New Biodegradable Hydrogels Based on Inulin and $\alpha,\beta$-Polyaspartylhydrazide Designed for Colonic Drug Delivery: In Vitro Release of Glutathione and Oxytocin

Delia Mandracchia, Nunzio Denora, Massimo Franco, Giovanna Pitarresi, Gaetano Giammona and Giuseppe Trapani

**Abstract**

Succinic derivatives of inulin (INU–SA) with two different degrees of derivatization (20% and 30%, mol/mol) were cross-linked with $\alpha,\beta$-polyaspartylhydrazide (PAHy) to obtain INUPAHy hydrogels. Cross-linking was performed using $N$-ethyl-$N$-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and $N$-hydroxysulfosuccinimide (NHSS) as coupling agents and by varying the reaction time (4 h, 8 h and 24 h). All samples prepared were characterized by FT-IR analysis and swelling measurements in different media. In vitro assays, performed in the presence of inulinase, demonstrated the degradability of the prepared hydrogels. Cell compatibility was evaluated using Caco-2 cells through both direct and indirect assay. Glutathione (GSH) and oxytocin (OT), both potential agents for the treatment of colonic inflammation, were entrapped into a INUPAHy hydrogel and their release was evaluated in simulated gastrointestinal fluids. The obtained results suggest that GSH- and OT-loaded INUPAHy hydrogels are potentially useful for the oral treatment of inflammatory bowel disease.

**Keywords**

Inulin, $\alpha,\beta$-polyaspartylhydrazide, hybrid hydrogels, biocompatible matrix, colon delivery, peptide release

**1. Introduction**

Targeting of drugs to the large intestine is therapeutically relevant for the treatment of colonic disorders, including inflammatory bowel disease (IBD), cancer and other pathologies [1, 2].

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Many systems have been proposed for delivering drugs specifically to the colon [3, 4] and among them, pH-sensitive or biodegradable hydrogels are very attractive [5, 6]. In this context, taking into account that the combination of a natural polymer with a synthetic one allows to obtain hybrid hydrogels with advantageous physico-chemical characteristics [5–7], in previous works we have prepared hydrogels by UV irradiation of polyaspartamide and dextran derivatives, chosen as synthetic poly(amino acid) and natural polysaccharide (degraded in the colon), respectively [5, 8].

Now, we have considered of interest to obtain other poly(amino acid)–polysaccharide hydrogels by using a different synthetic procedure (via chemical reaction) and polymers. In particular, we have chosen α,β-polyaspartylhydrazide (PAHy) as a synthetic, water-soluble and biocompatible poly(amino acid) [9] and inulin as a polysaccharide degraded in the colon. In fact, bacteria residing in the colon, and more specifically Bifidobacteria (that constitute up to 25% of the normal gut flora of both man and animals), are able to ferment inulin due to the production of inulinase. Therefore, inulin is considered an important prebiotic substrate [10, 11] and its bifidogenic activity causes various effects on the health, such as (1) a significant modification of the colonic microflora, because Bifidobacteria inhibit the growth of pathogenic bacteria present in the caecum–colon; (2) a protective effect against infection in the gastrointestinal tract and an amelioration in inflammation [12]; (3) a beneficial effect on the metabolism of carbohydrates and lipids, leading to a decrease in the cholesterol, triglyceride and phospholipid concentration in the blood, thus reducing the risk of diabetes and obesity; (4) a reduction in cancer risk, mainly gut cancer, due to an increase in cellular immunity [13, 14].

In addition, we have also considered that a great interest is turned to the use of therapeutic peptides or proteins to treat inflammatory bowel disease. In this context, glutathione (γ-glutamylcysteinylglycine) (GSH) and oxytocin (OT) seem promising. The former is a tripeptide able to protect cells against radical oxygen species (ROS) [15], the latter is a nonapeptide with a potent anti-inflammatory effect on the oxidative injury of the colon and it prevents the colonic glutathione depletion [16]. Due to their ability to prevent damaging cascade due to free radicals, these peptides represent potential agents for the treatment of colonic inflammation. It has been reported, indeed, that oxidative stress is one of the key elements of tissue injury in IBD and that intestinal damage is related to an increased production of free radicals, as well as to a low concentration of endogenous antioxidants [17–20].

