Cellulose-based hydrogels as body water retainers

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In this work the possibility of using hydrogels as body water retainers for a therapeutic aid in pathologies such as oedemas of various origins was explored. For such a purpose, the material requires a good compatibility and a controlled swelling capacity without altering the body electrolyte homeostasis. The hydrogel was designed to meet the swelling requirements with the physiological constraints and its biocompatibility was assessed either *in vitro* or *in vivo.* Absorption tests were performed in order to define the swelling behavior by varying the pH and ion content of the external solution. The hydrogel swelling capacity was assessed in the presence of various solvents, in order to evaluate its absorption capacity in solutions similar to biological fluids. In addition, the capacity of the gel to modify electrolyte homeostasis by adsorbing ions such as calcium, potassium and sodium was tested. In order to assess the gel biocompatibility after contact of the hydrogel with intestinal cells, arachidonic acid relase was determined. No significant intracellular increase of free arachidonic acid was found in the cells after up to 2 h of contact with the gel. The results suggest that, as far as brief periods are concerned, the gel does not cause an inflammatory response in intestinal cells.

1. Introduction

Hydrogels are three-dimensional polymeric networks that are widely used in many biomedical applications [1] such as drug delivery systems [2, 3], soft tissue substitutions, contact lenses [4], wound dressings and enzyme immobilization.

Polyelectrolyte hydrogels are characterized by the presence of ionic groups linked to the molecular network. This feature makes them particularly suitable for applications in which their sensibility to environmental conditions (e.g. pH, temperature, external solution composition, ion concentration) is required. In fact it has been observed that the absorbing capacity of polyelectrolyte gels varies widely if the external solution composition, pH [5, 6] and temperature [7] are changed, and decreases if the ion concentration is raised. Moreover, one of the most relevant features of the gels is their capability to absorb extremely large amounts of water, up to 5×10^3 times their initial weight [8].

In this work, a naturally based hydrogel has been developed with the aim of removing water from the body after ingestion via the faecal rout. Water loss is extremely important in the treatment of some pathological conditions such as chronic renal failure and oedemas of hepatic and cardiac origin. The latter two may show resistance to diuretics, thus requiring alternative routes for water elimination.

The hydrogel is meant to be administred orally and therefore must go through the oral cavity (for a very short time), the stomach, the small and the large intestine, and finally be eliminated. During this transit, it will encounter various environmental conditions, such as changes of pH and different ion concentrations. Because of this, the above-mentioned different environmental conditions were simulated *in vitro* in order to test the hydrogel

swelling capacities and to define the more suitable synthesis parameters for the application under investigation.

The tissue–material interactions are often followed by an acute or chronic inflammatory response, regulated by the release of many mediators such as cytokines and prostaglandins. However, little is known about the intracellular mechanisms that are involved in the response to a material.

Intestinal cells play a fundamental role as a barrier between the potential dangerous substances ingested and the internal organs; they are well known to produce inflammatory mediators such as prostaglandins and cytokines under the action of various stimuli. Early events, such as release of free arachidonic acid occurring after an inflammatory stimulus have been investigated as parameters of biocompatibility. Possible therapeutic use of the hydrogel will be discussed.

2. Materials and methods

2.1. Preparations of gels, swelling measurements and ion uptake

Cellulose-based hydrogels were synthesized starting from rough material, as described by Esposito *et al.* [9]. The swelling was measured by weighing samples after their immersion in water solutions for about 24 h. The influence of ion concentration on the absorbant capacity of hydrogels was evaluated by placing dessicated gel samples in solutions at three different NaCl concentrations ($0.01\,mol\,l^{-1}$; $0.5\,mol\,l^{-1}$ and $1\,mol\,l^{-1}$). Samples were weighed after 24 h. The same procedure was adopted for the analysis of the swelling capacity at different pH. Three buffer solutions of pH 3, 7 and 9 were utilized. NaCl was added to buffer solutions in order to keep the ion concentration at a fixed value of $0.4\,mol\,l^{-1}$. Swelling measurements were performed, also, in physiological (0.9% NaCl) and polysaline (commercially available, medical grade) solutions and in culture medium. In order to determine the ion uptake, ion concentrations were measured in the external solutions after putting the hydrogel in contact with the external solutions for 24 h using the Hitachi apparatus according to the manufacturer's protocol.

