Development and Characterization of Cellulose-Based Hydrogels for Use as Dietary Bulking Agents

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Received 3 December 2008; accepted 17 June 2009
DOI 10.1002/app.30956
Published online 7 October 2009 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: In the last decade, cellulose-based hydrogels have been receiving increasing attention for a number of applications because of their smart swelling behavior, biodegradability, and biocompatibility. Given the dramatic spreading of obesity and overweight in the industrialized countries and the lack of scientific consensus over currently available dietary supplements, it was recently proposed that such hydrogels might be used as orally administered bulking agents in hypocaloric diets, because the hydrogel swelling in the stomach may greatly reduce the space available for food intake, thus giving a sense of fullness. This study is focused on the synthesis of cellulose-based hydrogels, starting from pharmaceutical and food grade cellulose derivatives, and shows that such hydrogels possess good swelling properties in water solutions mimicking the environmental conditions of the stomach and the intestine, as well as a good biocompatibility. The crosslinking agent used was a "zero-length" crosslinker, that is, a water soluble carbodiimide, which is washed out from the gel after the synthesis and does not affect the gel compatibility, as shown by preliminary biocompatibility assays. The experimental results confirmed that cellulose-based hydrogels might be a scientifically valid dietary adjuvant in the treatment of obesity and overweight, and provide further scientific evidence for future experiments on humans. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 115: 1438–1444, 2010

Key words: hydrogels; swelling; stimuli-sensitive polymers; biocompatibility

INTRODUCTION

Hydrogels are three-dimensional polymeric networks that are able to absorb and retain large volumes of water (from at least 1.4 up to 5×10^3 times their initial weight).[1,2] Their high-water content provides them with excellent properties in terms of biocompatibility. In addition, smart hydrogels show a strong dependence of the swelling capability on the environmental conditions (e.g., temperature, ionic strength, and pH). The hydrogel swelling and deswelling processes are reversible and driven by diffusion of water and solutes in and out of the polymer network, respectively. This behavior makes hydrogels particularly attractive for their use in *in vivo* applications, especially in the pharmacological field.[2–9]

Previous studies[10–14] showed that cellulose based hydrogels, crosslinked from hydroxyethylcellulose (HEC) and carboxymethylcellulose sodium salt (CMCNa) using various crosslinking agents, display tunable swelling properties, depending on the total amount of cellulose derivatives used, the crosslinking agent concentration adopted and the weight ratio between CMCNa and HEC. HEC acts as a stabilizer of the polymer network, facilitating intermolecular rather than intramolecular crosslinking. More interestingly, the presence of the polyelectrolyte CMCNa in the hydrogel network provides a Donnan equilibrium[15] with the external solution, thus making the hydrogel sensitive to variations of the environmental ionic strength and pH.[10,11,16] These hydrogels were shown to be highly biocompatible and were investigated for use as body water retainers in the treatment of intractable oedemas.[17,18]

Among the possible bioapplications of such hydrogels, the development of novel dietary bulking agents was recently suggested. Obesity and overweight are the second cause of death after smoking, in the industrialized countries.[19] In cases where appropriate dietary regimes and physical exercise do not suffice to yield a significant weight loss, pharmacological therapies, and highly invasive surgical procedures (e.g., gastric bypass and intragastric balloon) are usually adopted for the control of obesity. Marketed slimming products fall within the dietary treatments and include several oral bulking agents,

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Contract grant sponsor: Italian Ministry of Research (MIUR).

which are claimed to swell inside the stomach, thus giving a sense of fullness. The use of such stomach fillers is particularly appealing, as gastric restriction is achieved, when needed, without requiring surgical interventions. However, there is no scientific consensus over the effectiveness of commercially available bulking agents.

