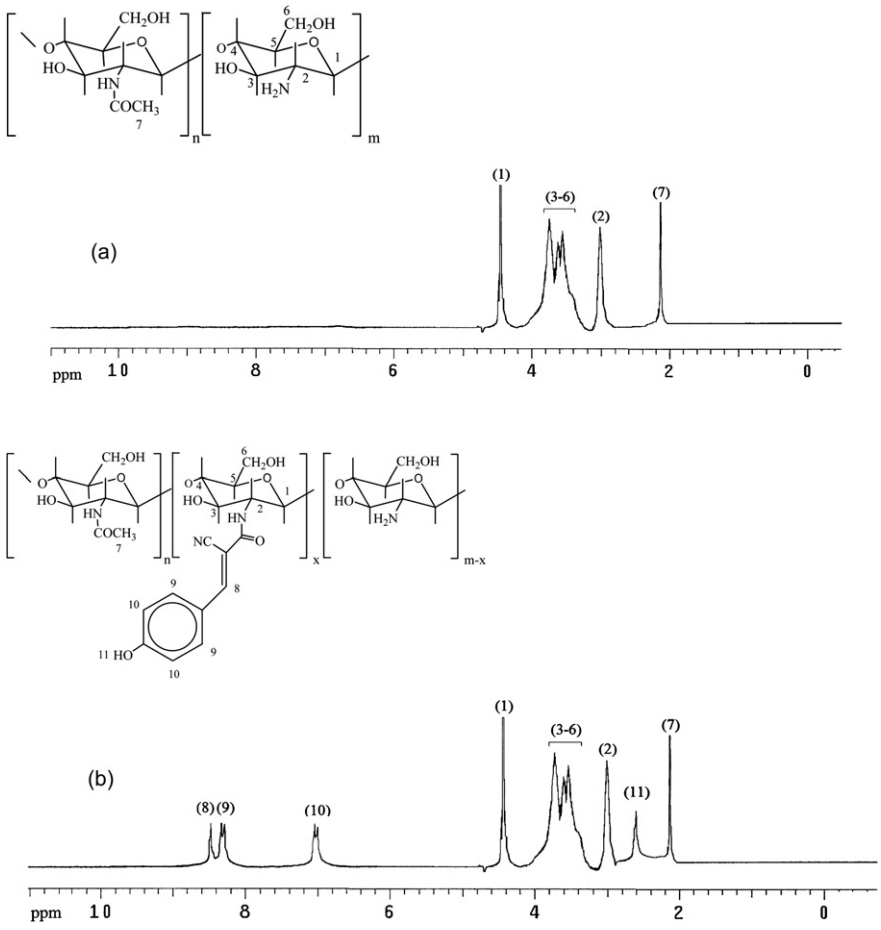


Fig. 2. ^{1}H NMR spectra of (a) chitosan (b) modified chitosan-ACHCA (CACN-5) with CD_3COOD/D_2O as solvent [19].

UV–vis. spectroscopy was used to monitor the kinetics of the photo-crosslinking process of chitosan-ACHCA membrane irradiated with multiband UV lamp 254/365 nm (Mineralight lamp model UV GL-25). All UV–vis spectrophotometric measurements were executed at room temperature on a Perkin–Elmer Bio UV–visible spectrophotometer.

Tensile strength–elongation tests were performed according to the standard method (ASTM D 882) using a Lloyd universal testing machine (model LR5K, UK). The test specimens were cut into strips 50 mm long and 15 mm wide, and the thickness of each strip (approximately 100 μm) was measured with digital vernier calipers. The relative humidity, temperature, gauge length and crosshead speed were set at 50 ± 5%, 25 °C, 30 mm and 5 mm min^{-1}, respectively. Eight replicate measurements were performed and the average of the six data points remaining after discarding the maximum and minimum values was calculated. The wet condition samples were prepared by immersed them into water for 2 h followed by removing the excess of water.

Thermogravimetric analysis (TGA) was performed on chitosan and modified chitosan by using a DuPont-2000 instrument. Experiments were performed with 2–3 mg of the sample under a dynamic nitrogen atmosphere flowing at a rate of 50 ml/min and at a heating rate of 10 °C/min.

Wide angle X-ray diffraction (WAXD) patterns of the hydrogels were recorded on X-ray diffractometer (D/Max2500VB2+/Pc, Rigaku, Japan) with Cu Kα characteristic radiation (wavelength $\lambda = 0.154$ nm) at a voltage of 40 kV and a current of 50 mA. The scanning rate was 5°/min and the scanning range of 2θ was from 5° to 55° at room temperature.

