A Cellulose-Based Hydrogel as a Potential Bulking Agent for Hypocaloric Diets: An *In Vitro* Biocompatibility Study on Rat Intestine

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ABSTRACT: A number of over-the-counter slimming products are currently available on the market. However, there is no scientific consensus over their effectiveness in promoting and sustaining weight loss. The need to develop an alternative dietary supplement for the treatment of obesity and overweight makes attractive a polyelectrolyte cellulose-based hydrogel, crosslinked through a water soluble carbodiimide, as a potential bulking agent or stomach filler for hypocaloric diets. The hydrogel is envisaged to be administered orally to absorb water in the stomach, thus swelling and giving a sense of fullness, and to be finally expelled by fecal way. To this purpose, a preliminary assessment of hydrogel swelling capacity in distilled water has been performed, and the biocompatibility of the material with respect to intestinal tissues has been evaluated *in vitro*. The direct contact with the intestinal mucosa *in vivo* has been simulated by contacting the hydrogel with the jejunum tract of rat intestine, and the capacity of the material to maintain the epithelial barrier integrity has been monitored by means of transepithelial electric resistance measurements and lactate dehydrogenase release assay. The reported results evidence that the hydrogel is well tolerated by the intestinal tissue during the expected time of contact *in vivo*. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 102: 1524–1530, 2006

Key words: hydrogels; swelling; microstructure; crosslinking

INTRODUCTION

As reported by the World Health Organization in 2003, more than 1 billion people worldwide are overweight, and at least 300 million of them are clinically obese.[1] Currently, obesity and overweight are the second cause of death after smoking, as major risk factors for several chronic diseases, such as type 2 diabetes, cardiovascular disease, sleep apnoea, hypertension, stroke, and certain forms of cancer. Moreover, being overweight or obese often has a dramatic impact on the psychological well-being, reducing the overall quality of life.[1–4]

Since weight gain depends on the energy balance between food intake and energy consumption, the direct causes of the recent rise in obesity, in particular among children, can be found in the increased consumption of nutrient-poor foods, which instead are rich in sugar and saturated fats, and in the reduced physical activity, which both characterize the modern lifestyle. Hence, the treatment of overweight and obesity usually consists of a supervised diet, often combined with adequate physical exercise. In the most serious cases, surgical procedures, that involve essentially gastric restriction, or particular drug treatments, may be required.[2]

Nevertheless, in the recent years, a number of dietary supplements and meal replacements have been developed and sold as over-the-counter slimming aids.[5] Although meal replacements can help to provide balanced nutrients and to control calories intake, dietary supplements are claimed to act either by binding fats and so reducing fat absorption, as reported for chitosan-based products, or by directly reducing the appetite, as for different natural fibers and herbal products, that seem to absorb liquids and swell inside the stomach, thus giving a sense of fullness.[5,6] The latter approach, based on the use of natural fillers or bulking agents, is very interesting for its great potential of reducing the amount of food intake by reducing the available space in the stomach, without the need of complex surgical interventions. However, there is no clear evidence of the effectiveness of currently available bulking agents in promoting weight loss, neither in the short-term nor in the long-term, whereas their adverse effects, usually including gastrointestinal symptoms, have been well documented.[5,6] Moreover, it should be taken into account that some fillers may be harmful, causing obstructions in the intestines, stomach, or esophagus, as reported for guar gum.[7]

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Therefore, the development of novel bulking agents, effective in promoting weight loss, is needed. In this direction, superabsorbent hydrogels, i.e., 3D polymeric networks that are able to swell several times their original size in aqueous solutions, are of particular interest, since not only can their swelling capacity be properly designed by controlling their chemical composition and physical microstructure, but it can also be modulated by changing the environmental conditions (e.g., pH, ionic strength, temperature).[8–15] Recently, superporous acrylate-based hydrogels, which swell very rapidly in aqueous solutions, have been proposed as an alternative to currently available dietary fillers.[16]

In this work, a novel cellulose-based hydrogel, obtained by crosslinking an aqueous mixture of hydroxyethylcellulose (HEC) and carboxymethylcellulose sodium salt (CMCNa) through a water soluble carbodiimide (WSC, EDC, or EDAC), was studied for the production of dietary bulking agents. The chemical structures of the cellulose derivatives and the carbodiimide used in this study are reported in Figures 1 and 2, respectively. Dry hydrogel-based pills are thought to be administered orally, to swell inside the stomach thus reducing the feeling of hunger, and to be finally expelled by fecal way. Therefore, this study aims at a preliminary assessment of the hydrogel biocompatibility, with respect to intestinal tissues and swelling capacity.