In a previous study,[20] cellulose-based hydrogels were shown to be promising for their possible application as dietary bulking agents, due to their biocompatibility, with respect to intestinal tissues, and high-water retention capacity. In particular, the use of a water soluble carbodiimide as crosslinking agent, with the resulting formation of ester bonds among cellulose chains, led to the synthesis of pure cellulose hydrogels, which were avoid of any crosslinker molecules thus resulting more attractive for biomedical applications.[13,21,22] In this work, the suitability of such hydrogels as bulking agents was further investigated. First of all, several hydrogel formulations were synthesized starting from pharmaceutical and food grade HEC and CMCNa, in view of future *in vivo* experiments on humans. Both acetone-dehydrated and oven-dehydrated samples were considered for further analyses. The hydrogel swelling capability was then tested in water and water solutions mimicking the stomach (pH = 2) and the intestine (pH = 4) environment. The biocompatibility of the samples was then assessed *in vitro* using human fibroblasts (HFs). Experimental results were encouraging for the envisaged application and provided scientific support for the use of cellulose-based hydrogels as dietary fillers.

MATERIALS AND METHODS

Hydrogel precursor materials

Cellulose derivatives meeting food and pharmaceutical standards were used for the hydrogel synthesis. CMCNa (Blanose 7HOF with $M_w \sim 7 \times 10^5$ Da, DS = 0.7, viscosity 1000–2800 cp [1%, 25°C]) and HEC (Natrosol 250 HR with $M_W \sim 10^6$ Da, MS = 2.5, viscosity 1500–2500 cp [1%, 25°C]) were both purchased from Eigenmann and Veronelli, Milano, Italy. The crosslinking agent used was 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC, Sigma-Aldrich, Milano, Italy). Citric acid of food grade was purchased from Dal Cin SpA, Milano and used as a catalyst. All the products were used as received.

Hydrogel preparation

Four different hydrogel formulations were synthesized for further investigation (Table I), by varying the cellulose concentration (3, 4, and 5 wt %) in the precursor solution and the amount of crosslinker (2 and 2.5 wt %). The CMCNa/HEC weight ratio was kept constant at 3/1. A mixture of CMCNa and HEC was first dissolved in distilled water by stirring gently at room temperature until a clear solution was obtained. First, HEC was easily dispersed, after which CMCNa was added. CMCNa dissolution was slow at the concentration adopted, requiring about 24 h. Once the cellulose solution was obtained, an aqueous solution of citric acid (1% *w/v*) was added as a catalyst. The carbodiimide WSC was finally added and allowed to dissolve and distribute homogeneously throughout the solution. The crosslinking reaction occurred in a few hours at room temperature, leading to the formation of a yellowish hydrogel.

TABLE I
Hydrogel Formulations Synthesized and Tested

Sample ID	Cellulose concentration (% *w/v*)	WSC concentration (% *w/v*)
E1	3%	2.5%
E2	4%	2.5%
E3	5%	2.5%
E4	5%	2%

Cellulose concentration indicates the total amount of cellulose derivatives dissolved in the precursor solution. The weight ratio between CMCNa and HEC was 3 : 1 for all samples.

To remove the unreacted chemicals, the partially swollen hydrogel samples were then soaked in a large amount of distilled water until equilibrium was attained. At this stage, the hydrogel samples were perfectly clear and transparent.

After the washing stage, dry products were obtained by desiccating the hydrogel samples by means of two different procedures: desiccation in oven at 45°C and desiccation by extraction with acetone, which is a nonsolvent for cellulose.[10] The morphology of both acetone and oven-dehydrated samples was then observed with scanning electron microscopy (SEM). The gel surface was analyzed in a variable pressure mode with a Zeiss EVO SEM.

Swelling measurements

Hydrogel sorption properties were evaluated both in distilled water and in water solutions mimicking the environmental conditions found in the stomach and the intestine. Simulating gastric and pancreatic solutions were prepared as follows:

- Simulating gastric fluid (SGF, pH = 2): 125 m*M* NaCl, 7 m*M* KCl, 45 m*M* $NaHCO_3$, 0.3% *w/v* pepsin (Sigma Aldrich).
- Simulating pancreatic fluid (SPF, pH = 4): 0.1% *w/v* pancreatin (Sigma Aldrich), 0.15% *w/v* Oxgall bile (Sigma Aldrich).