Another set of samples was used for swelling kinetic measurements in buffer solutions at pH 3 and pH 7. NaCl was added to the solutions to keep the ion concentration at $0.4\,mol\,l^{-1}$. Dessicated samples were placed in the solutions and then removed after 10 min, weighed and placed again in the same solution. The same procedure was repeated after 30, 60, 120, 240, 480 and 1440 min.

All the measurements were performed at ambient conditions. Samples were weighed with a Mettler balance (10^{-5} sensitivity). The NaCl was purchased from Aldrich; the buffer solutions from Fluka. All the products were used without any further purification.

2.2. Cell Culture

The CaCo2 human colon cancer cell line was kindly provided by Dr Cucciolla from the Second University of Naples. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Gibco Laboratories, Grand Island, NY) 1% non-essential aminoacids, penicillin ($100\,units\,ml^{-1}$), streptomycin ($100\,mg\,ml^{-1}$) (Sigma Chemical Co., St. Louis, MO) and fungizone ($50\,\mu g\,ml^{-1}$) (Gibco Laboratories). The cells were maintained at 37 °C in a 5% CO_2 95% air humidified atmosphere. Cells were seeded at a density of $5 \times 10^3\,cm^{-2}$ in 24-mm diameter Transwell-Col polycarbonate culture chambers (0.4 μm pore size, Costar, Cambridge, MA). The media were changed every 48 h. The cell growth in Transwell culture chambers was determined by the total protein determination according to the Bradford colorimetric assay method (BCA) on days 2, 5, 7, 9, 14 and 21. The experiments were performed on the cells on day 21.

2.3. Alkaline phosphatase assay

The alkaline phosphatase (ALP) activity was measured in whole cell lysates and served as a marker for enterocytic differentiation. At the designated time points duplicate cultures were washed three times with cold phosphate-buffered saline (PBS) solution (pH 7.4), scraped and collected into 2 mM Tris–HCl/50 mM mannitol (pH 7.2) (final volume 1 ml) and sonicated on ice and assayed (5 and 10 μl). ALP activity was measured by kinetic determination of *p*-nitrophenylphosphate hydrolysis using a commercial kit (Boehringer). The results are expressed in milliunits per mg of protein.

2.4. Transepithelial electric resistance (TEER) measurements

The transepithelial electric resistance (TEER) was measured in the Transwell chambers on day 21 using a Millicel electrode according to the manufacturer's protocol.

2.5. Biocompatibility tests

Six samples of the dry gel (weight ranging from 10 to 16 mg) were swollen overnight in 20 ml of culture medium under sterile conditions. A certain value of the swelling ratio of the gel was used as a control. The swollen gel was loaded into Transwell culture chambers containing differentiated enterocytes and removed after designated time points (10, 30 and 120 min). Assays were conducted in duplicate. No hydrogel was loaded into the chambers used as negative controls. Positive control cells were stimulated with $50\,ng\,ml^{-1}$ tumor necrosis factor-α (TNF-α; human recombinant) for 5, 10, 15 and 30 min.

2.6. Lipid extraction

At the designated time points the apical and basal media were removed from the Transwell chambers and the cells were fixed by adding 1 ml of cold methanol, scraped and collected into glass tubes in which 1 ml of chloroform and 0.9 ml of water were added according to the method of Bligh and Dyer [10]. The extraction was repeated three times. The chloroformic phase was drawn and evaporated under vacuum (Univapo 100 h, Uniequip). The dried lipids were re-suspended in 500 μl of

acetonitrile. Fifteen microliters of an acetonitrile solution containing $1\,mg\,ml^{-1}$ of 9-anthryldiazomethane (ADAM, Molecular Probes) were added to each sample. ADAM is a fluorescent reagent which derivatizes by esterification carboxylic groups. The reaction was allowed to proceed overnight at room temperature.

2.7. High pressure liquid chromatography (HPLC) analysis

HPLC analysis was performed in a Waters apparatus equipped with a 626 pump driven by Millenium software (v. 2.15), and a fluorescence detector Shimadzu RF-551. One hundred microliters of the derivatized solutions were injected in a reverse phase column (Bio-Rad C18 cm15 3 μ particles) and eluted at a flow rate of $0.8\,ml\,min^{-1}$ with a convex gradient (Curve num. 2 according to the manufacturer) by the following gradient: 7 min 100% A; 7–37 min 100% B, where the solvent A was 85% methanol and 15% water and the solvent B was 100% methanol. The elution was monitored in fluorescence at 412 nm upon excitation at 364 nm.

