EFFECTS OF PHYSICAL AND CHEMICAL MODIFICATION ON BIOLOGICAL ACTIVITIES OF CHITOSAN/ CARBOXYMETHYLCELLUSE BASED HYDROGELS

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ABSTRACT

Hydrogels network based on carboxymethylcellulose (CMC) and chitosan (CS), CC1 and CC2, have been prepared respectively in the presence or without the crosslinking agents [(N-hydroxysuccinimide (NHS)/N,N'-dicyclohexylcarbodiimide (DCC)] and characterized by FT-IR.

The swelling behavior in distilled water at 25°C and biological activities have been investigated. CC1 hydrogel revealed higher potential swelling than CC2. Hydrogels showed to possess an important antioxidant activity equal to 66.67% for CC1 and 57.27% for CC2, to scavenge hydroxyl radicals at 2mg/mL. And the values of their reducing power were approximately 53% and 57%.

From the hemolytic potential test the obtained materials were hemo-compatible. The anti-inflammatory activity exhibited that hydrogels were able to protect albumin from denaturation.

Keywords Chitosan; Carboxymethylcellulose; Hydrogels; Swelling; Biological activities.

1. INTRODUCTION

Hydrogels are hydrophilic polymers but they remain insoluble due to chemical or physical crosslinks between individual polymeric chains [1]. Both natural and synthetic polymers can be used for the synthesis of hydrogels, achieved by methods such as physical gelation, chemical cross-linking or self-assembly [2]. Hydrogels are used in various domains including the biomedical, biotechnology, pharmaceutical and separation technology. They are soft, pliable, non-toxic, biodegradable and wet materials [3].

Polysaccharides, the most abundant organic substances on the earth, are particularly important for preparation of hydrogels and play a role in domains involved in hydrophilicity, swelling, hydration, gelling...etc [4].

Chitosan (CS), [β-(1→4)-2-amino-2-deoxy-D-glucose], is biodegradable polysaccharide obtained from partial deacetylation of chitin [5,6]. Chitosan is readily soluble in dilute acids at pH<6 [7].The presence of polar groups (–OH and $–NH_2$ groups) in the chemical structure means it is able to form secondary interactions with other polymers. Due to its biocompatibility, biodegradability and antibacterial properties, it is used in the fabrication of hydrogels, films and microspheres [8], biomedical and cosmetic applications [9,10].

Sodium carboxymethyl celllose (CMC), [β-(1→4)-D-glucopyranose], is an important industrial polymer [11], produced by partial modification of cellulose with chloroacetic acid[12]. The presence of (—CH_2COO^-) groups conspicuously improve its solubility in water[13]. CMC is a typical weak acid polyelectrolyte (pKa 3.8). Due to the biocompatibility, biodegradability and non-toxicity of CMC, it is used in pharmaceutical and biomedical applications and pulp cell regeneration [14,15].

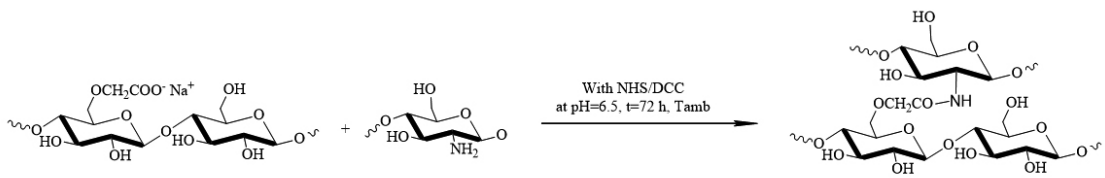
The aim of this work is the preparation and characterization of crosslinked hydrogels based on CMC and CS. Biological activities were examined by antioxidant, anti-inflammatory and hemolytic capacities of all these prepared biomaterials.

2. MATERIALS AND METHODS

Sodium CMC (MW= 250 kDa, 8% moisture Sodium content, average viscosity of 400–800 cps), chitosan with 65% N-deacetylation degree, DCC (N,N'-dicyclohexylcarbodiimide), NHS (N-hydroxysuccinimide) were purchased from Sigma-Aldrich. Other reagents were analytical grade and used without further purification.