2.3. Synthesis of chitosan-α-cyano-4-hydroxycinnamate (Chitosan-ACHCA)

Chitosan-ACHCA was prepared by the reaction of chitosan with ACHCA in presence of EDC and NHS which used as activating agents for the amide bond formation [31] according to our previous work [19] as shown in Scheme 1. The formulation details used in synthetic procedure are summarized in Table 1. First, 0.5 g chitosan was dissolved in 50 mL mixture of 2% wt acetic acid in water: acetonitrile (1:1) (sol A). Specific amounts of EDC, NHS and ACHCA were then charged in another vial that contained a 50 mL mixture of water: acetonitrile (1:1) and stirred for 5 min until full dissolution (sol B). Sol B was then added drop wise into sol A and the obtained solution was stirred for 24 h in dark at room temperature. The polymer was recovered with the addition of a large amount of acetone. FTIR, ^{1}H NMR, UV–vis. spectroscopy, TGA and WAXD were used to monitor the incorporation of ACHCA unites. The quantitative determination of DS of the final modified chitosan was calculated using ^{1}H NMR spectra.

The modified chitosan-ACHCA with DS values 28%, 43%, 54%, 69% and 78% were named respectively, as CSCN-1, CSCN-2, CSCN-3, CSCN-4 and CSCN-5.

2.4. Chitosan-ACHCA crosslinking

Chitosan-ACHCA was dissolved in 20 mL distilled water at room temperature. The solution was cast into a Petri dish (5 cm diameter), left in dark at room temperature, and dried overnight in a vacuum to obtain a uniform thin film. The film was irradiated

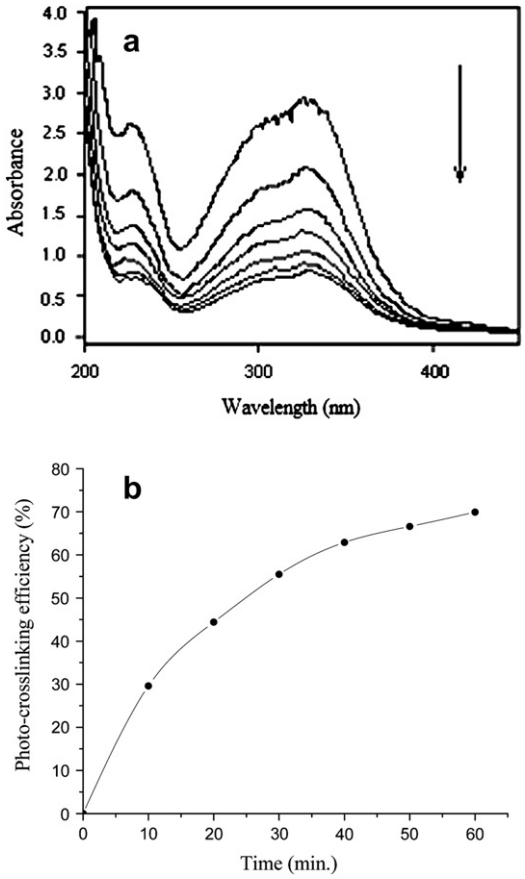


Fig. 3. (a) Changes in the UV spectral patterns of CACN-4 in thin film upon after UV irradiation time $t = 0$, 10, 20, 30, 40, 50 and 60 min. (b) The effect of irradiation time on the extent of crosslinking of a chitosan-ACHCA (CACN-4).

under a multiband UV lamp 254/365 nm (Mineralight lamp model UV GL-25). The kinetics of the photo-crosslinking process was monitored by measuring the disappearance of the absorbance band at 326 nm, which is characteristic of the α-cyano-4-hydroxycinnamoyl group [19]. Crosslinking efficiency at any time (ρ_t) was determined by calculating the percent conversion of photoactive chromophore using Eq. (1) [31]:

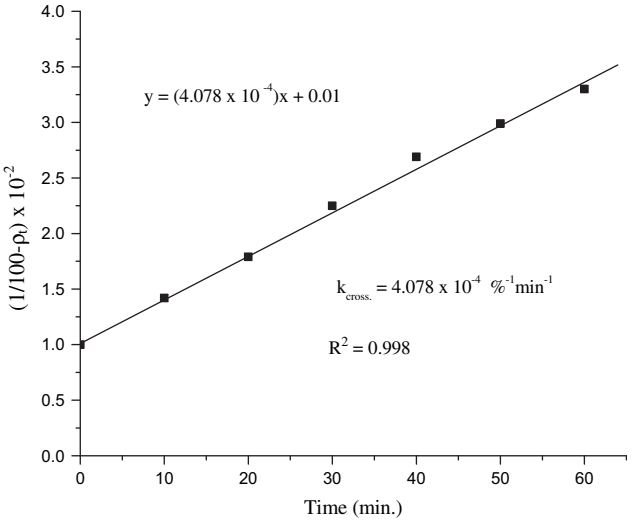


Fig. 4. Linear second order integral plot of the reciprocal of the percentage of the uncrosslinking against the time.