The pH of the two solutions was adjusted to the correspondent values by using a 35% v/v HCl solution.

Dry samples were weighed and immersed both in distilled water and in the gastric and pancreatic solutions. Once equilibrium was attained, the swollen samples were blotted with soft paper to remove excess liquid from their surface and weighed. The hydrogel swelling capability was quantified in terms of mass swelling ratio Q, defined as follows:

$$Q = \frac{W_s - W_d}{W_d} \quad (1)$$

where W_s is the weight of the sample swollen in a given solution and W_d is the weight of the same sample in the dry state.

Equilibrium swelling measurements were carried out for all samples using an electronic microbalance (Sartorius) with an accuracy of 10^{-5} g.

Hydrogel biocompatibility

Cell culture

HFs were obtained from gingival biopsies of healthy volunteers undergoing odontologic procedures. Briefly, specimens were cut in small pieces (about 10×10 mm^2) under sterile conditions, put in Petri dishes, covered with medium and placed in the incubator. After 10 days, specimens were removed and the adherent fibroblasts were collected and routinely cultured in Dulbecco's Modified Eagles Medium (DMEM, high glucose, with glutamax^{TM}), supplemented with 10% (v/v) fetal bovine serum (Gibco Laboratories, Grand Island, NY), 1% nonessential amino acids (Sigma Chemical Co., St Louis, MO), penicillin (100 units/ml), streptomycin (100 μg/mL), and fungizone (2.5 μg/mL) (Gibco Laboratories, Grand Island, NY). Cells were maintained at 37°C in a 5% CO_2, 95% air, humidified atmosphere. Media were changed every 48 h. For biocompatibility experiments, cells were used between passage 4 and 7.

Biocompatibility test-MTT assay

Two amounts of the dry gel (samples E1, E2, and E3) for each formulation (5 and 10 mg, in triplicate) were swollen at equilibrium under sterile conditions in the standard culture medium free of FBS and phenol red. HFs were seeded at a density of 1.5×10^4 cells/well in a 12-well plate in phenol red free medium and incubated (37°C, 5% CO_2) for 24 h before performing the biocompatibility assays. Then, the medium was replaced with fresh medium containing the various amounts of swollen hydrogels for 24 and 48 h. Cells treated and untreated with Triton X (1%) were used, respectively, as negative and positive controls.

To assess cell viability and proliferation after the contact with the gel for 24 and 48 h, MTT test was performed. The key component is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Mitochondrial dehydrogenases of living cells reduce the tetrazolium ring, yielding a blue formazan product which can be spectrophotometrically measured. The amount of formazan produced is proportional to the number of viable cells present.

MTT (5 mg/mL in DMEM without phenol red) was added to the wells at 10 vol % of the culture medium. After an incubation of 3 h at 37°C, the liquid was aspirated and the insoluble formazan produced was dissolved in HCl 0.1M in isopropanol. The absorbance of the obtained solutions was measured at 570 nm using a Beckman DU 640 spectrometer, Milan, Italy. The relative cell viability was calculated as percentage with respect to the maximal absorbance (control).

Statistical analyses

Two-way ANOVA was used to determine the effect of cellulose concentration and desiccation method (samples E1, E2, and E3) and the effect of WSC concentration and desiccation method (samples E3 and E4) on the hydrogel sorption capability in distilled water.

One-way ANOVA and Fisher's PLSD tests were applied to detect significant differences among groups of swelling data in physiological solutions. Paired t-tests were also performed to compare individual sets of data for a given sample. A probability value of 95% ($P < 0.05$) was used as the criterion for significance.