2.1. Amidation of CMC

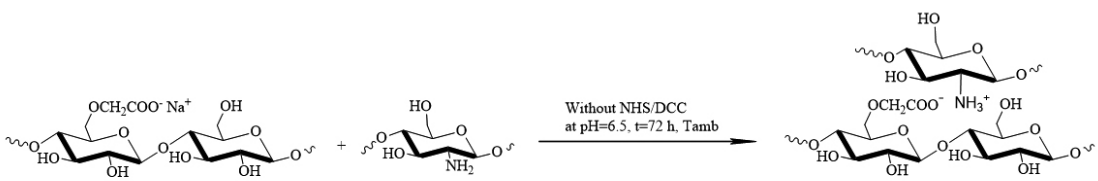
According to **Matute *et al.*,**[16] CC1 hydrogel was obtained by amidation reaction of CMC with chitosan in the presence of coupling agent (DCC and NHS) as shown below:



CMC **CS** **CC1** (Chemical network)

In a 200 ml beaker, 1 g of chitosan was dissolved in 100 mL of acetic acid (1%) with stirring for 24 h. 0.1 M of NaOH was added to adjust pH of the solution at 6.5. In the other hand, 5 mL of NHS (0.18g/mL) and 5 mL of DCC (0.3 g/mL) were added to 10 mL of an aqueous solution of CMC (0.15g/mL), under contentious stirring for 1 h. The obtained solution was mixed with chitosan solution and stirred for 72 h. The resulting hydrogel (CC1) was precipitated, washed with water/ethanol, isolated by centrifugation (300 rpm for 10 min), and air dried for three days.

The same procedure was followed to prepare the second hydrogel CMC/CS (CC2).



CMC **CS** **CC2** (Physical network)

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2.2. Determination of free amino groups

The number of amino groups was measured for CS, CC1 and CC2 by means of pH metric titration method[4]. 0.1 g of each sample was dissolved in (12.5 ml, 0.1 N) HCl and stirred for 24 h. Then, the solution was titrated with (0.1N) NaOH. The percentage of amino groups was calculated using equation (1)[17].

$$NH_2(\%) = \left[M_{NaOH}(V_2 - V_1) * \frac{161}{W}\right] * 100 \qquad (1)$$

where:

M_{NaOH} is the molarity of the NaOH solution, V_1 and V_2 the volume to neutralise the excess of HCl and the protonated amino groups, 161 is the molecular weight of the monomeric unit of chitosan and W is the mass of the sample in dry state before titration.

2.3. Potentiometric and conductometric titration

The contents of $–OCH_2COOH$ and $–NH_2$ in CC1 and CC2 were determined following the method described by Dhar *et al.,*[18]. Initially, the pH of each aqueous solution of hydrogel (1%) was increased to 11 by addition of NaOH (0,1M). The solution was then titrated dropwise with HCl (0.1N). The pH and the electrical conductivity were measured simultaneously until pH of the sample approached 2.

2.4. Fourier Transformed Infrared

Spectroscopy Analysis FTIR spectra were acquired in transmission mode from powders and dried films (1%). The spectra were recorded with a SHIMADZU 84005 spectrometer, in the range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1}. This analysis was conducted to observe changes of the functional group before and after interactions between polymer matrixes.

2.5. Swelling test

For swelling experiments, 80 mg (W_0) of each dried of prepared hydrogel were immersed in distilled water at 25 °C. The weight at different times (W_t) was determined and the swelling ratio (S) was calculated using the following equation:

$$S(\%) = \frac{W_t - W_0}{W_0} * 100 \qquad (2)$$

Where: W_t and W_0 are weights of the wet and dry samples, respectively.

2.6. Antioxidant activity assay

2.6.1. Scavenging effect on hydroxyl radical

The antioxidant activity of samples was evaluated using hydroxyl radical procedure [19]. Briefly, 0.2 mL of various concentrations (0.25-2 mg/ml) of (sample or ascorbic acid) was added into 0.2 mL of an aqueous solution of $FeSO_4$ (5 mM). Then, 0.2 mL of an aqueous solution of H_2O_2 (1% (v/v)) was added to the mixture, which was stirred again and incubated at 37°C. After 60 min, 1 mL of distilled water was added, and the absorbance was measured at 510 nm. The hydroxyl radical inhibition was calculated using the following equation:

$$\text{hydroxyl radical inhibition}(\%) = \left(\frac{A\ \text{control} - A\ \text{sample}}{A\ \text{control}}\right) * 100 \qquad (3)$$

Where, A_{sample} and $A_{control}$ are the absorbances at 510 nm of 0.2 mL of the sample (0.25–2 mg/ml) or 0.2 mL of distilled water with 0.4 mL of a solution (v/v) of $FeSO_4$(5 mM)/H_2O_2(1%).