In previous works[17,18] it has been demonstrated that the presence of the polyelectrolyte CMCNa significantly increases hydrogel swelling capacity when compared to pure HEC hydrogel, and this is due to its polyelectrolyte nature. In fact, it induces a Donnan type effect, resulting in an osmotic pressure that favors water to penetrate the hydrogel; moreover, a desiccation procedure by phase inversion with acetone allows for the formation of a microporous structure within the material, thus enhancing both the equilibrium swelling capacity and the rate of response to environmental stimuli.[19,20]

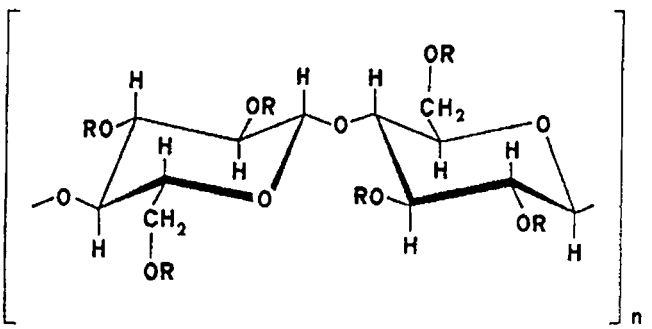


Figure 1 Repeating units of cellulose derivatives. For CMCNa, R = —H, —CH_2COONa and for HEC R= —H, CH_2CH_2OH, —$CH_2CH_2OCH_2CH_2OH$, —$CH_2CH_2OCH_2CH_2OCH_2CH_2OCH_2CH_2OH$.

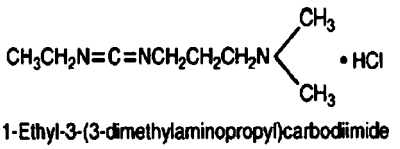


Figure 2 WSC molecule, employed as crosslinking agent for cellulose derivatives.

In this study, the use of the water soluble carbodiimide as a novel crosslinking agent improves the potential biocompatibility of the resulting hydrogel, since the WSC molecule is not incorporated into the crosslinking bonds but it is changed into an urea derivative, which displays a very low degree of cytotoxicity and can be easily washed out from the polymeric network.[21–25] The chemical reactions by which WSC induces the formation of ester bonds between polysaccharide macromolecules are well-known in the literature[21,22] and are schematically reported in Figure 3. As discussed in detail in a previous study,[26] the carboxyl groups provided by the CMCNa in an acidic environment can react with the WSC molecule as indicated in Figure 3(a), and an ester crosslinking bond between two cellulose macromolecules is finally obtained according to the reaction scheme in Figure 3(b).

The response of intestinal cells to the material under investigation has been assessed *in vitro* by placing the hydrogel in contact with the jejunum tract of rat intestine for a 4-h period and so simulating the expected time of direct contact between substances and the intestinal mucosa *in vivo*.

Transepithelial electrical resistance (TEER) measurements and lactate dehydrogenase (LDH) analysis have