RESULTS

Swelling measurements in distilled water

The swelling tests in distilled water were carried out for both oven and acetone dehydrated samples, and highlighted that the desiccation method strongly affects the hydrogel water holding capacity (Fig. 1; $P < 0.0001$ for desiccation method). In particular, the acetone dehydrated samples displayed higher swelling ratios versus the air-dried ones, which were about two-fold for samples E2 and E3 (respectively 4 and 5 wt % cellulose, 2.5 wt % WSC) and four-fold for sample E4 (5 wt % cellulose, 2 wt % WSC). However, the increase of the swelling capability between acetone- and air-dried specimens was not significant for sample E1 (3 wt % cellulose, 2.5 wt % WSC) (Fig. 1; $P = 0.11$).

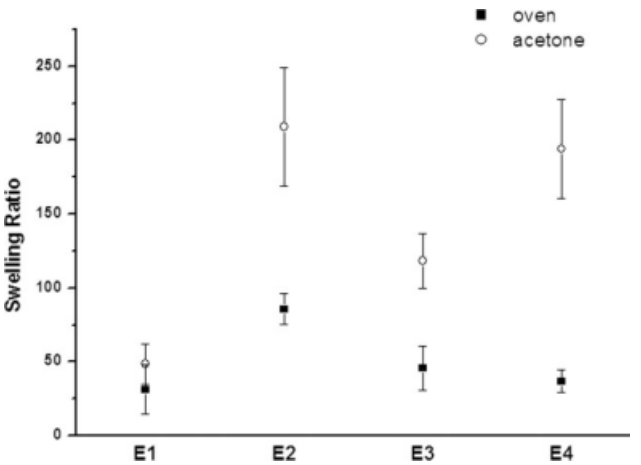

Figure 1 Hydrogel equilibrium swelling properties in distilled water. Results reported as mean ± standard deviation of the mean ($N = 5$ for each formulation).

Cellulose concentration in the precursor solution was also found to significantly affect the hydrogel sorption properties ($P < 0.0001$), with the highest swelling ratios obtained for sample E2 (4 wt % cellulose, 2.5 wt % WSC). When comparing samples E3 (5 wt % cellulose, 2.5 wt % WSC) and E4 (5 wt % cellulose, 2 wt % WSC), it was observed that WSC concentration could also affect significantly the equilibrium swelling capability ($P = 0.0026$), with higher swelling ratios yielded for sample E4 (acetone dried).

Swelling measurements in simulating gastric and pancreatic fluids

As acetone dehydrated samples showed the highest swelling capability in distilled water, such samples were chosen for further analysis. Swelling tests in physiological solutions mimicking the gastric (SGF) and pancreatic (SPF) environments were performed (Fig. 2). As expected, the swelling ratios obtained (~ 30–60) were much smaller than the analogous ones yielded in distilled water (~ 100–150), although such ratios might still be significant for application of the hydrogels as bulking agents in the stomach. Cellulose concentration was found to affect significantly the hydrogel swelling in SGF ($P = 0.0013$), but no significant difference could be detected among the hydrogel swelling ratios in SPF, for the number of samples tested ($n = 5$ for each formulation). With regard to WSC concentration, there was a significant difference between samples E3 and E4 in SGF ($P = 0.0035$), but not in SPF. The hydrogel swelling capability was higher in SPF than in SGF for all the formulations tested. However, only samples E1 and E4 displayed a significantly different swelling behavior in gastric and pancreatic solutions ($P = 0.015$ for sample E1, $P = 0.011$ for sample E4).

Biocompatibility test

The results from the MTT assays [Fig. 3(a,b)] showed that the hydrogel formulations tested did not significantly alter cell viability after both 24 and 48 h of incubation in culture medium, conditioned with two different amounts of hydrogel (5 and 10 mg).

Sample E1, both acetone and oven dehydrated, was the only one to be significantly different from the positive control ($P < 0.05$) after both 24 and 48 h of incubation, as it seemed to improve cell proliferation. A few samples displayed a slight reduction of cell viability after 48 h of incubation.