$$\rho_t = [(A_0 - A_t)/A_0] \times 100 \quad (1)$$

where A_0 and A_t are, respectively, the absorbance values at time 0 and, time t.

2.5. Determination of extractables in chitosan-ACHCA membranes

After 60 min of irradiation, the dry photo-crosslinked membranes were weighed then rehydrated in 10 mM HCl at room temperature. Samples were incubated for 24 h, after which the supernatant was aspirated and the samples were dried and reweighed, and the percent extractables calculated. Similarly, second and third extractions were also performed.

2.6. Swelling studies

The swelling properties of samples were studied by immersing the samples in a solution and at different periods of time at room temperature. The dried hydrogel membranes were cut into small squares with about 10 mm height. At predetermined time intervals, the squares were taken out from the solution, gently wiped with filter paper to remove the surface solution weighed and returned to the same container until equilibrium. The swelling ratios (SR) of these samples were calculated by using the Eq. (2). [32]:

$$\text{Swelling ratio(SR)} = (W_1 - W_0/W_0) \times 100 \quad (2)$$

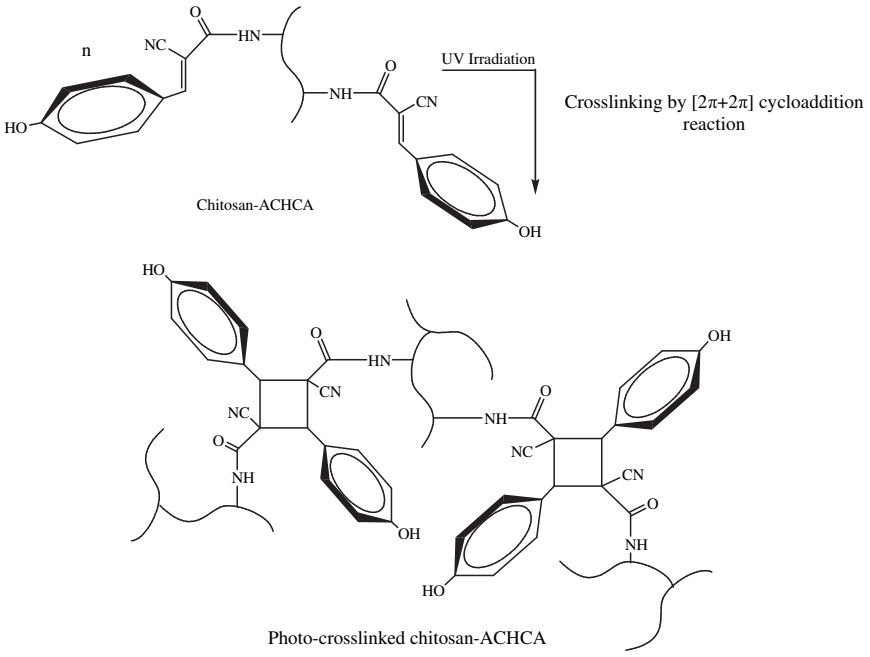
Where W_0 is the weight of dry gel W_1 is the weight of swollen hydrogel and at different swelling time. In order to check the behavior of chitosan hydrogels with the variation of pH it was used eight different solutions, acetic acid/acetate buffer pH 3, 4 and 5 a phosphate buffer with pH 6.0, and 7.0, ammonium hydroxide solution pH 9.0, and sodium hydroxide solution pH 11.0 and 12.0.