3. Theoretical background

The reduction of the chemical potential of the solvent in the swollen gel (which is related to the osmotic pressure generated inside the gel) is separable into three terms which represent the changes due to the mixing of polymer and solvent, to the mixing with the mobile ionic constituents, and to the elastic deformation of the network [11]. The related expressions are reported as follows

$$\Delta\mu = \Delta\mu_M + \Delta\mu_I + \Delta\mu_E \qquad (1)$$

where $\Delta\mu$ represents the difference between the water chemical potential in the mixture and the reference state water chemical potential (i.e. chemical potential of water in the external solution). The first term on the right of Equation 1 is the molar free energy of polymer solvent mixing and is affected by the affinity between polymer and solvent (Flory–Huggins Equation 1) [11]

$$\Delta\mu_M = RT[\ln(A - v_2) + v_2 + \chi_1 v_2^2] \qquad (2)$$

where R is the universal gas constant, T is the absolute temperature, v_2 is the volumetric fraction of polymer in the swollen gel and χ_1 is the polymer solvent Flory–Huggins interaction parameter.

This term is a function of the energetic affinity between polymer and solvent, which is accounted for by the value and the sign of the χ_1 parameter. For hydrophilic gels, $\Delta\mu_M$ contributes positively to the osmotic pressure.

The third term on the right of Equation 1 accounts for the retractile elasticity of polymer chain between crosslinks, in analogy with the theory of rubber elasticity [12]

$$\Delta\mu_E = RTV_1(\nu_e/V_0)(v_2^{1/3} - Sv_2) \qquad (3)$$

where V_1 is the molar volume of solvent, ν_e is the ''effective'' number of moles of network chains and V_0 is the volume of the dessicated gel.

The term (ν_e/V_0) is a structural parameter which determines the degree of crosslinking in the gel. The S parameter can assume the value of 0.5, 1 and 0, in accordance with the theories of rubber elasticity performed by Flory [13], Hermans [14] and James and Guth [15]. $\Delta\mu_E$ always contributes negatively to the osmotic pressure and is affected by the degree of crosslinking.

The term $\Delta\mu_I$ in Equation 1 is the contribution to molar free energy due to the Donnan effect resulting from the presence of ionic groups on the macromolecular chains. An expression [11] for the ionic contribution to the osmotic pressure resulting from the difference in ionic concentration between the gel and the external solution is

$$\pi_I = RT[ic_2/z_- - n(c^* - c)] \qquad (4)$$

where c_2 is the concentration of ionic groups fixed onto the polymer network, i is the fraction of polymer structural units (monomer) which were ionized, z_- is the charge of these ionic groups, n is the number of ions generated by the dissociation of one molecule of salt coming from the outer solution, c^* is the concentration of all the ionic species existing, at equilibrium, in the outer solution and c is the concentration of all the ionic species, included fixed charge, in the gel.

This term always contributes positively to the swelling of the gel and is affected by the pH and ionic strength of the external solution.

4. Results and discussion: *in vitro* testing

Under normal conditions, in its passage through the gastrointestinal tract, hydrogel is supposed to encounter, in the stomach, an acid milieu, in the intestine, luminally delivered macronutrients, minerals, electrolytes and water. Tests *in vitro* were performed in order to asses hydrogel swelling capacities in solutions differing for their ionic strength, the pH and composition, with the aim of reproducing the chemical characteristic of the *in vivo* environment described above.

Sorption kinetic tests at different pH were performed in order to better assess hydrogel synthesis parameters, and to guarantee that absorption takes place only in those sections which are selected for water elimination (intestinal tracts). The mucosal epithelium of the gastrointestinal tract forms a barrier to various dangerous substances. The inflammatory response in the gastrointestinal tract is a process mediated by the activation of the enzyme phospholipase A_2 and the subsequent mobilization of arachidonic acid from membrane phospholipids. Considering that the contact of the hydrogel with intestinal cells could lead to an inflammatory response of these cells, the relase of intracellular arachidonic acid was investigated as a possible marker of biocompatibility.