DISCUSSION

It was recently hypothesized that a cellulose-based, pH-sensitive hydrogel might work as an useful stomach filler, for the treatment of obesity and overweight.[20] The basic idea is that a xerogel-based pill is administered orally before each meal, and that the xerogel powder swells, once in the stomach. In such a way, the space available for food intake is reduced, giving a feeling of fullness. The swollen hydrogel is then eliminated from the body by the fecal way. In this perspective, the hydrogel is envisaged to pass through the gastrointestinal tract, thus it is supposed to encounter the different pH environments of the stomach and the intestine.

In this work, the swelling capability of different hydrogel formulations was tested in water solutions simulating the gastric and pancreatic milieus. The hydrogel biocompatibility was also assessed. The hydrogels were synthesized starting from cellulose derivatives meeting FDA and CE pharmaceutical

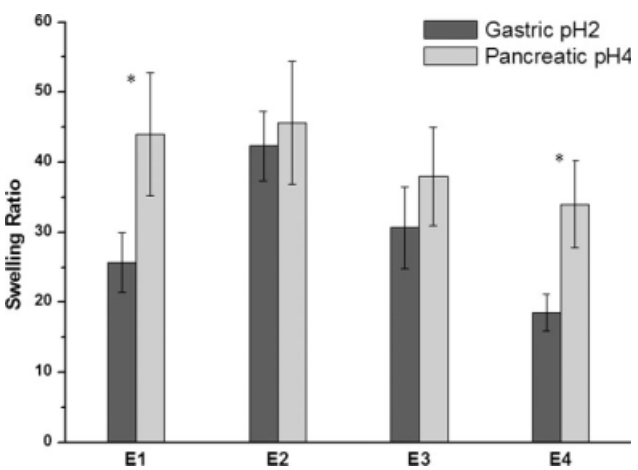

Figure 2 Equilibrium swelling properties of acetone dehydrated hydrogels in physiological solutions at different pH. Results reported as mean ± standard deviation of the mean ($N = 5$ for each formulation). *Significant difference between swelling ratios in gastric and pancreatic solutions.