3. Results and discussion

3.1. Synthesis of chitosan-ACHCA

Chitosan-ACHCA was synthesized in a controllable manner using an EDC/NHS conjugation method as presented in Scheme 1. The formulation details used in synthetic procedure are summarized in Table 1. FTIR spectra of chitosan, ACHCA and Chitosan-ACHCA modified polymer were shown in Fig. 1. The principal spectra absorptions bands of chitosan could be seen in the figure: 1076 cm^{-1} (O–C strech), 1159 cm^{-1} (bridge–O strech), 1313–1481 cm^{-1} (–CH bend), 1627 cm^{-1} (C═O strech), 2927 cm^{-1} (C–H strech) and 3431 cm^{-1} (O–H strech) [32]. The spectrum of ACHCA shows the characteristic carbonyl peak of the –COOH group at about 1700 cm^{-1} and sharp intense peak at 2370 cm^{-1} which correspond to the –CN group, whereas in the spectrum of the modified chitosan-ACHCA CACN-4 there is no peak corresponding to the carbonyl –COOH group of the ACHCA due to the amide bond formation between ACHCA and the amino group of chitosan. The prominent peaks at 1636 and 1550 cm^{-1} which are observed in the IR spectrum of the modified chitosan are assigned to the carbonyl stretching vibration of amides (amide I) and bending vibration of amides (amide II). Also, the appearance of the sharp peak at 2370 cm^{-1} corresponding to the –CN group confirm the incorporation of the ACHCA on the chitosan backbone.

The ^1H NMR spectra of chitosan in D_3C COOD/D_2O and chitosan-ACHCA in D_2O were shown in Fig. 2. The spectrum of chitosan (Fig. 2a) shows a small peak about at δ 2.03 ppm assigned to the presence of $-CH_3$ of the *N*-acetylated glucosamine residue. The signal at δ 3.08 ppm was assigned to H^2 of glucosamine and



Scheme 2. Photo-crosslinking of chitosan-ACHCA [19].

N-acetylated glucosamine, and the multiplet peaks from δ 3.6–3.9 ppm were attributed to H^3, H^4, H^5, and H^6 of glucosamine and *N*-acetylated glucosamine. There existed a peak at δ about 4.78 ppm because of the presence of H^1 of glucosamine and *N*-acetylated glucosamine [32].

As a representative example, the ^{1}H NMR spectra is presented of a chitosan-ACHCA (CACN-5) (Fig. 2b). The spectrum confirms incorporation of the ACHCA unites by the presence of phenolic proton peaks at δ 2.475 ppm, a doublet phenyl proton peaks at δ 6.630–6.647 ppm and at δ 8.208–8.225 ppm, which are characteristic for para disubstituted benzene derivatives, and vinyl proton peak at δ 8.332 ppm. Conveniently, the DS values can be determined by comparing the integrated intensity of the phenyl, vinyl or phenolic peaks of the ACHCA unites to the integral intensity of the H^2 of glucosamine and *N*-acetylated glucosamine.

3.2. Photo-induced crosslinking of chitosan-ACHCA

The photo-induced crosslinking of chitosan-ACHCA was followed by UV–vis. spectroscopy (Fig. 3a, b). Absorbance at 326 nm decreased with exposure time without appearance of an isobestic point, which is characteristic of a cis–trans isomerization mechanism for cinnamate crosslinking [31]. Within 60 min, crosslinking efficiency was 70%.

Integral method was used to account for the kinetics of the photo-crosslinking reaction. The best linear relationship was obtained by plotting the reciprocal of the percentage of the uncrosslinking ($1/100 - \rho_t$), where ρ_t is the crosslinking efficiency at time t, against the time (t) (Fig. 4). The rate constant of the photo-crosslinking reaction (k_{cross}) was evaluated from the slop of the linear plot ($4.078 \times 10^{-4}\%^{-1}$ min^{-1}). This implies that photo-crosslinking of

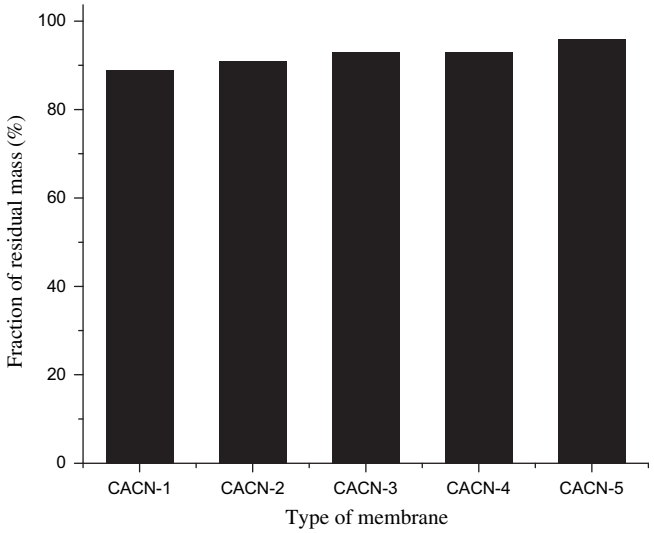


Fig. 5. Residual mass after serial extraction in 10 mM HCl of chitosan-ACHCA membranes subjected to 60 min of irradiation.

