# PREPARATION OF CELLULOSE-BASED HYDROGELS AND THEIR CHARACTERISTICS FOR CELL CULTURE

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A series of cellulose-based hydrogels were produced by crosslinking of hydroxypropylcellulose (HPC) and carboxymethylcellulose sodium salt (CMCNa) with citric acid (CA) and the gelation ability of the prepared crosslinked polymers was investigated in detail. It was found that the swelling ratio of the prepared hydrogels was closely related with their preparation conditions, such as reaction time and temperature. The gelators and resulting hydrogels were characterized by scanning electron microscopy (SEM), differential scanning calorimeter (DSC), and Fourier transform infrared spectroscopy (FTIR), respectively. The biocompatibility of the hydrogels was verified by *in vitro* cell viability studies on fibroblasts L929. The results showed that these crosslinked hydrogels can be used for potential biomaterial applications.

***Keywords***: cellulose, hydrogels, crosslink, biocompatibility

## INTRODUCTION

Cellulose is one of the most abundant natural polymers on earth, which consists of a linear chain of several hundred to over ten thousand linked D-glucose units. It is an important component of the primary cell wall of green plants. Some species of bacteria secrete cellulose and can form biofilms.[1] However, cellulose is insoluble in water or organic solvents, which greatly limits its applications. Functionalization/modification of celluloses can be carried out through their hydroxyl groups, which are partially or fully reactive with various reagents to afford derivatives like cellulose esters and cellulose ethers with useful properties.[2-4]

Recently, it has been found that cellulose hydrogels, typically forming three-dimensional network structures through covalent crosslinking, hold high promise for biomedical applications due to their ability to facilitate cell growth.[5-9] These natural hydrogels made from cellulose have been exploited as potential sorbent materials for dye and heavy metal removal as they are cost-effective, biocompatible, and biodegradable compared with synthetic hydrogels.[10-12] Chemical modifiers, such as glyoxal, have been used as crosslinkers to prepare cellulose hydrogels.[13] However, chemical modifiers used for improving bulk properties of polymeric materials become cytotoxic when they leach into biological environments. Therefore, besides conventional synthetic crosslinkers, new crosslinking agents based on natural biomaterials offer an interesting alternative to modify natural biomaterials for tissue engineering.[14-16]
Recently, citric acid (CA), a biocompatible and cost-effective crosslinking agent, has been successfully used in various cellulose derivative systems.[17-19] Liu *et al*. developed a new resistant

starch-citrate starch as a functional food ingredient based on CA.

Three types of starches, including normal, waxy and high amylose corn starches, were treated with CA (140 °C, 7 h) to increase their resistant starch content (>78%).[20] A similar strategy has been applied to initiate the crosslinking reaction of cellulose polymers by CA. The esterification mechanism based on the formation of an anhydride intermediate was proposed to explain the reaction of cellulose polymers with CA by Demitri *et al*.[21] Kim *et al*. also prepared glutarate starches using glutaric acid as a crosslinking agent.[22]

In this work, two cellulose derivatives, carboxymethylcellulose sodium salt and hydroxypropylcellulose, were used for hydrogel preparation with citric acid as crosslinking agent. CA was added in different concentrations to prepare hydrogels with various crosslinking degrees. We also investigated the physicochemical and structural properties of the crosslinked cellulose films. The biocompatibility of the hydrogels was verified by *in vitro* cell viability studies on fibroblasts L929.

## EXPERIMENTAL

### Materials

All water used in this experiment was deionized water. Carboxymethyl cellulose sodium salt (CMCNa) was obtained from BDH Ltd (Poole, England). Hydroxypropylcellulose (HPC, average Mw 100,000) was purchased from Aldrich. Citric acid (CA) was received from Chem-Supply Pty Ltd. All other chemicals were purchased from Sigma-Aldrich and Merck and used without further purification.

### General procedure for preparation of crosslinked CMCNa-CA-HPC films

The mechanism of the reaction is shown in Figure 1. Crosslinked CMCNa-CA-HPC films were prepared by the procedures described below.

(1) The solution was prepared by the following process. Firstly, HPC was added to water to make a clear solution under magnetic stirring. CMCNa was then added to the above clear solution until the mixture became clear again. The amount of CMCNa and HPC subjects to the calculation based on the mass percentage concentration. The HPC concentration was 2.0% (w/w water solution); CMCNa concentration was 2.0% (w/w water solution). Finally, CA was added in different concentrations (5.0%, 10% (w/w polymer)) to obtain samples with various crosslinking degrees. The reaction was kept for 24 hours at room temperature.