4.1. Effect of ion concentration, pH and composition of the external solution on the swelling ratio

A first set of samples was prepared in order to analyze the influence of ion concentration in the external solution on

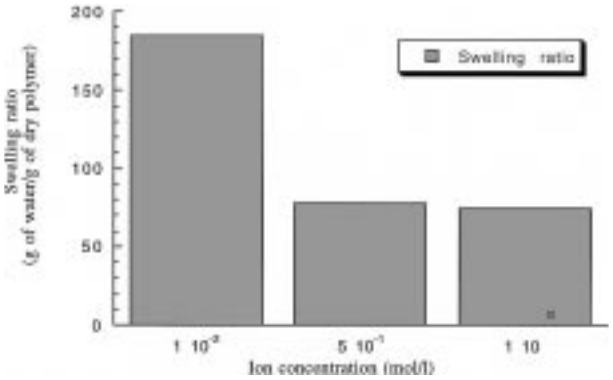

Figure 1 Equilibrium swelling at different ion concentrations.

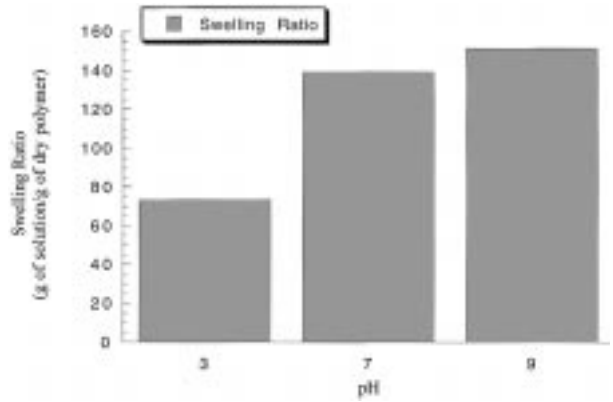

Figure 2 Equilibrium swelling at different pH.

the swelling ratio (SR) of hydrogels. This was defined as: SR = grams of water/grams of dry polymer. As shown in Fig. 1, an increase in ion concentration, which passes from 10^{-2} to $1\,\mathrm{mol\,l^{-1}}$, decreases the swelling ratio of the gel from 185 to about 80.

This can be explained by observing that when the ion concentration in the external solution is increased, the difference in concentration of mobile ions $(c^* - c)$ (see Equation 4) between the gel and the external solution increases, reducing the value of the osmotic pressure π_I and thus the gel's swelling capacity, as already discussed in the previous section.

Moreover, the screening of the fixed ionic charges on the network by the mobil ions coming from the solution reduces the electrostatic repulsion between the polymer chains, which contributes to the network expansion, and thus the swelling capacity of the gel.

Another set of samples was prepared for the study of the effect of changes in the pH of the external solution. The plot in Fig. 2 shows a high swelling ratio at pH = 9 and lower values at pH = 7 and pH = 3.

It has long been known that a crosslinked polymer gel bearing acidic and/or basic pendent groups will imbibe solvent to a certain extent which depends on the pH and the ionic composition of the solution bathing the gel. At low pH values, the chemical equilibrium of the dissociation of the carboxylic groups will change as H^+ ions will be associated with the carboxylate groups. Hence, the concentration of fixed charges in the gel will decrease, resulting in the formation of an uncharged gel [16]. In this case, the term $\Delta\mu_I$ in Equation 1 disappears, and the swelling capacity of the gel decreases. The opposite behavior occurs in a basic solution in the pH range 7–9: as the pH increases, the concentration of base cations in the outer solution will also increase. These cations will be attracted into the gel and will replace the mobile H^+ ions. The gel thus acts as an ion exchanger. New H^+ ions will be supplied by the yet undissociated carboxylic groups of the resin. The concentration of mobile ions in the gel will thus increase more rapidly than in the outer solution, and the ion swelling pressure will increase.

The supply of H^+ ions is, however, limited. Eventually, all the carboxylic groups of the resin will be dissociated. There will always be an excess of cations and a deficit of anions in the gel but these two contributions to the osmotic pressure will cancel each other out. With an increasing in the pH, the swelling pressure due to the presence of ionic charges linked to the gel structure (π_I in Equation 4), will begin the drop again, thus reducing the hydrogel swelling capacity.

Another set of samples was prepared in order to evaluate the swelling capacity of such materials in contact with solutions similar to biological fluids, such as physiological solutions, saline solutions or culture mediums.

Fig. 3 shows the equilibrium swelling ratio in these three different solutions. The swelling capacity in such a biological medium reaches a value of about 60–70. This value is much smaller than that obtained in distilled water; in fact, the ion concentration is higher in biological solutions than in distilled water and, as already mentioned, increasing the ion concentration decreases the swelling capacity of hydrogels.