2.6.2. Reducing power determination

The reducing power of samples was determined using the method described by **Yen & Chen** [20]. 1 mL of each sample with different concentrations (0.25-2 mg/mL) was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide ($K_3Fe(CN)_6$, 1%). After incubation at 50 ºC for 20 min, 2.5 mL trichloroacetic acid (TCA, 10%) was added and the mixture was centrifuged for 10 min at 1000 rpm.

2.5 mL of upper layer of solution was mixed with 2.5 mL of distilled water and 0.5 mL ferric chloride (1%), and the absorbance was measured at 700 nm. Vitamin C (Vc) was used as a positive control. The intensity of reducing power is directly proportional to the absorbance of the reaction mixture [21].

2.7. Hemolytic potential

According to **Fan *et al.,*** [22] hemolytic potential (PH) of samples was determined using the following formula.

$$PH(\%) = \frac{OD\ \text{of sample} - OD\ \text{of negatif control}}{OD\ \text{of positif control}} * 100 \qquad (4)$$

Where: OD is optical density.

2.8. *In- vitro* anti-inflammatory activity:

Anti-inflammatory activity of samples was evaluated by protein denaturation method as described by **Alhakmani *et al.,*** [23]. Diclofenac sodium (powerful non steroidal anti-inflammatory drug) was used as a standard drug. 2 mL of two concentrations (100 and 200 µg/mL) of samples or standard diclofenac sodium were mixed with 2.8 mL of phosphate buffered saline (pH6.4) and 2 mL of egg albumin (from fresh hen's egg). The obtained mixture was incubated at 27°C for 15 min, and induced at 70 °C in water bath. The absorbance was measured at 660 nm using distilled water as blank. 2 mL of egg albumin and 2.8 mL phosphate buffer saline (pH 6.4) were used as a control test. The percentage inhibition of protein denaturation was calculated using the following formula:

$$\text{inhibition}(\%) = \left(\frac{Asample - Acontrol}{Acontrol}\right) * 100 \qquad (5)$$

Where: A_{Sample}: absorbance of tested sample; $A_{control}$: absorbance of control

3. RESULTS AND DISCUSSION

3.1. Determination of free amino groups in the hydrogels

The amount of NaOH between the first and the second jumping point was attributed to the amount of free amino groups. **Fig. 1** shows the pH-titration of the CS and prepared hydrogels. The two steps at 2.5 and 12.5 ml of NaOH (0.1N) for chitosan are corresponding to 76.67% free amino groups which are insignificant in the case of CC1 and CC2 hydrogels. These results revealed the existence of interaction between CMC and CS.

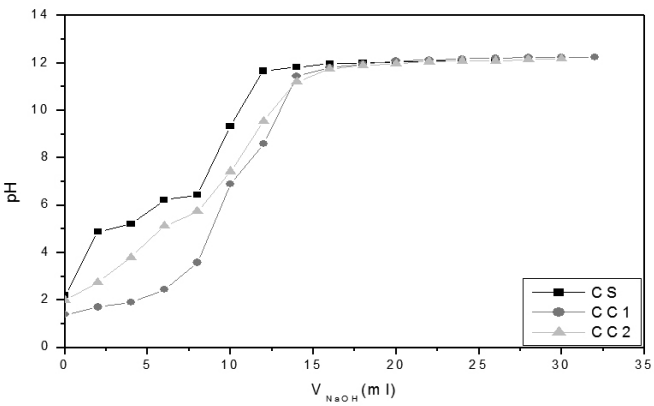

Fig. 1 pH titration of CS, CC1 and CC2.