(2) The solution obtained as described above was poured into a 2-mm thick polytetrafluoroethylene container. All samples were predried at 30 °C for 24 h to remove absorbed water. Finally, a thin film was formed after water evaporation.

(3) The films were kept at 70-120 °C for the crosslinking reaction with different reaction times.

### Biocompatibility study of the hydrogels

We cultivated fibroblasts L929 both on the surfaces of the hydrogels and bulk free-standing samples to investigate the biocompatibility of the hydrogels. Green-labeled cells represented viable cells, while red-labeled cells indicated dead ones. The experiment was conducted according to the method described in the literature.[23] The FTIR spectra were recorded on a NICOLET 6700 (Nicolet Instrument Co. U.S.A.) in the range of 400-4000 $cm^{-1}$. TGA was recorded in a TGA/SDTA851e (Mettler-Toledo, Switzerland). About 5.0 mg sample was placed in an aluminium pan, which was then sealed. The sample in the pan was heated from 30 to 500 °C at 20 °C/min. SEM was performed on a FEI Quanta 400F ESEM (FEI Co., USA). The samples were sprayed on a metal plate previously covered with double-sided adhesive and gold-coated using an Emitech K550 sputter coater (Ashford, Kent, UK) under vacuum. The samples were examined at 5.0 kV accelerating voltage.

## RESULTS AND DISCUSSION

### Preparation and characterization of CMCNa-CA-HPC polymers

Table 1 shows the reactivity of CA with cellulose at 100 °C with different reaction times, using a mixture of CMCNa and HPC with a weight ratio equal to 3/1.

### Characterization

The FTIR spectra of (a) CMCNa, (b) HPC, (c) predried CMCNa-CA-HPC film and (d) crosslinked CMCNa-CA-HPC film in the range of 500-4000 $cm^{-1}$ are shown in Figure 2. From FTIR results, the absorbance at 1700-1750 $cm^{-1}$ corresponded to the ester bond, which formed from the reaction of cellulose with CA (Fig. 1). Figure 2c shows no increase of the intensity in the range 1700-1750 $cm^{-1}$ as a function of absorption, which indicates that CA did not react with cellulose under predrying conditions. It was just a physical mixture film after predrying. After heating, however, the intensity of the 1700-1750 $cm^{-1}$ peak (ester bond) increased (Figure 2d), which indicates that CA reacted with cellulose. We can calculate the degree of reaction according to the ester groups. According

to the literature, due to the electrostatic repulsion between polyelectrolyte chains and the high degree of substitution of hydroxyl groups, poor crosslinking efficiency was reported when CMCNa was used alone.[24] Therefore, the presence of HPC in this work promotes intermolecular rather than intramolecular crosslinking.