Taking into account the beneficial role in inflammatory bowel disease of inulin [21–25], the aim of this work was to combine the interesting properties of this polysaccharide (as material to produce a hydrogel system, combined with PAHy) with the pharmacological effects of GSH and OT. The obtained hydrogels have been characterized, as far as their swelling in aqueous media, enzymatic degradability in the presence of inulinase, mucoadhesive properties and in vitro cell compatibility on Caco-2 cells are concerned. Finally, the ability of the prepared hydrogels to entrap GSH and OT and to release them in
simulated gastrointestinal fluid has been investigated in order to obtain information about their potential oral administration.

2. Materials and Methods

2.1. Materials

All reagents were of analytical grade, unless otherwise stated. DL-Aspartic acid, hydrazine hydrate, \(N,N\)-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), methanol, triethylamine (TEA), succinic anhydride (SA), fructose, \(N\)-ethyl-\(N\)-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), \(N\)-hydroxysulfosuccinimide (NHSS) and Trypan blue were purchased from Fluka. Inulin from Dahlia Tubers (INU, approx. 5000 Da), inulinase, anthrone, reduced glutathione (GSH), glutathione disulfide (GSSG), oxytocin (OT), L-glutamine, thiazolyl blue tetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) were obtained from Sigma-Aldrich.

Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and trypsin–EDTA were purchased from Euroclone.

2.2. Apparatus

The weight-average molecular weight and the polydispersity index of PAHy were evaluated by size-exclusion chromatography (SEC) analysis using a procedure reported elsewhere [9].

The weight-average molecular weights of INU–SA/A and INU–SA/B derivatives were determined by SEC using a Waters Associates apparatus Model 1515 HPLC isocratic pump, a differential refractometer (DRI) and a UltraHydrogel 250 column (size-exclusion range 1–50 kDa). Phosphate buffer solution (PBS, pH 7.2; 0.05 M) was used as a mobile phase, at 35°C, flow rate was 0.6 ml/min and dextran (molecular mass range 3–40 kDa) was used as size standard. Chromatographic data were processed by Waters Breeze software.

Centrifugations were performed with a Beckman Optima L-90K (Life Science) apparatus equipped with a 70Ti rotor and temperature control.

FT-IR spectra (KBr pellets) were recorded in the range 4000–400 cm\(^{-1}\) using a Perkin–Elmer 1600 IR Fourier Transform Spectrophotometer. The resolution was 1 cm\(^{-1}\). UV-Vis analyses were performed using a Perkin–Elmer Spectrometer Lambda Bio20. \(1\)H-NMR spectra were recorded using a Varian Mercury 300 MHz instrument.

2.3. Quantitative Analysis of GSH and OT

High-performance liquid chromatography (HPLC) analyses were performed with a Waters Model 600 pump equipped with a Waters 2996 photodiode array detector and a 20 µl loop injection autosampler (Waters 717 plus). Data were processed using Waters Empower software.
For GSH analysis, a reverse-phase Hydro Synergy C18 (250 mm × 4.6 mm; Phenomenex) column connected with a C18 pre-column was used. The mobile phase was acetonitrile/0.025 M PBS (pH 2.7) (1:99, v/v) in isocratic mode; flow rate 0.7 ml/min. The retention times of GSH and GSSG were 7.2 and 18 min, respectively. Calibration curves of GSH and GSSG were prepared using 0.025 M PBS (pH 2.7) as a solvent and they were linear ($r^2 > 0.999$) in the range of evaluated concentrations (0.02–1 mg/ml). Under these conditions, the quantification limit (LOQ), defined as the analyte concentration that could be analyzed with acceptable precision and accuracy, was determined to be 0.01 mg/ml for both GSH and GSSG.

For OT analysis, a reverse-phase Hypersil C18 (150 mm × 4.6 mm; Phenomenex) column with a C18 pre-column was used. The mobile phase was 80 mm PBS (pH 5)/acetonitrile (80:20, v/v) in isocratic mode; flow rate 1.5 ml/min. The retention time of OT was 7.5 min. The calibration curve of OT was linear ($r^2 > 0.998$) in the range of evaluated concentrations (1–30 U/ml). Under these conditions, the quantification limit (LOQ) was determined to be 0.5 U/ml.