4.1.2. Absorption kinetics

Gels were synthesized in spheres of 0.5 cm and 1.0 cm diameter in a partially swollen state, then dessicated and immersed in solutions of different pH. The kinetic weight gain was followed up to equilibrium was reached. The data are reported in Fig. 4. The figure shows the swelling kinetic at pH = 3 and pH = 7.

The swelling ratio, as well as the swelling kinetic, is always much higher at pH = 7 than at pH = 3, for both samples of 0.5 and 1.0 cm diameter. Of course, solubility is much higher for samples at pH 7 than for those at pH 3, as already stated in the previous sections and reported on

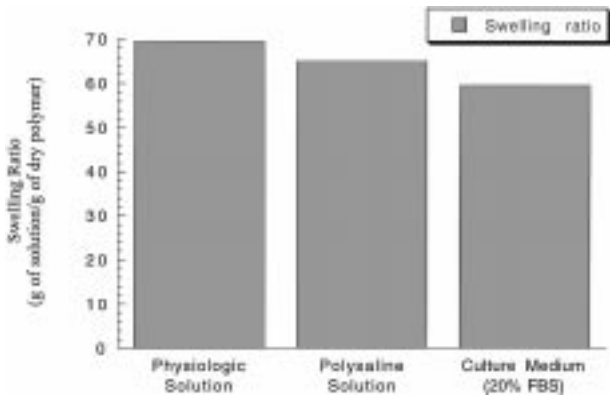

Figure 3 Equilibrium swelling in different solutions.

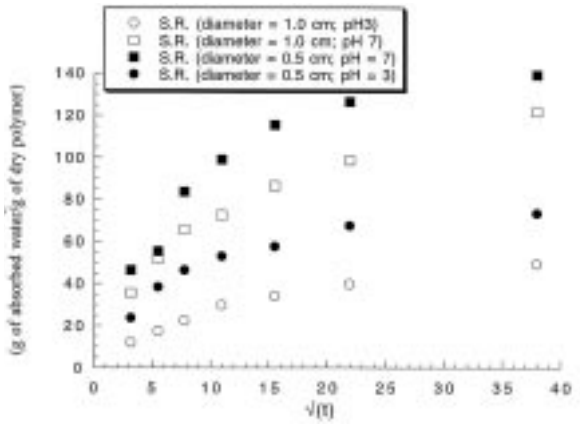

Figure 4 Absorption kinetic of a 0.5 and 1 cm diameter gel sphere at different pH.

the graph in Fig. 2. The equilibrium swelling value is reached after 24 h. Moreover, the equilibrium swelling ratio is higher for gel spheres of 0.5 cm diameter, and thus the dimension influences the swelling kinetic. Note that the non-zero intercept may be due to a water adsorption on hydrophilic groups on the polymer network.

Data show that absorption kinetic is fast. In fact, after only 10 min, the hydrogel absorbs about the 25–30% of the total water quantity absorbed at equilibrium. Differences in the swelling kinetic between samples of different dimensions could be due to either: (a) the material's microstructure being different in samples of different dimensions or (b) an absorption mechanism taking place which is different from the standard one, in which a Fickian diffusion is involved. Further studies are in progress for a complete characterization of such phenomena. One should take into account that such behavior gives one a more adjustable parameter for material modeling, and thus for the production of a more suitable product for the particular application.