3.2. Potentiometric and conductometric titration

The potentiometric and conductometric titration curves of CC1 and CC2 are shown in **fig. 2**. The presence of different transition points are corresponding to $–OCH_2COOH$ and $–NH_2$ functional groups on the hydrogels. CC1 curve exhibits three transition points at pH equal to 9.5, 4.8 and 3.8 and four different regions which are corresponding to the neutralization of the excess of NaOH, protonation of OCH_2COO^- on carboxymethylcellulose chains in the hydrogel, protonation of $-NH_2$ groups of chitosan and excess acid present in the solution at pH > 9.5, (9.5-4.8), (4.8-3.8) and (3.8-2), respectively. By measuring the moles of HCl reacted for each functional group (second and third transition regions), the amounts of $-OCH_2COOH$ and $-NH_2$ were 0.25, 0.78 meq/g respectively in CC1 and 0.33, 0.83 meq/g in CC2.

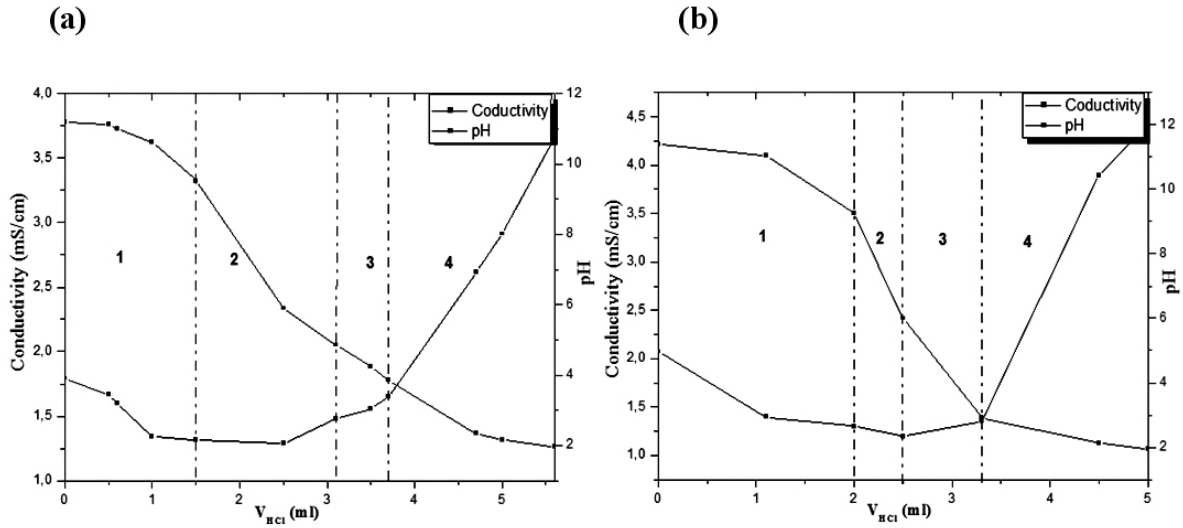

Fig. 2 pH and conductivity titration curve of (a):CC1, (b):CC2.

3.3. Fourier transformed infrared (FTIR) spectroscopy

The FTIR spectra (**fig. 3)** of CMC, chitosan, CC1 and CC2 show major changes in the functional groups of CMC due to the interaction with chitosan.

The CMC spectrum (**fig. 3.a**) exhibits bands at 3453, 2925, 2856, 1600, 1416 and 1323cm^{-1} attributing to stretching vibration of OH, asymmetric and symmetric stretching vibration of the aliphatic CH_2 asymmetric and symmetric of the carboxylate groups (-COO-), and bending vibration of CH, respectively [24] .The bands due to primary alcoholic –CH_2OH stretching modes appeared at 1076 cm^{-1}.

FTIR spectrum of chitosan (**fig. 3.b**) presents a broad O-H stretching band at 3445 cm^{-1}. Characteristic bands for amide groups were found at 3445, 1650 cm^{-1} [25].The bands at 2925, 2848 and 1380 cm^{-1} were assigned to C-H links on chitosan chain [26]**.** The region from 1152 to 1081cm^{-1} is the characteristic absorption of C–O–C linkage. Finally, C–N fingerprint band appeared at 896cm^{-1} [27]**.**

The IR Spectrum of CC1 (**fig. 3.c**) is completely different. New pics at 1625 cm^{-1} and 1571 cm^{-1} are attributing to (υ (C=O)) and (υ (C–N)) vibrations of amide I and amide II respectively [28]**.** Also, the band at 3322 cm^{-1} is corresponding to the axial stretching of the O–H and N–H [29]. The wide visible band at 631 cm^{-1} is due to N-H out of the plane. This confirms the amidation of CMC by CS (i.e., conversion of COOH to CONHR) **(fig. 4).**