2.4. Synthesis of PAHy

$\alpha,\beta$-Polyaspartylhydrazide (PAHy) was prepared by polycondensation of DL-aspartic acid in the presence of $H_3PO_4$ at 180°C followed by reaction with hydrazine hydrate in DMF solution. PAHy was isolated by filtration and purified as reported elsewhere [9]. Analytical and spectral data of PAHy were in agreement with literature values [9]. The molecular mass of PAHy was 23.5 kDa (polydispersity index (PDI) = 1.78).

2.5. Synthesis of INU–SA Derivatives

The reaction of INU with SA was performed following an already reported method and its modifications [26–28]. Briefly, an appropriate amount of INU (1 g) was dissolved in 14 ml anhydrous DMF under argon and suitable amounts of TEA and SA were added according to the following molar ratios: $X = 0.20$ or $0.30$; and $Y = 0.35$, where $X$ indicates the ratio mol SA/mol INU repeating units and $Y$ indicates the ratio mol TEA/mol INU repeating units.

Two INU–SA derivatives with a different degree of derivatization in succinic groups (named as INU–SA/A and INU–SA/B derivatives at 20 and 30% (mol/mol) in SA groups, respectively) were obtained and characterized by FT-IR and $^1$H-NMR spectroscopy.

Their weight-average molecular mass, determined by SEC analysis, was 5800 (PDI = 1.20) and 6100 Da (PDI = 1.65) for INU–SA/A and INU–SA/B, respectively.

2.6. Preparation of INUPAHy Hydrogels

INU–SA/A or INU–SA/B derivative (150 mg of each) was dissolved in double-distilled water and the pH was adjusted to 6.0 by addition of 0.1 M NaOH solution. EDC and NHSS were added in equimolar amount in the order give a molar ratio, $Z$,...
equal to 0.8, where \( Z = \text{mol EDC (or NHSS)}/\text{mol INU–SA repeating units} \). The pH was maintained at 6.0 by addition of 0.1 M HCl solution and the reaction mixture was kept in a thermostatic bath at 25°C for 1 h. Then, PAHy aqueous solution was added in order to have a molar ratio, \( Z' \), equal to 0.6, where \( Z' = \text{mol PAHy repeating units}/\text{mol INU–SA repeating units} \). The final concentration of each INU–SA derivative was 8.6% (w/v). After PAHy addition, reaction was carried out for 4, 8 and 24 h. For each time, the formation of a hydrogel occurred. Each sample was purified by several washes with double-distilled water, centrifuged at \( 12 \times 10^3 \) rpm at 4°C for 15 min and recovered as lyophilized powder. The hydrogels obtained were named as INUPAHy/A and INUPAHy/B, respectively. Each experiment was performed in triplicate and the obtained yields were determined on the basis of the initial weights of INU–SA and PAHy.

2.7. Swelling Studies

The swelling ability of INUPAHy/A and INUPAHy/B hydrogels was determined after 24 h of immersion in double-distilled water or PBS (pH 6.8) (simulated intestinal fluid) and after 2 h of immersion in 0.1 M HCl (pH 1.0) solution (simulated gastric fluid), according to a procedure reported elsewhere [29]. In particular, 30 mg of each lyophilized hydrogel was immersed through a tared sintered glass filter (10 mm diameter; G2 porosity), in the swelling medium and incubated at 37°C for the established time. The excess of liquid was removed by percolation under atmospheric pressure and the filter, after centrifugation at 3000 rpm for 5 min, was weighed. The weight swelling ratio \( q \) of the hydrogel was calculated as follows:

\[
q = \frac{W_s}{W_d},
\]

where \( W_s \) and \( W_d \) are the average weights of the swollen and dry samples, respectively.

Each experiment was performed in triplicate and the results are reported as mean ± SD.

2.8. Enzymatic Hydrolysis Studies with Inulinase

INUPAHy/A \((t=24 \text{ h})\) and INUPAHy/B \((t=24 \text{ h})\) hydrogels (30 mg) (i.e., hydrogels obtained after 24 h of reaction) were incubated with 6 ml PBS (pH 4.5) in the absence or in the presence of inulinase (final enzyme concentration 10 U/ml), under continuous stirring (100 rpm) at 37 ± 0.1°C for 24 h. The enzyme solution was prepared immediately before the experiment.