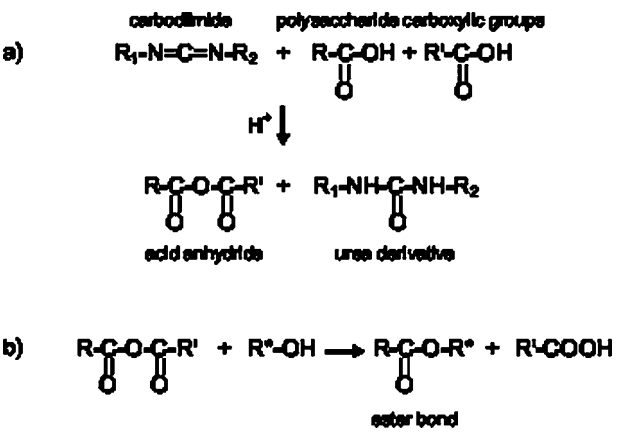


Figure 3 Reaction scheme of WSC with polysaccharide molecules: (a) in acidic aqueous environment, the carbodiimide induces the intramolecular or intermolecular formation of an acid anhydride between two polysaccharide carboxyl groups, changing itself into a nontoxic urea derivative; (b) the acid anhydride reacts with a hydroxyl group to yield an ester crosslinking bond between two polysaccharide molecules.

revealed that the hydrogel does not affect negatively the epithelial barrier integrity.

Hydrogel microstructure has been analyzed by means of scanning electron microscopy (SEM), and its effect on the material's equilibrium sorption capacity has been tested in distilled water, as a function of different WSC concentrations. Further studies on hydrogel sorption capacity in water solutions mimicking the actual environmental conditions in the stomach and the intestinal tract will be performed.

MATERIALS AND METHODS

Materials

The hydrogel under investigation has been obtained by crosslinking a mixture of carboxymethylcellulose sodium salt (CMCNa) and hydroxyethylcellulose (HEC) by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC) as crosslinking agent in acid water solution. CMCNa (MW, 700,000; DS, 0.9; viscosity, 3400 cp [c = 1%, H_2O at 25°C]), HEC (MW, 250,000; MS, 2; viscosity, 80–125 cp [c = 2%, H_2O at 25°C]), WSC, and citric acid were purchased from Sigma-Aldrich (Milan, Italy) and used as received.

Hydrogel synthesis

The hydrogel has been prepared according to the following procedure. A mixture of CMCNa and HEC is dissolved in distilled water by gently stirring at room temperature, until a clear and homogeneous solution is obtained. The final polymer content is 3 wt %, with a weight ratio CMCNa/HEC of 3/1. After the addition and mixing of either 5 or 2.5 wt % of the carbodiimide WSC as crosslinking agent, an aqueous solution 1 wt % of citric acid is added as a catalyst, in order for the crosslinking reaction to occur in a few hours at atmospheric conditions. The resulting hydrogel is in a partially swollen state; therefore, the unreacted chemicals (e.g., the catalyst, the substituted urea derived from WSC) are removed from the polymer network by simply washing with distilled water, until the equilibrium swelling is reached. Microporous xerogel is finally prepared by desiccating swollen hydrogel samples by phase inversion with acetone, as reported for different cellulose-based hydrogels.[19,20] A further desiccation stage at 30°C for about 1 h allows the elimination of the residual acetone.

Hydrogel samples desiccated in air atmosphere at room conditions have been prepared for the sake of comparison.

Swelling studies

Equilibrium swelling measurements in distilled water were performed to assess the hydrogel sorption capacity.

The water uptake is expressed in terms of swelling ratio Q, defined as follows:

$$Q = \frac{W_{\text{swollen}}}{W_{\text{dry}}}$$

where W_{swollen} is the weight of the hydrogel swollen at equilibrium and W_{dry} is its weight in the dry state. Reported values of equilibrium water uptakes for each different WSC concentration and desiccation procedure have been averaged over 3 independent measurements.

In brief, preweighed xerogel samples, obtained from distinct batches, were soaked in a large amount of distilled water, and their weight gain was monitored until the equilibrium was reached, after about 24 h. Before weighing, the swollen hydrogel samples were gently blotted with soft paper to remove excess water from their surface.

All the measurements were carried out using an electronic microbalance (Sartorius) with an accuracy of $\pm 10^{-5}$ g.

Morphological analysis

The microstructure of hydrogel samples, desiccated both by phase inversion in acetone and in air atmosphere at room conditions, was analyzed by means of a JEOL JSM-6500 F SEM. The dry samples were placed directly onto the sample holder without the need of prior sputter coating.