4.2. Hydrogel–cell interactions

In order to assess ion uptake or release, the hydrogel was swollen in physiological and polysaline solutions and in culture mediums, as previously described in Materials and Methods. The data reported in Table I show that the hydrogel swelling did not alter the electrolyte concentration of the solutions assayed, thus suggesting a possible application of the gel in the treatment of pathological situations such as oedema, renal failure and diuretic resistance. Moreover the hydrogel cell compatibility was evaluated. For this purpose we chose, as the *in vitro* model, the human-derived carcinoma cell line CaCo2. These cells undergo spontaneous enterocytic differentiation after several days in culture showing microvilli on the apical side, tight junctions and enzymatic activities of brush border hydrolases which are characteristic of the mature small intestinal epithelium [17, 18]. Furthermore, CaCo2 cells, when grown on Transwell polycarbonate membranes, represent an useful model for the study of the transport system of the small intestinal epithelium [19]. Intestinal cells are known to respond to various inflammatory stimuli via the activation of the enzymes phospholipase $A_2(PLA_2)$ and prostaglandin endoperoxide synthase 1 and 2, also referred as cyclo-oxygenase 1 and 2 (COX-1 and COX-2; for a complete review, see Williams and Dubois [20]. After the activation of PLA_2, free arachidonic acid is released from membrane phospholipids and converted to prostaglandins (PGs) via cyclo-oxygenase and peroxidase activity. PGs mediate important functions, including mucosal restitution and immonoresponsiveness [21] in the intestine. Thus we evaluated the release of arachidonic acid and other fatty acids by the CaCo2 cells after their contact with the hydrogel, using cells stimulated with TNF-α as a positive control. TNF-α is known to induce PLA_2 activation and the release of arachidonic acid from membrane phosphatidylcholine and phosphatidylethanolamine pools [22–24] and COX-2 expression [25–27] in several cell types and in some intestinal cell lines [28]. We used TEER and ALP specific activity measurements as a parameter for cell differentiation: after 21 days' culture in Transwell chambers the average TEER value was $352.2 \pm 17.8\,\text{m}\Omega$ and the ALP activity was 526 ± 60.3. These data complied with those previously reported elsewhere [29, 30].

In order to evaluate the pro-inflammatory activity of the gel, it was weighed and swollen in the culture medium under sterile conditions and, after 24 h, loaded into Transwell culture chambers in contact with differentiated CaCo2 cells (21 days' culture). The production of free fatty acids (arachidonic, oleic and palmitic) in the cells and in the culture medium was evaluated using the HPLC method as described in Materials and Methods. Fig. 5 shows a typical chromatogram obtained with this method. Upon incubation of cells with the gel in a time ranging from 10 to 120 min, no appreciable variation in the free fatty acids pattern was found, whereas a 30 min TNF-α stimulation, used as a positive control, led to a marked increase in fatty acid release, as shown in Fig. 6. The maximum production of free fatty acids was obtained at 30 min

TABLE I Data are equilibrium ion concentrations in the solutions swelling the gel. Starting solutions were used as control

	Physiological solution	Polysaline solution	Culture medium
Ca^{++}(mEq l^{-1})	0	4.85	3.9
Control	0	5.0	4.1
Na^{+}(mEq l^{-1})	159.0	141.0	153.0
Control	159.0	141.0	153.0
K^{+}(mEq l^{-1})	0.26	10.0	6.22
Control	0.26	10.35	6.20
Cl^{-}(mEq l^{-1})	155.0	105.0	121.0
Control	154.0	104.0	121.0

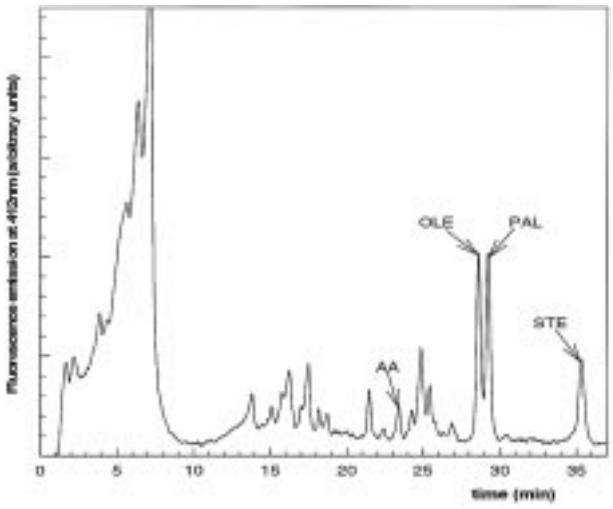

Figure 5 HPLC analysis of fluorescent derivatized fatty acids. Free fatty acids were derivatized with ADAM and separated by HPLC as reported in Materials and methods. Each peak was identified using authentic standards. AA: arachidonic acid; OLE: oleic acid; PAL: palmitic acid; STE: stearic acid.