After the incubation time, samples were neutralized, centrifuged at \( 12 \times 10^3 \) rpm at 4°C for 15 min and the supernatants were separated. Samples were washed five times with double distilled water under continuous stirring for 1 h to extract enzyme, soluble degradation products and electrolytes entrapped within the network. Then, the remaining hydrogels were dried, weighed and their weight swelling ratio, \( q \), was evaluated after 24 h of immersion in double distilled water.
Degradation (%) was expressed as:

\[
\text{Degradation (\%, w/w)} = \frac{\text{Weight}_{\text{start}} - \text{Weight}_{\text{after hydrolysis}}}{\text{Weight}_{\text{start}}} \times 100.
\]

The degradation of INUPAHy/A\((t=24 \text{ h})\) hydrogel was also evaluated after incubation with inulinase (10 U/ml) from 1 until 24 h and by using the anthrone method, following a procedure reported elsewhere [27].

Each experiment was performed in triplicate and the results are reported as mean ± SD.

2.9. Interaction with Mucin

Mucin (10 mg) was dispersed in PBS (pH 6.8) (10 ml) and the mixture was kept at 37°C for 24 h under stirring (150 rpm). Then, appropriate amounts of PAHy, INU–SA/A, PAHy/INU–SA/A physical mixture or poly(acrylic acid) (chosen as a positive control) were added to the mucin dispersion (mucin/polymer weight ratio equal to 1) and each sample was incubated at 37°C under stirring (150 rpm) for 2, 5, 8, 15 or 24 h. After each incubation time, the transmittance (%) of the samples was recorded at 500 nm. Each experiment was performed in triplicate and the results are reported as mean ± SD.

2.10. Cell Culture

Caco-2 cells (a kind gift from Professor Nicola Colalufo, Dpt Farmaco-Chimico, Università degli Studi di Bari, Italy) were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml) (complete medium). Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ and were fed every day. Cells were seeded in 96- and 24-well plates at a density of approx. 1 × 10⁴ and approx. 3 × 10⁵ cells/well, respectively. After 24 h of incubation, medium was changed with complete medium (for untreated cells) or with medium supplemented with the investigated sample (see below), then cells were incubated for 24 or 48 h.

2.10.1. Cell Viability Analysis by MTT Conversion and Trypan Blue Exclusion Assays

The number of living cells was determined by quantitative colorimetric MTT assay. In particular, 100 µl of cell suspension was plated in each well of 96-well plates at a density of approx. 1 × 10⁴ cells/well. After 24 h of incubation at 37°C in a humidified atmosphere with 5% CO₂, the culture medium was replaced with complete medium containing different amounts of INUPAHy/A\((t=24 \text{ h})\) hydrogel (range 0.01–1 mg sample/ml medium) (direct method) or with a ‘conditioned medium’ wherein INUPAHy/A\((t=24 \text{ h})\) hydrogel was previously dispersed and swelled (indirect method). For the indirect method, the hydrogel was incubated in complete medium for 24 h at 37°C. Next, the medium was centrifuged at 3800 rpm for 10 min and later steriley filtered in order to remove the hydrogel, thus giving the ‘conditioned medium’. Untreated cells were used as positive control and
cells incubated with a 2% (w/v) SDS solution were used as negative control for both direct and indirect method. After the incubation period (24 or 48 h), 10 µl of a 0.5% (w/v) MTT/PBS solution were added to each well and the incubation was prolonged for 4 h. After this period, medium was removed and replaced by 150 µl of a DMSO/ethanol (1:1) solution per well. The absorbance at 492 nm was measured by a microplate reader (Wallac Victor³, Multilabel Counter, Perkin–Elmer) and cell viability data were calculated. Cell viability (in percentage) was expressed as (Abs492 treated cells/Abs492 control cells) × 100.