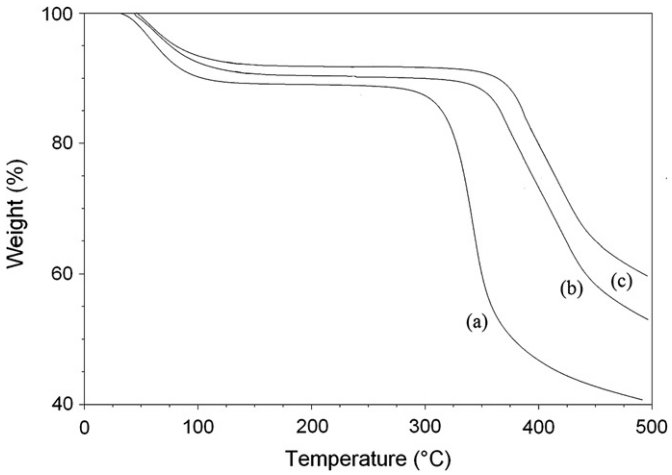


Fig. 6. TGA thermogrames of (a) chitosan, (b) photo-crosslinked CACN-2 and (c) photo-crosslinked CACN-4.

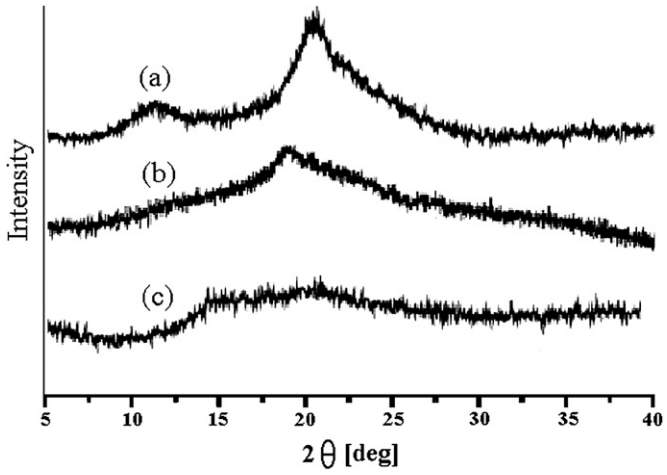


Fig. 7. WAXD patterns of (a) chitosan, (b) photo-crosslinked CACN-1, and (c) photo-crosslinked CACN-5.

modified chitosan occurs mainly through a $[2\pi + 2\pi]$ bimolecular second order electron cycloaddition reaction (Scheme 2).

The relative degree of intra-or intermolecular chitosan-ACHCA crosslinking was determined by measuring the extractable mass fraction of the hydrogels membrane. In principle, the extractable component should be negligible if all chitosan-ACHCA molecules were fully crosslinked by intermolecular reactions. As seen in Fig. 5, photo-crosslinked hydrogels membrane retains 89–96% of the initial mass fraction.

3.3. Thermal gravimetric analysis

The TGA results of pure chitosan and photo-crosslinked membranes CACN-2 and CACN-4 were displayed in Fig. 6. Pure chitosan, CACN-2 and CACN-4 exhibited an initial weight loss up to about 100 °C which may be due to presence of moisture. However, for pure chitosan, no weight loss occurred at the later stage, *i.e.* up to about 300 °C a rapid weight loss of about 50–55% is observed between 290 and 370 °C and this may be attributed to the splitting of the saccharide rings. These results were in accordance with previous report [32]. For the photo-crosslinked chitosan-ACHCA CACN-2 and CACN-4, the weight loss was observed at a later stage, *i.e.* about 360 °C and 372 °C for CACN-2 and CACN-4 respectively. However, the modified chitosan-ACHCA polymer had a lower weight loss and higher residue of about 53% and 60% for CACN-2 and CACN-4 respectively until 500 °C. This supports that modification of chitosan by photo-crosslinking after loading of ACHCA through the amide bond formation renders chitosan thermally more stable. This may be due to the formation of a rigid polymer network, making it thermally more stable.