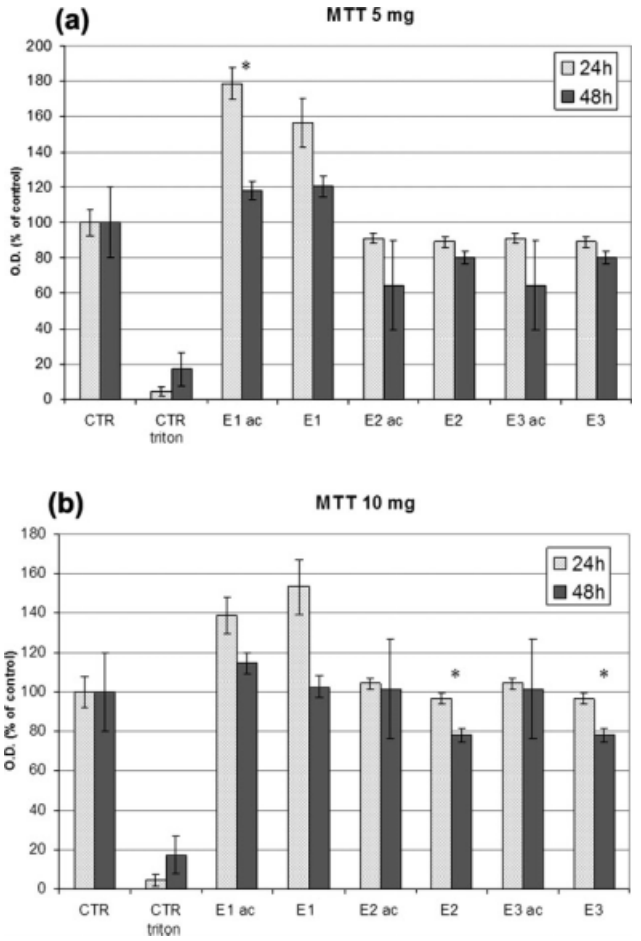

Figure 3 MTT assays. Human fibroblasts were incubated for 24 and 48 h in contact with either 5 mg (a) or 10 mg (b) of dry hydrogel swollen at equilibrium, either oven or acetone dehydrated (the latter indicated as "ac"). Results are reported as mean ± standard error of the mean of three independent determinations. No significant differences were detected between each sample and the positive control (untreated cells), with the exception of samples E1 and E1ac. *Significant reduction of cell viability when comparing the data for 24 and 48 h of incubation ($P < 0.05$).

standards, to directly use the experimental results as preliminary data for future tests on humans.

The swelling capability in distilled water was firstly evaluated and was found to be greatly affected by the desiccation method adopted to obtain the dry xerogel powder. As expected, the samples dehydrated by phase inversion in acetone, which is a nonsolvent for cellulose, displayed significantly higher swelling ratios compared with the samples dried in oven (Fig. 1), with the only exception of sample E1 (3 wt % cellulose, 2.5 wt % WSC), for which acetone- and air-dried xerogels showed similar sorption ratios. The different swelling capability was ascribed to the different microstructure achieved in acetone versus oven-dehydrated samples. Indeed, the rapid extraction of water by acetone leads to the formation of dry samples with a microporous structure,[10] which further contributes to the hydrogel swelling by means of capillarity effects [Figs. 1 and 4(a)]. Conversely, air drying in oven results in samples possessing a compact and dense microstructure, thus able to absorb lower amounts of water [Figs. 1 and 4(b)].

With regard to the effect of different cellulose concentrations on the hydrogel swelling capability (samples E1, E2, and E3, obtained by 3, 4, and 5 wt % cellulose respectively and 2.5 wt % WSC), sample E2 displayed the highest swelling ratios, both in distilled water (Fig. 1) and simulating gastric and pancreatic solutions (Fig. 2). To interpret this result, it is worth considering the key role played by polymer concentration in the crosslinking process. Indeed, two counteracting mechanisms can lead to different degrees of crosslinking, even though the crosslinking agent to polymer ratio is kept constant: (1) an increase of the polymer concentration in solution favors the formation of entanglements, which, after chemical crosslinking, become effective as additional crosslinking points, thus reducing the water sorption capability[13]; (2) a very low concentration of polymer in solution reduces the probability of chemical crosslinking, simply because the concentration of the reactive sites is decreased and the rate of reaction is proportional to the concentration of reactive sites. In