The IR spectrum of CC2 (**fig. 3.d**) exhibits the possibility of physical interaction between the positive charges derived from CS (CS-NH_3^+) and the negative charges from CMC (CMC-COO^-). A strong band at 1600 cm^{-1} is attributed simultaneously to asymmetric angular deformation of COO^- ion and $NH^+_{3\ groups.}$

3.4. Swelling

The swelling ratios (S%) of hydrogels are represented in **fig. 5**. CC1 hydrogel exhibits maximum swelling (S_{max}%) ≈ 677% at 4 h and slightly decreases to 525% after 24 h. However, CC2 hydrogel shows S_{max} equal to 542.5% during 1 h and then decrease rapidly to 350% due to, the network was discontinuous and the links between polymer chains of CMC and CS are physical and electrostatic.

3.5. Antioxidant activity

3.5.1. Scavenging effect on hydroxyl radical

Scavenging activity of hydrogels on hydroxyl radical was investigated using Fenton reaction. It was already known that hydroxyl radical generated by Fenton reaction was the most reactive of all the reduced forms of dioxygen and is thought to initiate cell damage *in vivo* [30]**.** According to results of scavenging effect on hydroxyl radical presented in **fig.6**, all the samples show a maximum values higher than 50% at 2mg/mL: 66.67(CC1), 57.27%(CC2), 73% (CMC and CS), and 94.63% (ascorbic acid). CC1 and CC2 reveal relatively lower anti-hydroxyl radical activity than the rest samples. Also, the scavenging effect of all our samples including ascorbic acid increased with increasing the concentration.

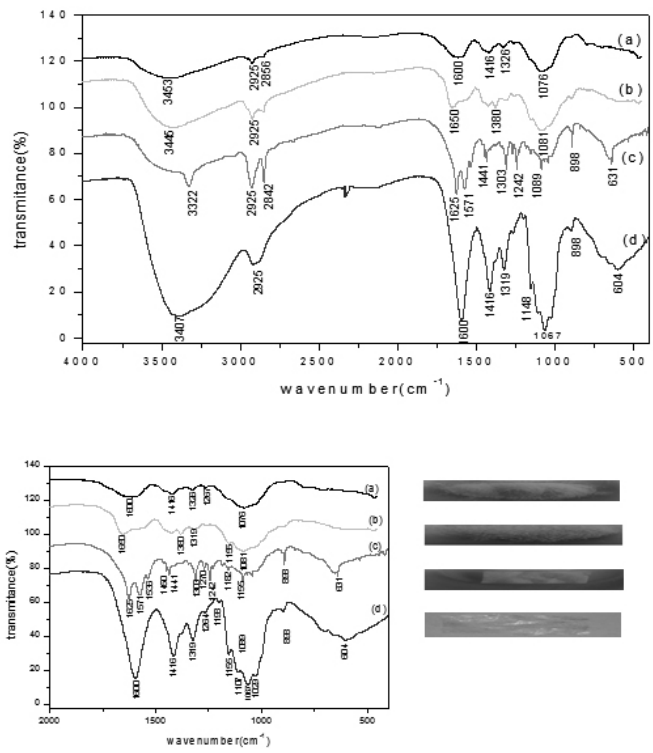

Fig. 3 FTIR spectra of (a): CMC, (b): CS, (c): CC1, (d): CC2.

As shown previously in different works, the presence of carboxyl [19] and amino group [31] on polysaccharide chains take undoubtedly part in free radicals scavenging and contributed to the antioxidant activity. So, the chemical and physical bonds between these groups decrease the scavenging activity probably due to the chelating affect of metal ions (Fe^{2+}).

5.2.2. Reducing power determination:

The antioxidant activity has been reported to have a direct, positive correlation with the reducing power [32] and to be a simple, fast and reproducible test [33]. The reducing capacity of samples may serve as a significant indicator of its potential antioxidant activity. It was proved that the presence of the reducing agents caused the reduction of Fe^{3+}/ferricyanide complex to ferrous form (Fe^{2+}) which was monitored by measuring the formation of Perl's Prussian blue at

700 nm [34]. As shown in **fig. 7**, the reducing power of samples correlated well with increasing concentration. The reducing power values of ferrocyanide ion, ($[Fe(CN)_6]^{3-}$), to ferrocyanide ion, ($[Fe(CN)_6]^{4-}$) for tested samples were determined: 0.64 (CMC and CS), 0.53(CC1) and 0.57(CC2), these results are in good agreement with scavenging effect on hydroxyl radicals.