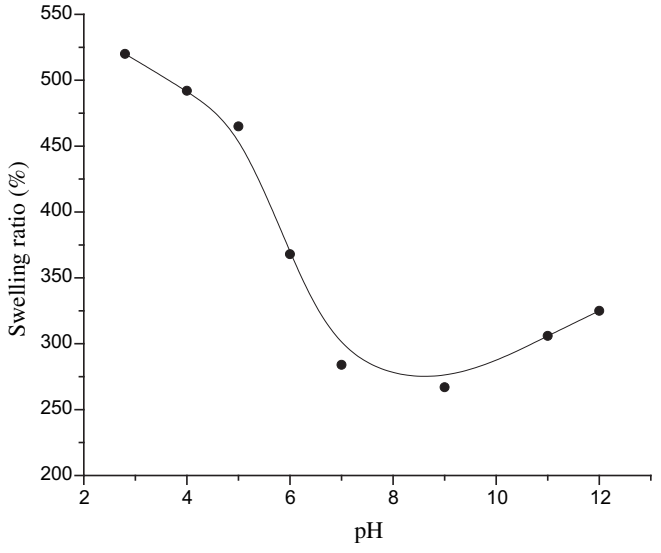


Fig. 8. Swelling behaviors of photo-crosslinked chitosan-ACHCA (CACN-4) hydrogel membrane as a function of pH.

3.4. X-ray diffraction pattern

Crystallinity of chitosan, photo-crosslinked CACN-1 and CACN-5 chitosan-ACHCA modified polymers were characterized by WAXD pattern (Fig. 7). Chitosan itself exhibited typical peaks that appeared at $2\theta = 10°$ and 20°. These peaks were assigned to be a mixture of (001) and (100), and (101) and (002), respectively [32]. As presented in Fig. 7, it is clear that the crystallinity decreases with increasing the DS value and hence, with increasing the extent of crosslinking. This lowering in crystallinity can be attributed to the amide bond formation between amino groups of chitosan and carboxyl groups of ACHCA, as well as the formed network after the photo-crosslinking process which may led to breaking the hydrogen bonding between amino groups and hydroxyl groups in chitosan, resulting in an amorphous structure. Similar discussions for the deformation of crystal structure had been explained in previous reference [32].

3.5. Swelling of photo-crosslinked hydrogel membranes

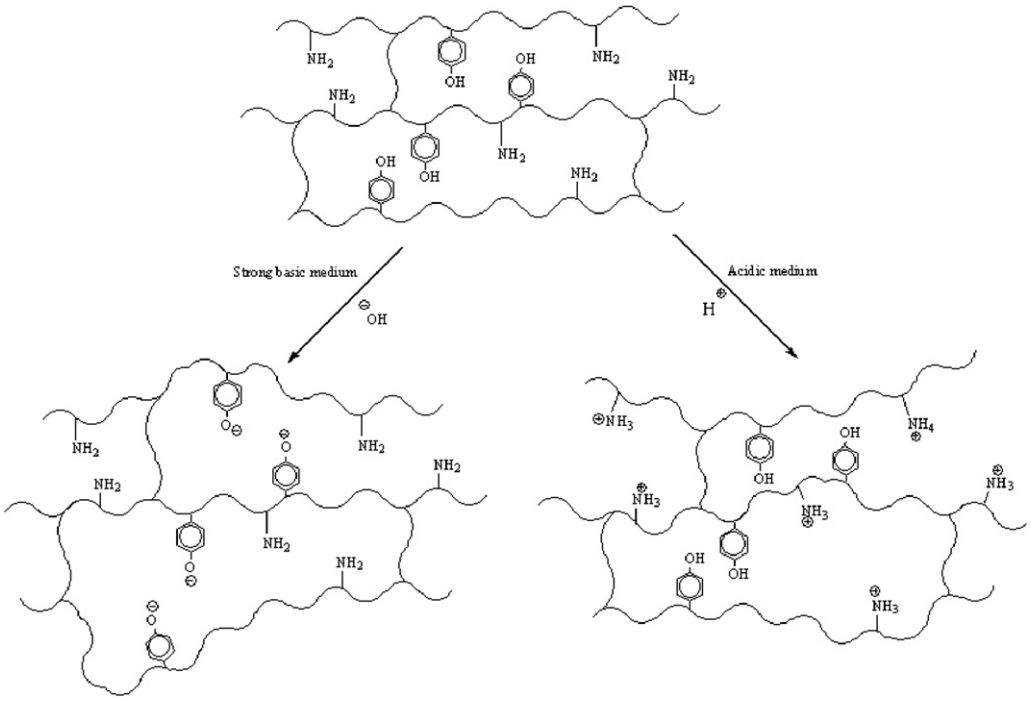
The equilibrium swelling of hydrogels is a result of the balance of osmotic forces determined by the affinity to the solvent and the network elasticity. Fig. 8 shows the swelling capacity of the photo-crosslinked hydrogel membrane of chitosan-ACHCA (CACN-4), as a function of pH.