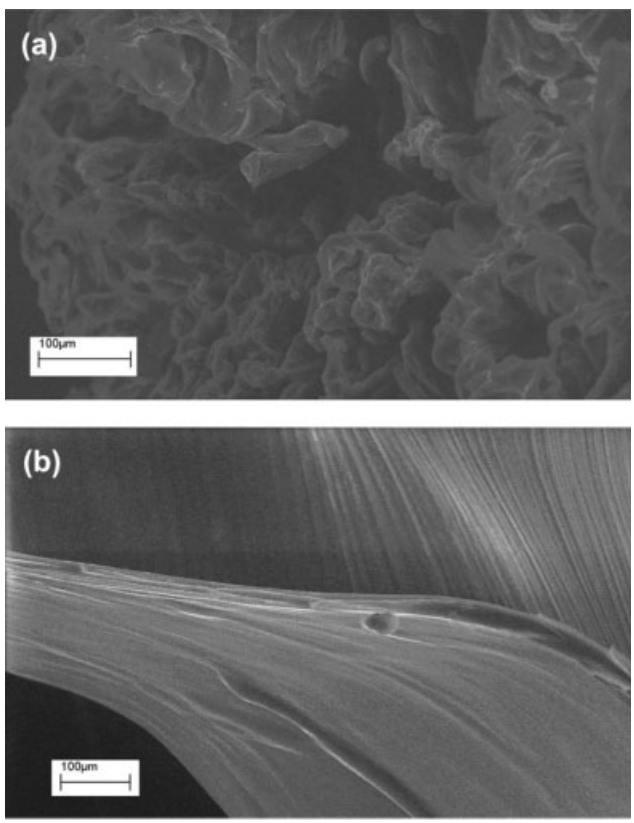

Figure 4 SEM micrographs of the surface of sample E2: (a) acetone dehydrated and (b) oven dehydrated. The scale bar is 100 µm.

the latter case, unstable and loosely crosslinked hydrogel networks are obtained, which show poor swelling properties compared with well-crosslinked networks. The former mechanism, that is the formation of physical entanglements, might explain the reduction of the swelling ratio detected when increasing the polymer concentration from 4 to 5 wt % (samples E2 and E3), whereas a low concentration of reactive sites is likely to explain the low-sorption properties of sample E1 (3 wt % cellulose), when compared with sample E2 (4 wt % cellulose). Moreover, the loose crosslinking achieved for sample E1 might also justify the fact that its swelling properties do not significantly depend on the desiccation method used. Based on the swelling in distilled water and in simulating gastric and pancreatic fluids, it was concluded that, for the specific cellulose derivatives used, a cellulose concentration of 4 wt % is the optimal one to be adopted for the hydrogel synthesis.

It is worth noting that the values of the hydrogel swelling ratio in distilled water reported in this work differ greatly from those reported in a previous study.[20] For an analogue of the acetone-dehydrated sample E1 (3 wt % cellulose, 2.5 wt % WSC), the equilibrium swelling ratio in distilled water was reported to be about 200, as opposed to the value of 50 found in this study. Such a striking difference might be ascribed to the different quality of cellulose derivatives used for the synthesis. Indeed, the hydrogel synthesis and the properties of the final polymer network are greatly affected by the starting materials, for example, by their molecular weight, degree of substitution and viscosity in water solutions. However, changing the source of the precursor materials in the hydrogel production, shifting from chemical grade precursors to pharmaceutical grade ones, was necessary to target the biomedical field.

With regard to the hydrogel swelling capability in physiological solutions, it was expected that the cellulose-based hydrogels were sensitive to variations of the pH of the external solution, as well as to variations of the ionic strength, because the CMCNa used is a polyelectrolyte species that makes the hydrogel polyanionic. It has long been known that a crosslinked polymer gel, bearing acidic and/or basic pendant groups, is able to imbibe solvent up to a certain extent, which depends on the pH and the ionic composition of the solution bathing the gel.[15] For ionic hydrogels, the swelling driving force (i.e., the total osmotic pressure) presents two additional contributions if compared with neutral ones, ascribed to the presence of fixed charges on the polymer backbone. A Coulombian contribution accounts for the electrostatic repulsion established between charges of the same sign tethered on the polymer network, which causes the polymer chains to stretch to a more elongated state, thus increasing the swelling capability. The second ionic contribution accounts for the different concentration of mobile counterions found between the hydrogel and the external solution, with more counterions present in the gel, which induces more solvent to enter the network. The counterions are indeed trapped into the gel to ensure macroscopic electrical neutrality. This effect is explained in terms of a Donnan type equilibrium[15] established between the gel and the surrounding solution. Therefore, as the ion concentration in the external solution changes, the ionic hydrogel responds in terms of different swelling.