Cell viability was also evaluated by Trypan blue exclusion assay. Cells were seeded in 24-well plates at a density of approx. 3 × 10⁵ cells/well and incubated at 37°C in a humidified atmosphere with 5% CO₂ for 24 h. After incubation, medium was removed from each well and replaced with the ‘conditioned medium’ or with medium containing different amounts (range 0.01–1 mg sample/ml medium) of the investigated sample for the indirect and direct method, respectively. In both methods, after the incubation period (24 or 48 h), cells were detached using a trypsin–EDTA solution (200 µl/well). The resulting cell suspension was diluted with fresh complete medium (1 ml/well). Next, 10 µl of the resulting suspension was diluted (1:1) with a 0.4% (w/v) Trypan blue solution and then transferred into the cell-counting chamber. Cell viability was determined by counting under a light microscope, the viable cells in each well appeared as bright, not blue-stained, cells. Each experiment was performed in triplicate and the results are reported as mean ± SD.

2.11. Drug Loading by Soaking Procedure

A concentrated solution of each drug (GSH dissolved in PBS (pH 4.5), OT dissolved in water) was added to INUPAHy/A(t=24 h) hydrogel; the mixture was maintained at room temperature under magnetic stirring for 24 h. After this time, the solvent was removed by filtration, and the sample was rapidly washed (with PBS (pH 4.5) for GSH and with water for OT) in order to remove drug molecules adsorbed on the surface. Hydrogel sample loaded with GSH or OT was dried at 0.1 mmHg in the presence of P₂O₅ until constant weight.

2.12. Determination of GSH or OT Amount Entrapped into INUPAHy Hydrogel

Drug-loaded INUPAHy/A(t=24 h) hydrogel (50 mg) was extensively extracted at room temperature with 30 ml of PBS solution (pH 4.5) for GSH or 30 ml of water for OT. The extraction liquids were collected and lyophilized. The obtained residues, dissolved in PBS solution (pH 4.5) for GSH or water for OT, were assayed by HPLC for the quantitative determination of drugs. The amount of GSH entrapped in INUPAHy/A(t=24 h) hydrogel was found to be 21.4% (w/w) and the amount of OT was found to be 19.7% (w/w).

2.13. Drug Stability at pH 1.0 and pH 6.8

The chemical stability of GSH and OT was evaluated at pH 1.0 and pH 6.8. Each drug (10 mg) was incubated at 37°C in 10 ml 0.1 M HCl or PBS (NaCl, Na₂HPO₄, NaH₂PO₄, Na₂HPO₄, Na₃PO₄).
KH$_2$PO$_4$ pH 6.8 solution. At scheduled time intervals, samples were withdrawn, filtered (0.45 µm Millipore filter) and analyzed by HPLC. The degradation was evaluated by determining the final concentration of each drug with respect to the starting value.

2.14. Drug Release at pH 1.0 and pH 6.8

Samples (10 mg) of drug-loaded INUPAHy/A$_{(t=24\ h)}$ hydrogel were dispersed in flasks containing 0.1 M HCl (pH 1.0, simulated gastric fluid) or PBS (pH 6.8) (simulated intestinal fluid) and maintained under magnetic stirring (100 rpm) at 37.0 ± 0.1°C for 2 h and 24 h, respectively. Sink conditions were maintained during the experiments (drug concentration was always below 10% of drug solubility). Then, at established time intervals, each sample was filtered through a 0.45 µm Millipore filter and analyzed by HPLC. Each experiment was carried out in triplicate and the results are reported as mean ± SD.

3. Results and Discussion


To prepare new hydrogels based on inulin (INU) cross-linked with α,β-polyaspartylhydrazide (PAHy), potentially useful for intestinal delivery of therapeutic peptides such as glutathione (GSH) and oxytocin (OT), at first the polysaccharide was derivatized with succinic anhydride (SA) as reported elsewhere [27, 28], in order to introduce carboxyl groups able to react with hydrazide groups of PAHy and, at the same time, to confer a pH dependent swelling to the resulting hydrogels.

Briefly, INU was derivatized with SA in an organic phase (anhydrous DMF) for 24 h at 25°C using TEA as a catalyst and two different ratios ($X$) between mol SA and mol INU repeating units, equal to 0.20 and 0.30. The resulting INU–SA derivatives (named as INU–SA/A and INU–SA/B, for $X$ equal to 0.20 and 0.30, respectively) were obtained in high yield (98–99% (w/w), based on the starting INU), and they were purified by cationic exchange resin. The FT-IR spectrum of each INU–SA derivative showed a strong band at 1732 cm$^{-1}$, attributable to the stretching of the C=O group of both the ester bonds of SA residues linked to INU and the carboxyl groups of SA. The presence of these latter was also confirmed by the stretching characteristic bands at 1575 cm$^{-1}$ and at 1416 cm$^{-1}$ of carboxylate groups (data not shown).