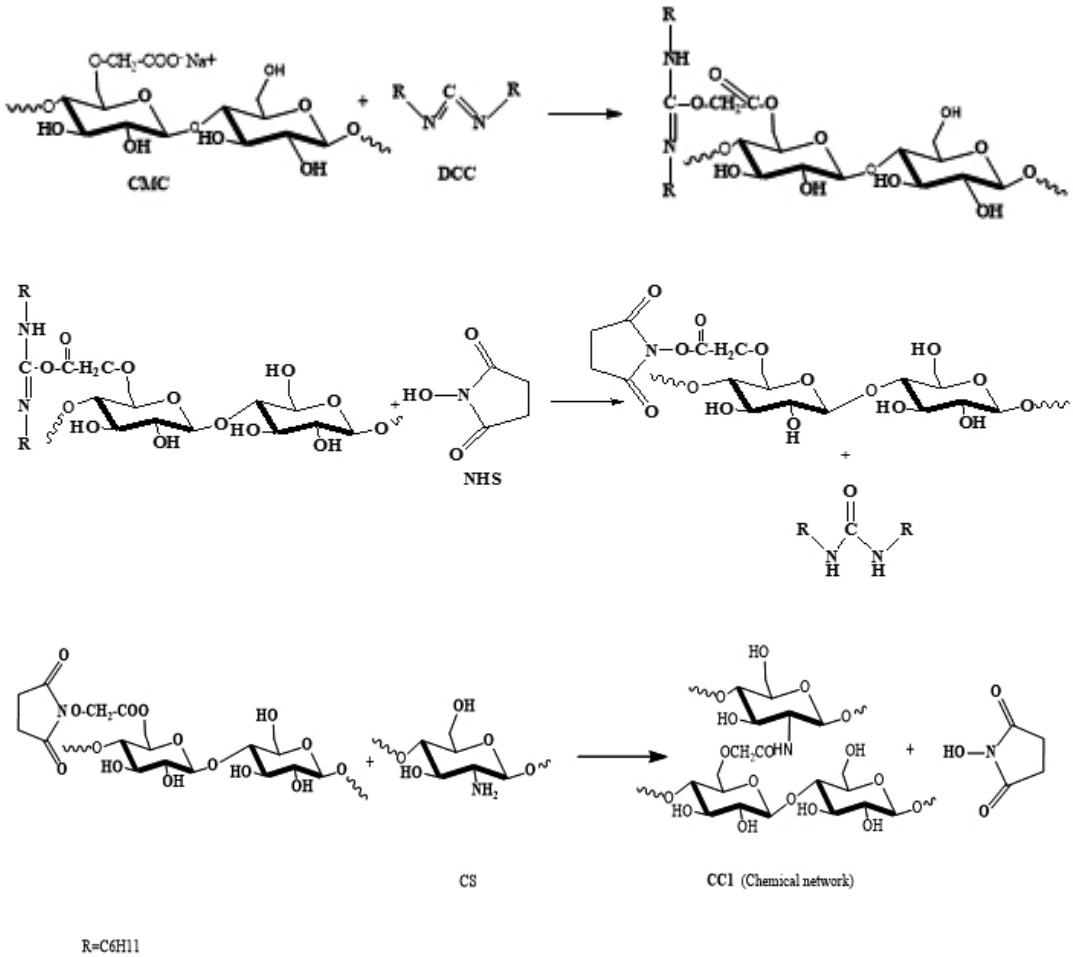

Fig. 4 Amidation of CMC by CS in presence of NHS/DCC.

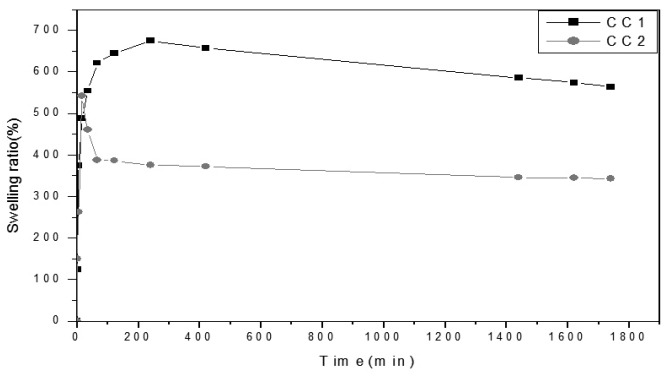

Fig. 5 Swelling ratio of prepared hydrogels at 25°C.