Tests on rat intestine: TEER measurements and LDH

With the aim of evaluating the effects of the cellulose-based hydrogel on the intestinal tissue, different segments of rat jejunum intestine were placed in direct contact with either the hydrogel (WSC 5 wt %, desiccated in acetone) or the crosslinker for a 4-h incubation time, and the TEER and the LDH release were measured during and after the contact respectively, as significant indicators of cell damage. A 4-h incubation time is expected to approximate the real time of contact *in vivo* where food passes through the duodenum and jejunum over ~3 h. The jejunum represents the gastrointestinal segment where most digestion and absorption occur.

The experiments were carried out on native tissues mounted in a Ussing chamber set up.[27] This experimental approach has advantages in surveillance of the mucosa functionality compared to other *in vitro* techniques.[28]

All animal procedures were carried out using institutionally approved protocols. Three male Wistar rats (weighing about 350 g) were used for the experiment. From each animal, three adjacent segments (1.5 cm length) of the jejunum tract were isolated, cut along the

intestine longitudinal axis to produce a mucosal sheet, and mounted vertically in a Ussing chamber.

After mounting, each half chamber was filled with 6 mL Krebs-buffer (NaCl, 124; KH_2PO_4, 1.25; $MgCl_2$, 1.8; KCl, 1.75; $CaCl_2$, 1.6; $NaHCO_3$, 26; glucose, 10 (in m*M*)), bathing the intestinal tissue on both the mucosal and serosal side. The exposed tissue surface area was 0.3 cm^2. The Krebs-buffer was continuously oxygenated with O_2/CO_2 (95/5%) and stirred by gas flow in the chambers. The equilibrium between CO_2 and $NaHCO_3$ maintained the pH of the Krebs buffer at the constant value of 7.4.

The temperature of the Krebs solution was kept constant at 28°C all through the experiment. Tissues were connected to an automatic short-circuit current device (SH-89, Tecnovision, Milan, Italy) by four electrodes: two Ag/AgCl electrodes directly placed in the solutions and two calomel electrodes, which made contact with the bathing solutions via agar-Krebs filled agar bridges.

In particular, the three segments were treated as described in the following: segment (1) was taken as a control, i.e., simply bathed with physiological solution; segment (2) was placed in contact with 20 mg of hydrogel, swollen to equilibrium in the physiological solution (6 mL), and added from the luminal side of the epithelium; and segment (3) was treated with the carbodiimide, by adding 0.42 g of WSC to the bathing solution (6 mL) from the luminal side of the epithelium.

Either in control or test intestinal segments, the transepithelial potential difference, which is expression of the ion transport function of the intestine, was constantly monitored as tissutal viability index by the two calomel electrodes. All through the experiments, the transepithelial voltage showed the constant value of 1.5 ± 0.2 mV, which is typical for this tract of rat intestine.[29]

The TEER was determined every 5 min by passing pulses of current (33 μAcm^{-2}, 500 ms) across the tissue via Ag/AgCl electrodes and measuring the resulting transepithelial voltage deflection.

At the end of the experiment, the LDH leakage into the mucosal and serosal bathing solutions was monitored for control as well as test jejunum segments. LHD activity was measured according to the spectrophotometric method described by Wroblewski and La Due.[30] This method is based upon the ability of LDH to catalyze the reduction of pyruvate to lactate by oxidation of NADH to NAD^+. The decrease of NADH absorbance at 340 nm using sodium pyruvate as a substrate was recorded by a Beckmann Coulter DU 6405 spectrophotometer.

LDH activity was expressed as enzymatic units per milliliter (UmL^{-1}) and was calculated according to the following equation. One enzymatic unit corresponds to the amount of enzyme capable of oxidizing one micromole of NADH per minute at 25°C, pH 7.3

$$\text{LDH activity (U mL}^{-1}) = \frac{\Delta A(\text{min}^{-1}) \times 3.15 \times \text{df}}{\varepsilon \times 0.1 \times 1}$$

where ΔA min^{-1} is the rate of absorbance change at 340 nm, 3.15 is the total reaction volume in milliliters, df is the dilution factor, ε is the millimolar extinction coefficient of NADH at 340 nm = 6.2 mM^{-1} cm^{-1}, 0.1 is the sample volume in milliliters, and 1 is the path length in centimeters. Triplicate determinations were performed for each sample.