The $^1$H-NMR spectra of INU–SA derivatives showed a signal at δ 2.57 (4H, s, –CH$_2$–CH$_2$–) attributable to the presence of the succinic residues in these samples (data not shown). Spectral data of INU–SA derivatives were in agreement with literature values [27]. The derivatization degree in succinic residues (DD$_{SA}$) was determined by $^1$H-NMR analysis and resulted to be 20 ± 2 and 30 ± 1% mol/mol for INU–SA/A and INU–SA/B derivatives, respectively.

INU–SA derivatives were water soluble at 25°C, unlike the native INU, presumably due to the presence of carboxyl groups.
3.2. Preparation and Characterization of INUPAHy Hydrogels

INU–SA/A and INU–SA/B derivatives were chemically cross-linked with PAHy in the presence of EDC and NHSS (Scheme 1). For each INU–SA derivative, the reaction with PAHy was carried out for 4, 8 and 24 h. All INUPAHy networks were obtained in high yield (90–95%, w/w) and as soft and transparent hydrogels.

The FT-IR spectrum of each hydrogel showed the absence of the peak at 1416 cm\(^{-1}\), attributable to the carboxylate groups of INU–SA, as well as the presence of characteristic bands at 1530 cm\(^{-1}\) and 1732 cm\(^{-1}\), attributable to the amide II of PAHy and the stretching of C=O group, respectively (data not shown).

Scheme 1. Schematic structure of INUPAHy hydrogel obtained after cross-linking reaction between INU–SA and PAHy in aqueous solution.
Table 1.
Values (means ± SD, n = 3) of weight swelling ratio, $q$, determined in various media for INUPAHy/A and INUPAHy/B hydrogels obtained after 4, 8 and 24 h of cross-linking

<table>
<thead>
<tr>
<th>$q$</th>
<th>INUPAHy/A hydrogel</th>
<th>INUPAHy/B hydrogel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 h</td>
<td>8 h</td>
</tr>
<tr>
<td>In double-distilled water</td>
<td>27.7 ± 0.2</td>
<td>24.7 ± 1.1</td>
</tr>
<tr>
<td>In PBS (pH 6.8)</td>
<td>8.1 ± 0.1</td>
<td>5.6 ± 0.9</td>
</tr>
<tr>
<td>In HCl (pH 1.0)</td>
<td>3.4 ± 0.2</td>
<td>2.2 ± 0.1</td>
</tr>
</tbody>
</table>

3.2.1. Swelling Studies

Since the swelling ability of a hydrogel affects cell compatibility, rate of chemical or enzymatic hydrolysis, as well as drug release, the weight swelling ratio, $q$, of INUPAHy/A and INUPAHy/B hydrogels obtained after 4, 8 and 24 h was evaluated in various media. In particular, double-distilled water, PBS (pH 6.8) and HCl (pH 1.0) solutions were chosen in order to evaluate the potential pH-sensitive swelling, due to the presence of succinic residues linked to INU. Swelling data are reported in Table 1.

As expected, in all the investigated media weight swelling ratio decreases by increasing the cross-linking time from 4 to 24 h, because of the increase in inter- and intra-molecular cross-linking that produces more compact networks with a consequent decrease in water uptake. On the other hand, when the cross-linking time is shorter, less compact hydrogels with a higher swelling ability are obtained. Data reported in Table 1 show a good affinity of all prepared hydrogels toward the aqueous media and a pH-sensitive behaviour; in fact, when pH is 6.8 (simulated intestinal fluid) the swelling is about 2.5–4.5-times greater than the swelling at pH 1.0 (simulated gastric fluid). These results suggest that, in INUPAHy hydrogels, some carboxyl groups of INU–SA do not react chemically with PAHy, but they remain as pendant acid groups in the network, thus causing a pH-sensitive swelling, overall when INU–SA/B is employed (it contains a greater amount of carboxyl groups).