In particular, anionic hydrogels, such as the ones described in this work, tend to deprotonate and swell when the external pH is higher than the dissociation constant pK_a of their ionizable groups, that is when a large amount of ions are tethered to polymer network. At low pH values (e.g., in the simulating gastric fluid at pH = 2), the chemical equilibrium of the dissociation of the carboxylic groups anchored to CMCNa chains changes, as H^+ ions will be associated to the carboxylic groups. The concentration of fixed charges in the gel will thus decrease, leading to lower swelling ratios and eventually resulting in the formation of an uncharged gel at very low pHs.[11] Conversely, as the pH increases, the concentration of cations in the outer solution will increase. These cations will replace the mobile H^+ ions into the gel, so that new H^+ ions will be supplied by the yet undissociated carboxylic groups of the cellulose. The concentration of mobile ions in the gel will thus increase more rapidly than in the outer solution, leading to higher ion swelling pressures. This is the main reason why the swelling capability of the cellulose-based hydrogels was higher at pH = 4 rather than pH = 2, for all the formulations tested, although the difference was not significant for samples E2 (4 wt % cellulose, 2.5 wt % WSC) and E3 (5 wt % cellulose, 2.5 wt % WSC). The supply of H^+ ions is, however, limited, because eventually all the carboxylic groups of the hydrogel will be dissociated. Therefore, at a given value of pH, the ionic swelling pressure will begin to drop again, thus reducing the hydrogel swelling capacity.[11] Because of the different pH as well as to the presence of several ions in the external solutions, the hydrogel swelling capacity in the physiological media was much smaller than that obtained in distilled water. However, values of the swelling ratio around 50–60, such as those obtained for sample E2, might be ideal for the intended application of the hydrogel as stomach filler.

In terms of hydrogel responsiveness to pH variations, it is worth noting the likely effect of different amounts of crosslinking agent in the precursor solution, which was accounted for by comparing

samples E3 and E4 (5 wt % cellulose, 2.5 and 2 wt % WSC, respectively). As WSC mediates the formation of ester bonds between the carboxyl and hydroxyl groups of the cellulose derivatives,[13] the amount of WSC used in the hydrogel synthesis might affect not only the resulting degree of crosslinking, but also the concentration of carboxyl groups (i.e., the ionizable ones) in the hydrogel network, which determines the hydrogel responsiveness to different pHs. This might explain the significantly higher swelling ratios in distilled water detected for acetone dehydrated sample E4 (2 wt % WSC) versus sample E3 (2.5 wt % WSC), as well as the higher sensitivity of sample E4 to pH variations, at least in the range of pHs studied (Fig. 2). Indeed, when decreasing the pH of the swelling medium, the sorption capability of sample E4 drops more sharply than that of sample E3, so that at pH = 2 sample E3 shows a higher solution uptake.

In view of the possible application of the hydrogel as stomach filler, HFs were used to assess the gel's biocompatibility, to obtain more reliable information (compared with the use of immortalized cell lines) about cellular response after the contact with the gel. The preliminary biocompatibility assays showed that the hydrogel formulations, disregard of the desiccation method used, are well tolerated by the cells even after 48 h of incubation, thus suggesting that no toxic substances are released from the xerogels on swelling. The slight reduction in cell viability induced by some samples was probably due to the fact that the gel could have acted mechanically on the cell layers; however even at 48 h of contact all the samples tested showed an optimal biocompatibility in terms of cell proliferation. Further experiments should be carried out to better characterize the cells' behavior with respect to the contact with the gel.

CONCLUSIONS

In this work, cellulose-based hydrogels obtained from cellulose derivatives of pharmaceutical grade were investigated as dietary bulking agents for the treatment of overweight and obesity. Several formulations were prepared, differing for the cellulose and/or the crosslinker concentration, with the crosslinker being a water-soluble carbodiimide, and for the desiccation method. *In vitro* swelling tests performed in water and in physiological solutions mimicking the environment of the gastrointestinal tract showed that all hydrogel formulations were sensitive to pH variations and yielded degrees of swelling suitable for the envisaged application. Moreover, the hydrogel swelling capability was easily tunable, being it particularly affected by the cellulose concentration, the amount of crosslinking agent used, and the desiccation method. *In vitro* biocompatibility assays provided evidence for the nontoxicity of such hydrogels, thus supporting their possible use as stomach fillers *in vivo*.

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