RESULTS AND DISCUSSION

Swelling studies

With the aim of investigating the effect of different degrees of crosslinking on the material's sorption properties, equilibrium swelling measurements in distilled water were performed on hydrogel samples, obtained from two different WSC concentrations in the reactive polymer solution, 2.5 and 5 wt %, respectively. Moreover, the swelling capacity of samples desiccated both by phase inversion in acetone and in air atmosphere at room conditions has been evaluated for each WSC concentration. The resulting swelling ratios are reported in Figure 4.

As expected, the use of a higher concentration of WSC in the starting solution leads to a lower sorption capacity, for both the desiccation techniques used; indeed, a higher concentration of crosslinking agent induces the formation of a polymeric network with a higher number of crosslinking bonds per unit volume (i.e., higher crosslink density), which contribute negatively to its swelling capability.[18] In particular, for the acetone-dried samples, doubling the WSC concentra-

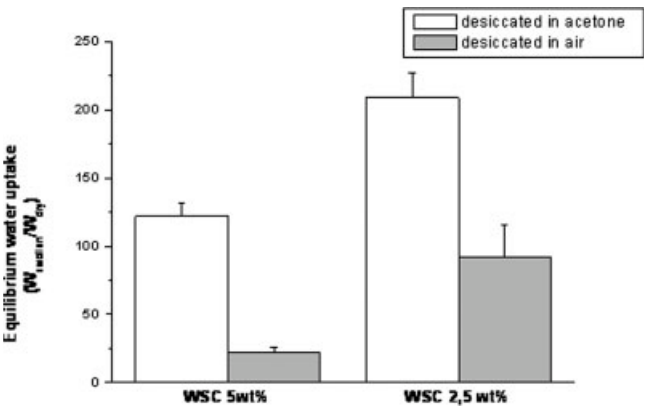


Figure 4 Hydrogel equilibrium swelling capacity in distilled water: influence of different WSC concentrations and desiccation procedures. Reported results, expressed as mean ± standard error, have been averaged over three independent measurements.

tion from 2.5 to 5 wt % reduces the swelling capacity from about 210 to 120 g water/g dry polymer.

Moreover, it can be observed that the samples dehydrated by phase inversion in acetone display higher water uptakes when compared with the same samples desiccated in air at room conditions. This finding can be explained considering that the desiccation by phase inversion in acetone, which is a nonsolvent for cellulose, leads to the formation of a microporous network within the material, which gives rise to a further capillary retention of water.[19,20]

It is worth noting that the hydrogel swelling capacity, dependent on both the degree of crosslinking and the hydrogel microstructure, can be modulated by changing also the weight ratio CMCNa/HEC. Indeed, the presence of fixed charges on the polymer backbone due to the polyelectrolyte CMCNa improves the material's swelling capacity when compared with pure HEC hydrogel, owing to an increased osmotic pressure (Donnan effect).[17,18] In particular, being the osmotic pressure proportional to the difference in concentration of charges between those contained in the gel and those in the external environment, a polyelectrolyte hydrogel is expected to be particularly sensitive to changes of the ionic strength or pH of the external solution.[17–20]

With the aim of developing a dietary bulking agent, the hydrogel sorption properties in water solutions mimicking the strong environmental conditions typical of the stomach will be assessed in future studies.