Moreover, the $q$ values in HCl (pH 1.0) and in PBS (pH 6.8) solutions are lower than those found in double distilled water, probably due to the ionic strength and osmotic pressure of these media.

3.2.2. Enzymatic Hydrolysis Studies with Inulinase

Enzymatic degradation studies in the presence of inulinase (10 U/ml) have been performed on samples showing the most pronounced pH-sensitive behaviour, i.e., INUPAHy/A$_{(t=24\text{ h})}$ and INUPAHy/B$_{(t=24\text{ h})}$ hydrogels. Table 2 reports the values of degradation (%) and weight swelling ratio (determined in double-distilled water) of the hydrogels recovered after 24 h of incubation with inulinase. The data clearly show a pronounced enzymatic degradation on both hydrogels (in the absence of enzyme the degradation is negligible, data not shown). This result confirms that inulinase can diffuse into these networks where it is able to degrade INU also after its
Table 2.
Degradation and weight swelling ratio, \( q \), for INUPAHy/A\(_{t=24\text{ h}}\) and INUPAHy/B\(_{t=24\text{ h}}\) hydrogels determined after incubation with inulinase (10 U/ml) for 24 h

<table>
<thead>
<tr>
<th>Hydrogel</th>
<th>Degradation (% w/w)</th>
<th>( q ) (in double-distilled water after enzymatic degradation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INUPAHy/A(_{t=24\text{ h}})</td>
<td>48.0 ± 0.7</td>
<td>37.5 ± 1.2</td>
</tr>
<tr>
<td>INUPAHy/B(_{t=24\text{ h}})</td>
<td>33.0 ± 1.2</td>
<td>32.0 ± 1.1</td>
</tr>
</tbody>
</table>

Values are means ± SD (\( n = 3 \)).

Figure 1. Enzymatic degradation of INUPAHy/A\(_{t=24\text{ h}}\) hydrogel by inulinase (10 U/ml) as a function of time: quantitative determination by the anthrone method. Left ordinate, amount of detected fructose; right ordinate, % of degradation. aAmount (%) of detected fructose relative to the total fructose in the sample.

derivatization with SA and cross-linking with PAHy. In contrast, other authors have demonstrated that when INU is derivatized, for example, by acetylation or methylation, it is not biodegradable, probably because of a change in polymer conformation due to derivatization [30]. In addition, since it is known that INU degradation products in the colon aid the amelioration of inflammatory bowel disease [21–25], the enzymatic degradation of INUPAHy hydrogels represents a very important property for a potential use of these hydrogels in pharmaceutical field.

Taking into account that INUPAHy/A\(_{t=24\text{ h}}\) undergoes the greatest enzymatic degradation after 24 h of incubation with inulinase, we have chosen this sample for further studies.

Thus, enzymatic degradation of this sample was also evaluated after different times of incubation with inulinase (10 U/ml) and by using the anthrone method [31] which is based on the quantitative determination of the fructose resulting from the INU degradation. The results, shown in Fig. 1, clearly show that degradation by
Figure 2. Transmittance values (%) at 500 nm of mucin dispersions in the presence INU–SA/A, PAHy, INU–SA/A and PAHy physical mixture or poly(acrylic acid) (chosen as a positive control) as a function of the incubation time.

Inulinase is time dependent, reaching about 40% after 24 h of incubation, according to data reported in Table 2.

3.2.3. Interaction with Mucin

Bioadhesion is an important property for obtaining a prolonged interaction between a polymeric system and a biological surface, useful for improving drug delivery in the site of action or absorption such as the intestinal mucosa [32, 33]. Therefore, mucoadhesive properties of starting polymers employed to prepare INUPAHy/A\(_{t=24\ h}\) hydrogel, have been evaluated. In particular, interaction studies between INU–SA/A or PAHy (alone or in physical mixture, see Materials and Methods and mucin were performed in PBS (pH 6.8) by a turbidimetric method (already used successfully by other authors [34, 35]), by employing poly(acrylic acid), as a positive control [36]. Due to the formation of macro-aggregates, as a consequence of polymer–mucin interaction, the transmittance value decreases by increasing this interaction [34, 35]. As shown in Fig. 2, transmittance values for INU–SA/A alone or in physical mixture with PAHy are lower than those found for poly(acrylic acid) and, therefore, these samples show an interaction with mucin stronger than that of the positive control. Moreover, it is evident that after 8 h of incubation with mucin, the transmittance value of each sample is about constant, thus suggesting that the maximum polymer–glycoprotein interaction is reached. It is reasonable to suppose that also INUPAHy hydrogels could have a mucoadhesive behaviour potentially useful for a prolonged oral drug delivery.

