BIOLOGICAL ACTIVITIES OF CHITOSAN/CARBOXYMETHYLCELLULOSE 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)]. 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.*

Key words: *Chitosan; Carboxymethylcellulose; Hydrogels; Biological activities.*

Introduction

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[1].

Chitosan (CS), [β-(1→4)-2-amino-2-deoxy-D-glucose], is biodegradable polysaccharide obtained from partial deacetylation of chitin [2]. Chitosan is readily soluble in dilute acids at pH<6 [3]. 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[4], biomedical and cosmetic applications[5].

Sodium carboxymethyl celllose (CMC), [β-(1→4)-D-glucopyranose], is an important industrial polymer [6], produced by partial modification of cellulose with chloroacetic acid [7]. The presence of (—CH_2COO^-) groups conspicuously improve its solubility in water [8]. 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 [9].

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

1.- 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.

1.1.- Amidation of CMC

According to MATUTE *et al* [10] CC1 hydrogel was obtained by amidation reaction of CMC with chitosan in the presence of coupling agent (DCC and NHS) as shown below:

The same procedure was followed to prepare the second hydrogel CMC/CS without theses coupling agent (CC2).

1.2.- Antioxidant activity assay

1.2.1.- Scavenging effect on hydroxyl radical

The antioxidant activity of samples was evaluated using hydroxyl radical procedure [11]. 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:

$$\text{hydroxyl radical inhibition}\,(\%) = \left(\frac{\text{A control} - \text{A sample}}{\text{A control}}\right) * 100 \qquad (1)$$

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%).

1.2.2.- Reducing power determination

The reducing power of samples was determined using the method described by YEN and CHEN [12]. 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

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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 [13].

1.2.- Hemolytic potential

According to FAN *et al.*, [14] hemolytic potential (PH) of samples was determined using the following formula.

$$PH(\%) = \frac{\text{OD of sample} - \text{OD of negatif control}}{\text{OD of positif control}} * 100 \qquad (2)$$

Where: OD is optical density.

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

Anti-inflammatory activity of samples was evaluated by protein denaturation method as described by ALHAKMANI *et al.* [15]. 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 (10%) (from fresh hen's egg 10%). The obtained mixture was incubated at 27°C for 15 min, and induced (denaturation) at 70 °C in water bath. The absorbance was measured at 660 nm using distilled water as blank. 2 mL of egg albumin (10%) 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:

$$\text{inhibition}(\%) = \left(\frac{\text{Asample} - \text{Acontrol}}{\text{Acontrol}}\right) * 100 \qquad (3)$$

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

2.- Results and discussion

2.1.- Antioxidant activity

2.1.1.- 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* [16]. According to results of scavenging effect on hydroxyl radical presented in figure 1, all he 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.

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As shown previously in different works, the presence of carboxyl and amino group [17] 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+}).

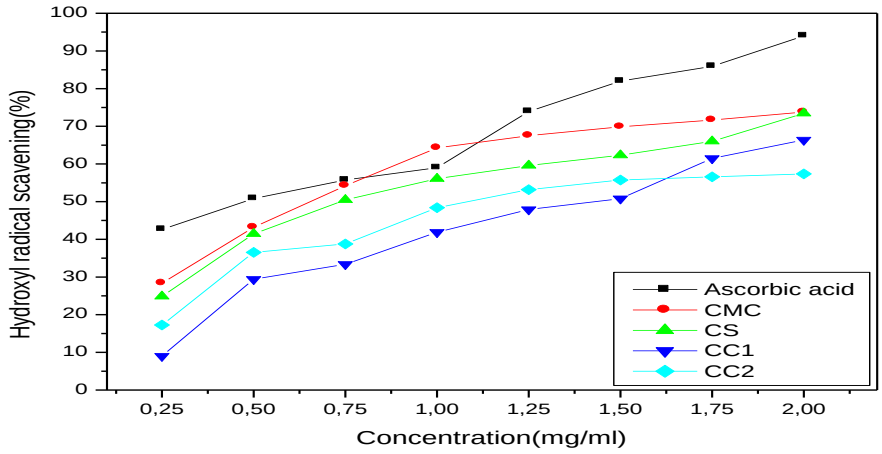

Figure 1.- Scavenging effect on hydroxyl radical

2.1.2.- Reducing power determination

The antioxidant activity has been reported to have a direct, positive correlation with the reducing power [18] and to be a simple, fast and reproducible test [19]. 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 [20]. As shown in figure 2, 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.

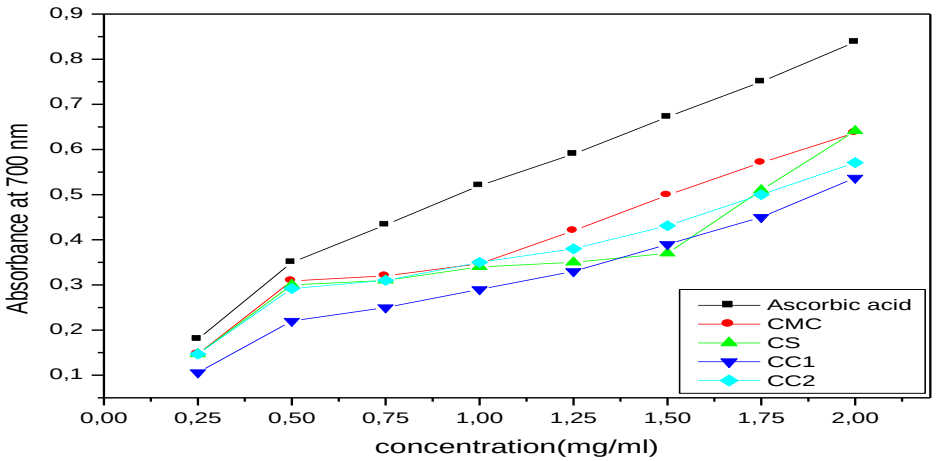

Figure 2.- Reducing power of samples and ascorbic acid

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2.2.- 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 [21]. The Lysis of erythrocytes percentage of different samples at 37 °C for 1 h was less than 10%, as presented in table. I. Consequently, all tested samples were non hemolytic.

Table I.- Hemolytic potential of samples

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

2.3.- *In vitro* Anti-inflammatory activity

It has been demonstrated [22] 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. Table II 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. So, it can be foretold that upon topical or systemic application of our materials onto the inflamed living tissue, an anti-inflammatory activity take place.

Table II.- *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

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.

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

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would be useful in tissue engineering.

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