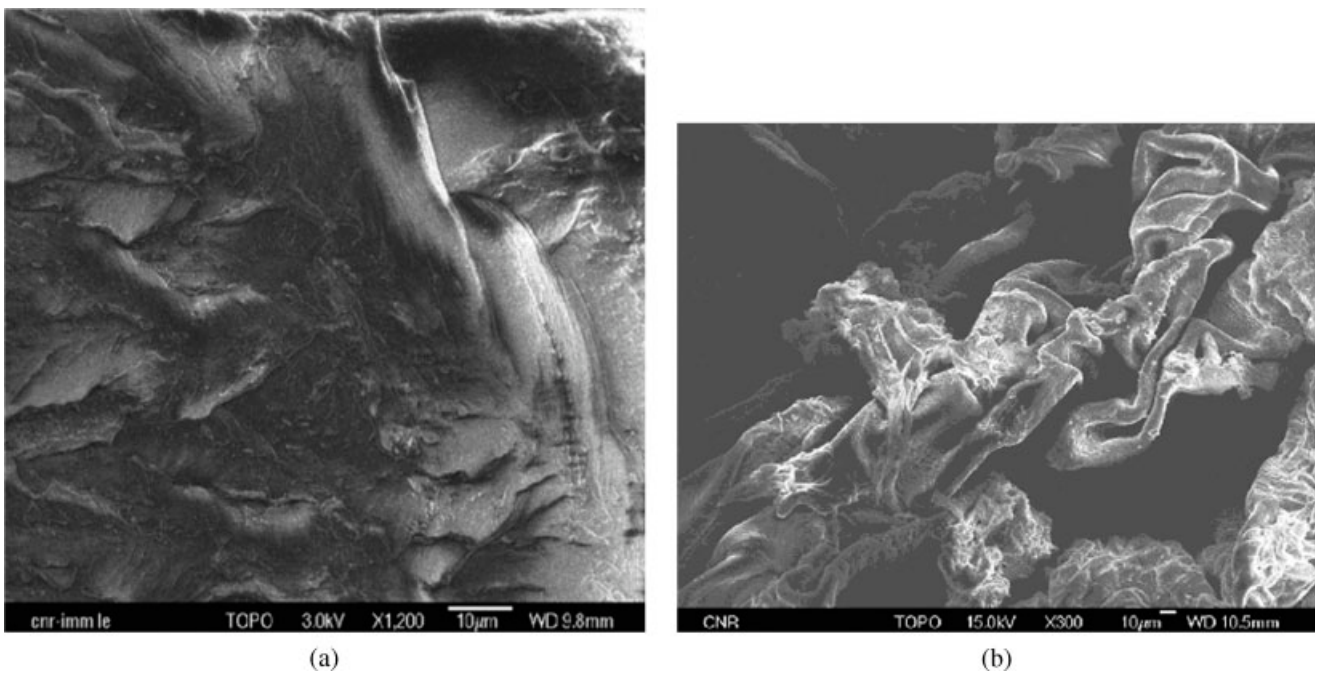
Morphological analysis

Scanning electron micrographs were performed on hydrogel samples, dehydrated both by phase inversion in acetone and in air atmosphere at room conditions, to assess the effect of the desiccation technique on the hydrogel morphology. For the sake of comparison, Figure 5 reports the different micrographs obtained for a 5 wt % WSC-concentrated hydrogel. As expected, the sample desiccated in air atmosphere displays a compact structure when compared with the same sample dried in acetone, which conversely shows a highly irregular and nonhomogeneous surface. This difference in hydrogel microstructure is ascribed to the more rapid extraction of water from the hydrogel when placed in contact with acetone. Indeed, a microporous network is formed within the material as the fast expulsion of water occurs.[19,20]

TEER measurements

The gastrointestinal epithelium normally functions as a selective barrier that permits the absorption of nutrients, electrolytes, and water. The epithelial barrier function is provided by the epithelial cells and the tight junctions (TJs) that connect them. The integrity of TJs is fundamental for the functional integrity of the tissue and can be electrophysiologically expressed by the TEER.