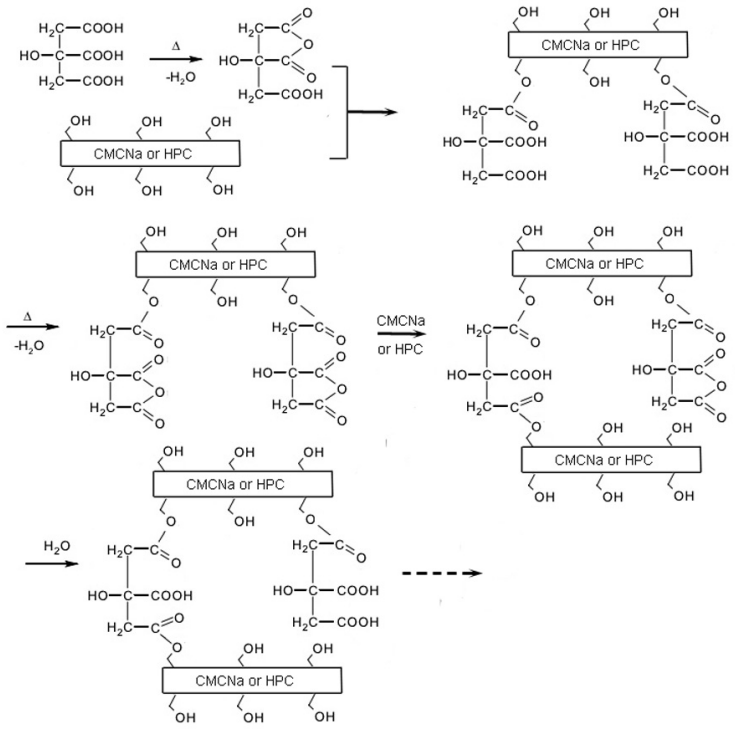


Figure 1: Crosslinking reaction mechanism of CA with cellulose

The TGA thermogram of the obtained crosslinked cellulose (c), in comparison with those of precursor HPC (a) and CMCNa (b), is shown in Figure 3. The TGA curve of HPC exhibits an onset of weight loss at 360 °C. The maximum weight loss rate was reached at the temperature of 430 °C. The TGA curve of CMCNa exhibits an onset of weight loss at 270 °C. The maximum weight loss occurred at the temperature of 320 °C. In contrast, crosslinked cellulose (c) exhibited two obvious weight loss stages. The first started at 80 °C and was caused by the loss of water, which can contribute to the further ester reaction between HPC, MMC and CA.

Table 1
Experimental conditions for CMCNa-CA-HPC polymers

| Sample | Ratio of CMCNa/CA/HPC | Reaction time (h) |
|---|---|---|
| CMCNa-CA-HPC-1 | 3/0.2/1 | 0.1 |
| CMCNa-CA-HPC-2 | 3/0.2/1 | 0.2 |
| CMCNa-CA-HPC-3 | 3/0.2/1 | 0.5 |
| CMCNa-CA-HPC-4 | 3/0.2/1 | 1 |
| CMCNa-CA-HPC-5 | 3/0.2/1 | 2 |
| CMCNa-CA-HPC-6 | 3/0.4/1 | 0.5 |

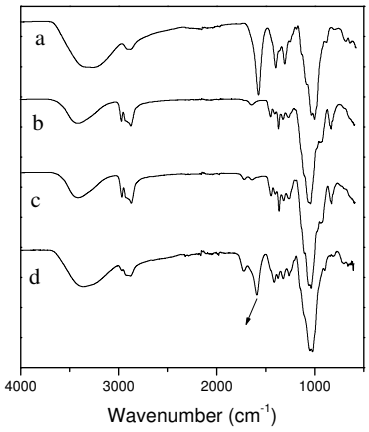


Figure 2: FTIR spectra of compounds (a) CMCNa, (b) HPC, (c) predried sample of CMCNa-CA-HPC and (d) crosslinked CMCNa-CA-HPC-3

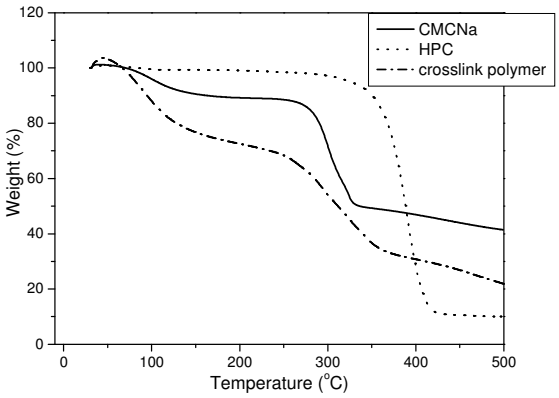


Figure 3: TGA of compounds (a) CMCNa, (b) HPC and (c) crosslinked CMCNa-CA-HPC-3

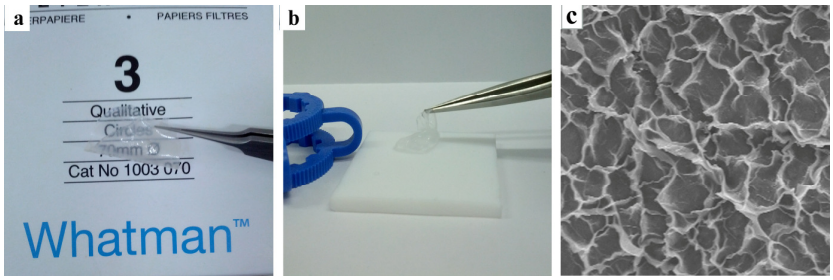


Figure 4: Photographs of crosslinked cellulose films, (a) before swelling in water, (b) after swelling in water, (c) SEM images of hydrogels after freeze drying

The second stage started at 250 °C, and the maximum weight loss rate was reached at the temperature of 350 °C, respectively. In addition, during thermal decomposition, the crosslinked cellulose presented a medium residual amount compared to those of the two precursor celluloses. The TGA profiles indicated that the crosslinked cellulose presented a new thermal character compared with the two precursors.