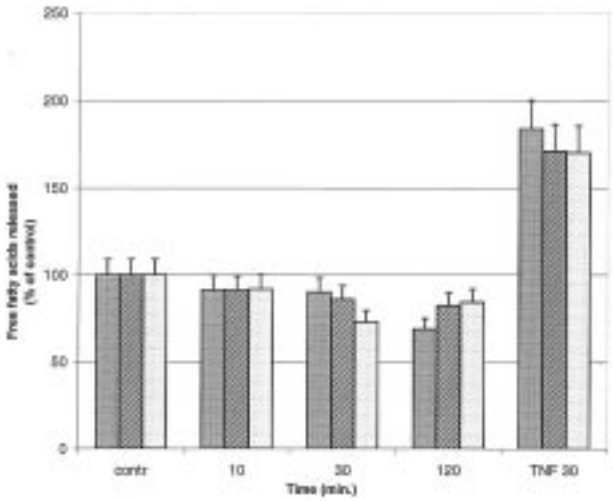

Figure 6 Free fatty acid release. The hydrogel was put in the Transwell chambers in contact with cells at day 21 of culture. After designated time points, free fatty acids were extracted, and derivatized and separated by HPLC. TNF-α-induced fatty acids release was used as positive control. First column: arachidonic acid; second column: oleic acid; third column: palmitic acid.

incubation time with TNF-α (data not shown). We did not detect any appreciable amount of free fatty acids either in the apical or in the basal medium.

The data obtained showed that the hydrogel does not alter the electrolytic concentrations of the swelling fluids. This makes it a suitable candidate as an adiuvant in the treatment of diseases where water loss is required. Preliminary studies on the release of inflammatory mediators showed that, at least for short times, an appreciable increase in free fatty acid or prostaglandin release was not detectable, neither in the cells nor in the culture medium. This indicates that the gel has a good cell compatibility since it did not lead to an inflammatory response after its contact with cells.

5. *In vivo* testing: preliminary studies

To test the efficiency of the hydrogel in absorbing water *in vivo,* 2% by weight hydrogel powder was added to rats' diet. The hydrogel powder was mixed with the diet chow and compressed molded in discs of 1 cm diameter. Water (5% by weight) was added before compression in order to confer more consistency to the chow–hydrogel mixture; discs were dried under vacuum after compression.

Preliminary data on *in vivo* capacity to trap water were performed by giving the described diet to six rats (body weight 201 $\pm$ 27 g). The experiment was divided into three periods (A, B, C) each 2 weeks long. Period A before the treatment, period B administering chow pellets with the hydrogel and period C giving the chow without the mixed hydrogel. The rats were put into a metabolic box and the water ingested per day and the faecal volumes were monitored.

During period A, the daily average volume of water ingested by the rats was 57 $\pm$ 5 ml each; instead, during period B, the ingested volume of water was 72.5 $\pm$ 6 ml, while during period C the daily average was 57 $\pm$ 5 ml each. Considering that the rats of the experimental group consumed 15 $\pm$ 2 g of fodder per day and the concentration of the gel in the fodder was 2% by weight, the total amount of polymer ingested by the rats was 0.3 $\pm$ 0.04 g per day.

Thus, considering that these rats consumed in the period B an average volume of water of 15.5 ml more than during the period A, it was stated that 1 g of hydrogel retains 51.7 ml of water *in vivo*. The faecal weight changed between period A and B from an average of 33 $\pm$ 0.4/48 h to an average of 42.06 $\pm$ 0.3/48 h. Further studies on rats are in progress.

6. Conclusions

Cellulose-based hydrogels were tested for their potential use in biomedical applications. Swelling measurements were carried out in order to check their absorbant capacity when immersed in solutions at different ion concentration and pH. A reduction of the swelling capacity was observed with an increase in the ion concentration of the solution or a decrease in the pH. Kinetic measurements were also performed, and gel sample dimensions were responsible for a different swelling behavior of hydrogels. Such a phenomenon makes these materials more suitable for the design of the final product. It was decided by our group to investigate the events occurring after the cell contact with materials at the intracellular level: in fact, in agreement with van Kooten *et al*. [31], our group believes that more efforts should be done to study biocompatibility in terms of cell functions. These kind of studies (e.g. biochemistry, molecular biology) can provide more information about cell response to biomaterials, leading to a better understanding of the mechanisms underlying cell–material interactions. This hydrogel's main clinical application may be the treatment of oedemas of cardiac, hepatic and renal origin, which are resistant to diuretic therapy. We are currently evaluating the long-term gel compatibility, also in terms of morphological variations, toxicity and carcinogenic potential.

Our long-term research goal is to formulate a material able to replace all the intestinal functions, in particular,

the capability of uptake of nutrient substances which are no longer bioreabsorbable by exeresis of intestinal tracts.

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