In the present work, TEER measurements were carried out to assess the effect of the cellulose-based



(a) (b)

Figure 5 Scanning electron micrographs of the hydrogel surface; the pictures refer to the hydrogel obtained from a reactive solution with 5 wt % concentration of WSC and CMCNa/HEC weight ratio equal to 1/1: (a) air-dried sample and (b) acetone-dried sample.

hydrogel on the integrity of intestinal epithelium. The results are reported in Figure 6 as percentage of the initial value of TEER (corresponding to 20.2 ± 1.2 Ωcm^2), measured before the incubation of the epithelium with the hydrogel or the crosslinking agent (WSC). Data are expressed as average of three independent experiments. It can be observed that the contact of the hydrogel or the crosslinker with rat intestine does not significantly affect the TEER values, when compared with the control (segment 1, perfused with physiological solution). The statistical significance of the data was tested by Dunnett's test after transforming percentage data in corresponding arcsen values. These results suggest that neither the hydrogel nor the crosslinker significantly affect the integrity of gastrointestinal epithelium or alter its permeability in *in vitro* conditions.

LDH assay

Enzyme leakage, especially cytosolic LDH, is widely used as a sensitive measure of cellular damage such as cell membrane injury.[31]

The results of LDH activity measured in the mucosal and serosal perfusates, collected after the 4-h period of incubation of rat intestine, are reported in Table I, as mean ± standard error of three experiments. No significant release of LDH is detected for both the gel-treated and the crosslinker-treated segments either in the serosal or mucosal bathing solution with respect to the control tissues, as stated by Dunnett's test. These results suggest that neither the hydrogel nor the crosslinker induce detectable injury to intestinal cells during their contact with mucosa.

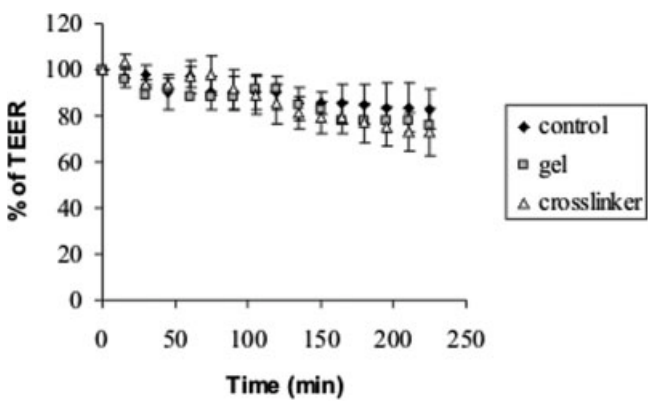


Figure 6 TEER of rat intestine jejunum, monitored during the contact with the hydrogel or the crosslinker. Data are expressed as percentage of the initial value measured before the incubation with the hydrogel or the crosslinker. The time of contact of about 4 h is expected to approximate the real time of contact *in vivo*. Results represent mean ± standard error of three independent experiments.

TABLE I
Lactate Dehydrogenase (LDH) Release by Rat Intestinal Cells after 4-hour Incubation with the Hydrogel or the Crosslinker

	Control (mU/mL)	Gel (mU/mL)	Crosslinker (mU/mL)
Mucosal solution	4.94 ± 1.35	6.79 ± 2.16	4.63 ± 1.10
Serosal solution	5.25 ± 0.82	4.40 ± 2.10	6.48 ± 2.23

The release of LDH has been measured both in the mucosal and serosal perfusates, as explained earlier. The reported results are expressed as mean ± standard error ($n = 3$).

CONCLUSIONS

A novel cellulose-based hydrogel, crosslinked by means of a water soluble carbodiimide, has been presented as potential bulking agent for hypocaloric diets. Equilibrium swelling measurements in distilled water revealed that the hydrogel swelling capacity can be modulated by changing the chemical composition of the reactive mix and the desiccation process. Moreover, the polyelectrolyte nature of the polymer network, due to the presence of CMCNa, is expected to allow hydrogel swelling as a function of the environmental conditions (e.g., pH, ionic strength). Dry hydrogel-based pills are thus envisaged to be administered orally, to swell at the strong acid pH of the stomach, thereby giving a sense of fullness, and to be finally expelled by fecal way.

To test this potentiality, a preliminary assessment of the hydrogel biocompatibility has been carried out by studying the biological effects of the direct contact of the material with the jejunum tract of rat intestine *in vitro*. The reported results evidence that the hydrogel does not reduce significantly the TEER of intestinal tissue nor increases the release of LDH by intestinal cells, thus maintaining the epithelial barrier integrity.

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