According to Fig. 8, the swelling capacity decreases by increasing the pH value until about pH 10 then slightly increases at higher pH values, this can be explained as in the following. Under pH 6, the majority of the free unsubstituted amino groups will be protonated and exist in form of a positively charged $-NH_3^+$ groups, and hence the electrostatic repulsive force between the charged sites causes increasing in swelling. On the other hand, at higher pHs (between 6 and 10) the amino groups exist in non-ionized $-NH_2$ forms so hydrogen bonding between amine and amide carbonyl may lead to a kind of crosslinking followed by a decreased swelling. At very high pH values (above 10), the phenolic –OH groups of the introduced ACHCA moieties become ionized and the electrostatic repulsive forces between the negatively charged phenoxide sites ($-O^-$) causes increase in the swelling as presented in Scheme 3.

The hydrogel swelling kinetics, Fig. 9, represent the dynamic swelling behavior of CACN-4 photo-crosslinked hydrogel membrane in water. Initially, the rate of water uptake sharply increases and then begins to level off. The equilibrium swelling was achieved after 60 min. The swelling corresponds to the crosslinking behavior. In this study, the hydrogel membrane shows the maximum water-swelling ratio of 328%.

3.6. Swelling behavior of photo-crosslinked chitosan-ACHCA membranes as a function of DS value

The equilibrium degrees of swelling of the chitosan-ACHCA hydrogel membranes as a function of DS value are shown in Fig. 10. The Figure indicates a typical swelling-DS relationship. The



Scheme 3. Schematic presentation of photo-crosslinked chitosan-ACHCA hydrogel membrane behavior in acidic and basic medium.

swelling of hydrogel membranes was decreased by increasing the DS values. As usually swelling is highest just after the gelation and decreases with increasing the DS value because the increasing of the DS will in turn increase the degree of the photo-crosslinking which will lower the swelling.

3.7. Mechanical properties

The tensile strength and elongation curves of chitosan-ACHCA membranes with various DS values are presented in dry (Fig. 11a) and wet conditions (Fig. 11b). Under dry conditions, the tests were carried out at room temperature. As expected, tensile strength increases with increasing DS value, whereas elongation shows an opposite trend. The mechanical properties of photo-crosslinked chitosan-ACHCA membranes undergoes a conversion to much more brittle behavior with increasing DS value due to the greater degree of crosslinking in the membrane. Similar observations were previously reported [33]. On the other hand, under wet conditions the same trend was observed but the elongation of chitosan-ACHCA membranes shows a significant increases while the tensile strength shows a significant decreases. This can be explained as in the following: the water molecules will act as a plasticizer and embedding themselves between the crosslinked chains of the photo-crosslinked chitosan-ACHCA membrane

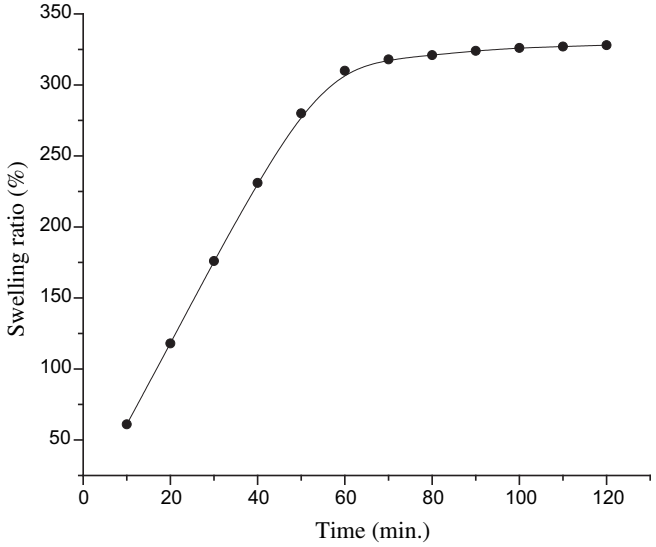


Fig. 9. Swelling behavior of photo-crosslinked chitosan-ACHCA (CACN-4) hydrogel membrane as a function of time.