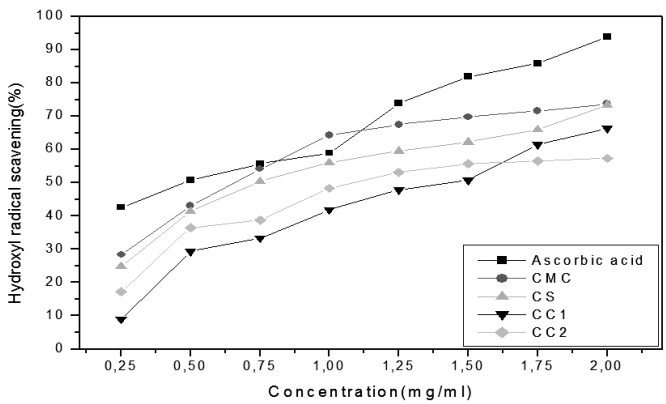

Fig. 6 Scavenging effect on hydroxyl radical.

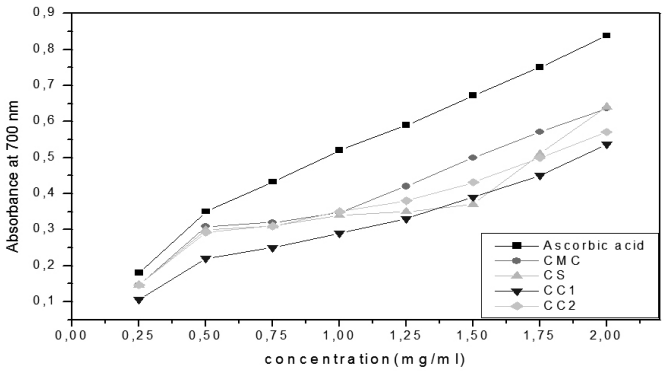

Fig. 7 Reducing power of samples and ascorbic acid.

3.6. Hemolytic potential

Hemolytic activity of materials was defined as the measure of the extent of hemolysis that may be caused by the material against normal human erythrocytes [35]. The Lysis of erythrocytes percentage of different samples at 37 °C for 1 h is less than 10%, as presented in **table.1**. Consequently, all tested samples are non hemolytic.

Table 1 Hemolytic potential of samples.

Sample	CMC	CS	CC1	CC2
Hemolysis (%)	0.3	5.07	4.35	4.85

3.7. *In vitro* Anti-inflammatory activity

It has been demonstrated [36] that the prevention of protein denaturation by medicinal agents under an *in vitro* experimental condition is an indication of anti-inflammatory effect, and consequently, this test would be worthwhile to use routinely for the preliminary screening of anti-inflammatory effect during the development process of studied product.

The mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding [37]. **Table.2** presents the percentage inhibition values of albumin denaturation of samples and diclofenac sodium (reference) at (100 and 200ug/mL). It has been observed that the percentage inhibition increase with concentration.

Table 2 *In-vitro* anti-inflammatory effect of polymeric matrix/Diclofenac sodium.

Samples	% inhibition (100 µg/ml)	% inhibition (200 µg/ml)
CMC	16.3	20.58
CS	6.2	6.81
CC1	2.23	2.38
CC2	3.1	4.75
Diclofenac sodium	50.8	81

4. CONCLUSION

The physical and chemical crosslinking of CMC with Chitosan was successfully obtained in aqueous solution by casting method. The chemical network was prepared using DCC/NHS.

Infrared spectrophotometric analysis revealed the different interactions between CMC and CS. The swelling data of CC1 hydrogel in distilled water at 25°C exhibit a higher stability than CC2 reaching S_{max} 677%.

In addition, biological assays showed that the prepared materials might be a potential candidate as antioxidant material due to the important hydroxyl radical scavenging and reducing power activities.

Therefore, all used hydrogels have no hemolytic effect against normal human erythrocytes. Thus, Owing to inhibitory effect of protein denaturation, these matrices would be useful in tissue engineering.

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