Due to the good solubility of HPC, CMCNa and CA in water, the resulting films can be readily coated homogeneously on TEFE substrates. Importantly, the hydrogels maintained excellent optical properties. Figure 4a shows that they formed transparent films, through which the background could be clearly observed. Also, the resulting films were bendable. On the contrary, HPC or CMCNa films were fragile and broke easily during bending. All these results indicated that the obtained crosslinked celluloses presented good mechanical properties (Fig. 4a). After being immersing in distilled water for about 24 h, the films can reach equilibrium swelling. Cellulose-based hydrogels typically form three-dimensional network structures through covalent crosslinks, which makes them robust and can be lifted with tweezers (Fig. 4b). We used a microbalance to measure the swelling ratio (SR) by weighing the samples before and after swelling, according to the reference method.[21,25] For the *in situ* observation of hydrogels, the samples were rapidly cooled down in liquid nitrogen and then dried by a freeze drier. The structures were observed under scanning electron microscopy (SEM) imaging (Fig. 4c). SEM images provided information regarding pore size and geometry, as well as relevant information regarding the homogeneity of the hydrogel network.

The crosslinking conditions were also observed by studying the swelling ratio during the reaction progress. Figure 5 shows the swelling ratio as a function of the reaction time at 100 °C. The ratios for the mixture of CMCNa and HPC (3/1) with CA are listed in Table 1. According to the results presented in Figure 5, the swelling ratio of CMCNa/HPC (3/1) crosslinked with 5% of CA decreased as reaction time increased, which means the crosslinking degree increased. When 10% of CA was added to the celluloses, the swelling ratio curve was similar to that for the addition of 5% CA. The crosslinking reaction was observed by measuring the swelling ratio at the temperature ranging from 70 °C to 120 °C for 0.5 hour (Fig. 6). From the comparative study above, it was concluded that temperature and reaction time were effective in controlling the degree of crosslinking. Increasing the reaction temperature or reaction time can improve the crosslinking degree of the films.

Figure 7 shows SEM photomicrographs of crosslinked cellulose-based films. The films were generally homogenous without pores or cracks and the cellulose chains were well dispersed. However, for different CA contents the granules had different morphologies. As shown in Figure 7a, the granules were partially fragmented when an amount of 5% CA was added.

However, when the CA content was further increased to 10%, the granules became strip-shaped and could not be clearly observed by SEM (Fig. 7c).

We suspected that the strip-shaped granules were due to granular swelling in the concentrated solution of CA early in the reaction and collapse occurred during cooling.

**Live and dead assay of hydrogels**

An interesting property of these hydrogels was their excellent biocompatibility. In order to verify their biocompatibility, live and dead assays were carried out for *in vitro* cell viability studies of fibroblasts L929.[26] Bulk free-standing samples, as well as thin hydrogel layers, were used as substrates to study the viability, morphology and proliferation of cells cultivated on the surfaces of samples. Figure 8 shows images of survived cells on CMCNa-CA-HPC-3 hydrogel after 1 day of culture (Fig. 8a, 8b). Compared to the cells in control wells (Fig. 8c, 8d), an obvious improvement in the number of live cells on the hydrogel surface was observed. The cells were shown to stay viable for 1 day (green), which indicated that no toxic effects were caused by the material or possible residual crosslinking agents. However, the cells seeded on the hydrogels were not able to survive after the treatment with alcohol (Fig. 8e, 8f).

Most of the cells died (red) in one day. Directional growth of the cells can be observed in Figure 9. The cells were fully developed on the surface of the hydrogels after approximately 5 days of cultivation. The cells survived well on the CMCNa-CA-HPC-3 hydrogel (Fig. 9a, 9b), compared to those in control wells (Fig. 9c, 9d). However, they died after the hydrogel was treated with alcohol (Fig. 8e, 8f). Time imaging revealed that the cells showed a highly dynamic formation, which indicated no toxic effects from the material or possible residual crosslinking agents. The microscopic observation indicated that L929 cells were well spread on the surface of the hydrogels. The results showed that these cellulose-based hydrogels offer an easy approach for cell culture.

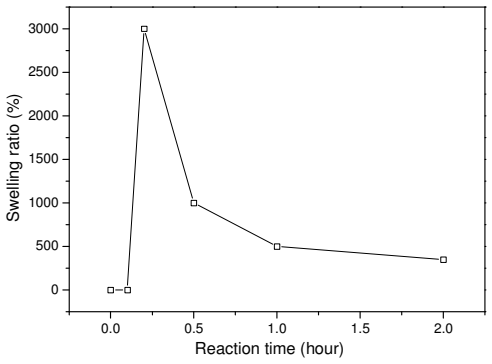


Figure 5: Swelling ratio as a function of reaction time at 100 °C (CMCNa/CA/HPC=3/0.2/1)