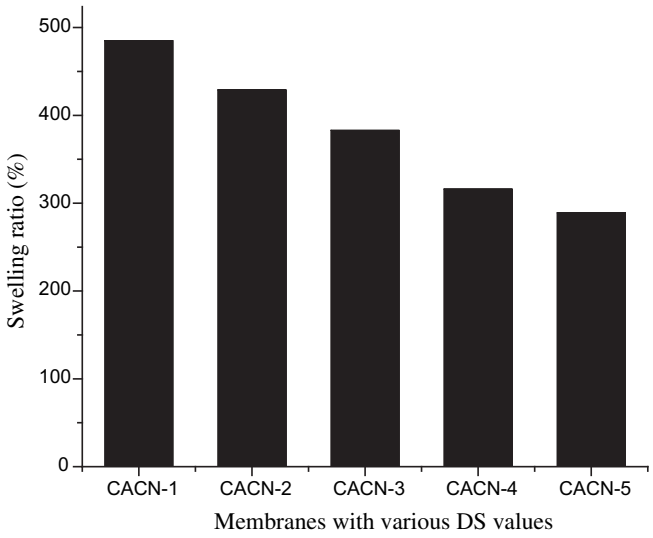


Fig. 10. Swelling behaviors of different chitosan-ACHCA hydrogel membranes as a function of degree of substitution.

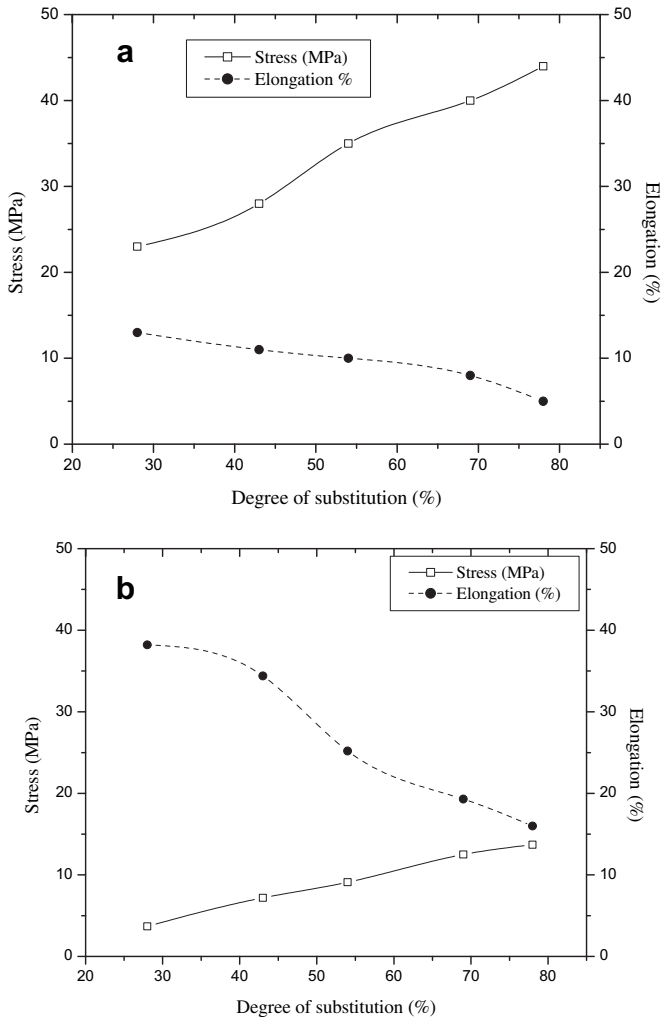


Fig. 11. Stress and elongation of chitosan-ACHCA membranes on (a) dry condition and (b) wet condition.

network, spacing them apart, increase the free volume and thus significantly lowering the glass transition temperature for the membrane and increase the flexibility, though its strength and hardness will decrease as a result of it. These results are in accordance with the swelling behavior studies, by increasing the degree of crosslinking, the swelling decreases and hence, the amount of water embedded between the photo-crosslinked membrane network will decreases, so the flexibility will decreases and tensile strength increases. Similar results were previously reported [33]. These results indicated that these membranes are useful in some biomedical applications such as drug delivery systems or enzyme immobilization.

4. Conclusions

Synthesis, characterization, and photo-crosslinking of α-cyano-4-hydroxycinnamated chitosan derivatives were investigated and proved to be a facile route for enhancing the mechanical properties of chitosan hydrogel membranes. Specifically, chitosan with varying degrees of α-cyano-4-hydroxycinnamated derivatization was synthesized using an EDC/NHS conjugation method. The WAXD studies of the modified chitosan hydrogel membranes showed that the incorporation and photo-crosslinking of α-cyano-4-hydroxy-cinnamat unites decreased the crystallinity of chitosan. The maximum tensile strength of modified chitosan hydrogel membranes on wet condition was found when the degree of substitution was 78%. These modified chitosan hydrogel membranes showed good thermal, swelling and mechanical properties.

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