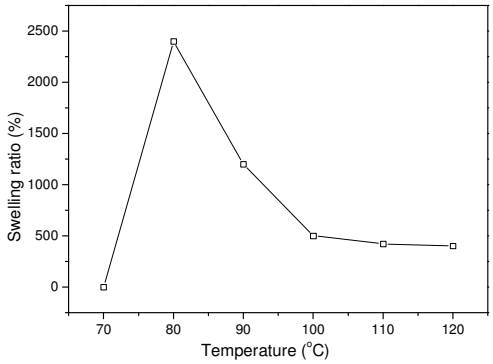


Figure 6: Swelling ratio as a function of reaction temperature (0.5 h), (CMCNa/CA/HPC=3/0.2/1)

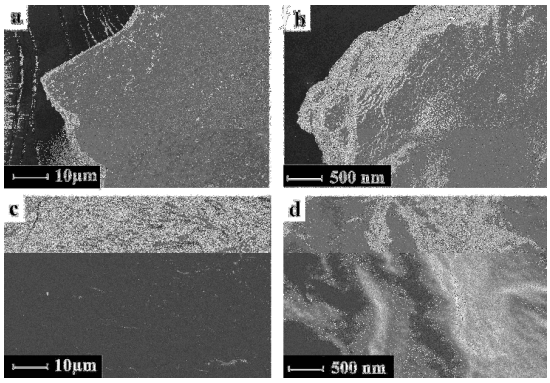


Figure 7: Scanning electron micrographs of crosslinked cellulose-based films with different CA contents: (a), (b), 5% CA; (c), (d) 10% CA

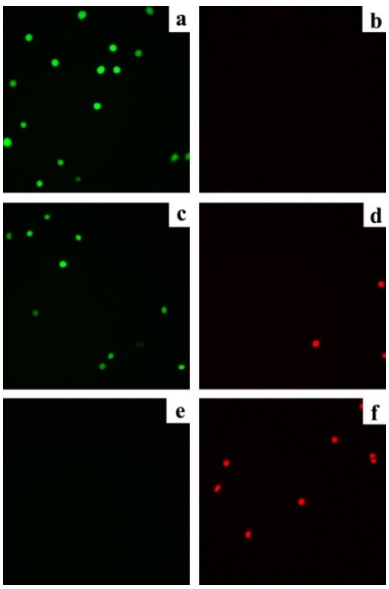


Figure 8: L929 cells cultivated on bulk samples and thin layers of hydrogels after 1 day cultivation time; live cells (green) and dead cells (red) (a), (b), on CMCNa-CA-HPC-3 hydrogel; (c), (d) in control wells; (e), (f) after alcohol treatment of the hydrogel

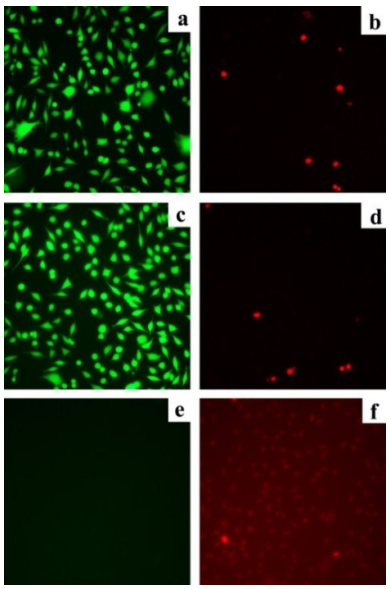


Figure 9: L929 cells cultivated on bulk samples and thin layers of hydrogels after 5 days cultivation time; live cells (green) and dead cells (red) (a), (b), on CMCNa-CA-HPC-3 hydrogel; (c), (d) in control wells; (e), (f) after alcohol treatment of the hydrogel

## CONCLUSION

We have prepared transparent, high water content, flexible hydrogels from two celluloses, which can successfully form a hydrogelator by using CA as crosslinking agent. The reaction conditions for the formation of the hydrogelator were optimized. With 5% (wt) CA loading, the swelling ratio of the hydrogel was improved to 3000% at 100 °C. In addition, an obvious change in the swelling ratio of the hydrogel was achieved when varying the reaction times and temperatures. By reacting with citric acid, a crosslinked cellulose structure was formed, which could be observed by TGA, SEM and FTIR analyses. Cell monitoring was possible after cells were seeded and they adhered to these modified cellulose hydrogels. Compared with bulk free-standing samples, after approximately 1 day and 5 days of culturing, the survival rate of the cells supported by the hydrogel was greatly improved. The new gel system obtained in this work is promising for cell culture studies.

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