3.3. Cell Viability Analysis

A fundamental property of a hydrogel system is its biocompatibility. For this reason, we have performed a preliminary in vitro study to have information about the compatibility of INUPAHy/A\(_{t=24\ h}\) hydrogel towards human intestinal model cells, such Caco-2 cells. Direct and indirect methods (see Materials and Methods) have
Figure 3. Viability (%) of Caco-2 cells evaluated by MTT assay after 24 or 48 h of incubation with INUPAHy/A_{t=24 \text{ h}} hydrogel (direct method, 1 mg sample/ml cell-culture medium) or with the growth medium conditioned by INUPAHy/A_{t=24 \text{ h}} hydrogel (indirect method, 1 mg sample/ml cell-culture medium).

been performed; the first consists of the direct contact of the hydrogel (investigated amounts 0.01–1 mg/ml medium) with the cells for 24 or 48 h, while in the indirect method, the growth medium was firstly conditioned for 24 h at 37°C by the hydrogel and then added to the cells. For both the methods, cell viability was assessed by MTT and Trypan blue exclusion assays. Results were compared with those obtained from cells incubated in the growth medium in the absence of the hydrogel (control) and they were expressed as percentage of cell viability. It has been found that a different incubation times does not induce a time-dependent inhibitory effect on cell viability that was similar to that of control. At the maximum amount tested (1 mg/ml medium), cell viability was always higher than 80%, as shown in Fig. 3.

These results suggest that INUPAHy/A_{t=24 \text{ h}} hydrogel does not release, in the growth medium, substances which interfere, in a negative manner, with cell viability and it does not cause a decrease in the cell viability after direct contact with cells. Similar results have been obtained using the Trypan blue exclusion assay (data not shown).

3.4. Drug Release at pH 1.0 and pH 6.8

Taking into account cell compatibility, enzymatic degradation, potential mucoadhesive behaviour and pH-sensitive swelling shown by INUPAHy/A_{t=24 \text{ h}} hydrogel, it is reasonable to suppose its potential use for intestinal release of peptides, such as GSH and OT, for the treatment of bowel inflammation.

For this reason, both these peptides were loaded into the chosen hydrogel by a soaking procedure. Drug loading resulted to be 21.4% (w/w) and 19.7% (w/w) for GSH and OT, respectively.

Figure 4 shows the drug-release profiles, expressed as percentage of drug delivered (related to the entrapped total dose) as a function of time in 0.1 M HCl (pH 1.0) solution (simulated gastric fluid) for 2 h or in PBS solution (pH 6.8) (simulated in-
Figure 4. Glutathione (GSH) and oxytocin (OT) release from INUPAHy/A (t=24 h) hydrogel at 37.0 ± 0.1°C in 0.1 M HCl solution (simulated gastric fluid) from 0 to 2 h and in PBS solution (pH 6.8, simulated intestinal fluid) from 0 to 24 h.

As can be seen, the release profile of GSH and OT at pH 1.0 shows an initial slight burst effect; in fact, after 30 min about 10% of OT and 20% of GSH were released and these values slightly increased until 2 h in acidic medium. When the release medium was PBS solution (pH 6.8), a greater amount of both drugs was released, according to the pH-sensitive swelling of the hydrogel. It is interesting to observe that in both the investigated media, the final amount of GSH released was higher than OT. The observed release profiles may be due to the different molecular mass of two peptides (307 Da for GSH and 1000 Da for OT) that causes a different diffusion rate from the matrix. In addition, it is also possible
that the electric charge of the two peptides in the release medium could affect the drug release. GSH is positively charged at pH 1.0, but negatively charged at pH greater than its isoelectric point (pI, 5.77) [37]. In contrast, since the pI of OT is 7.7 [38], this peptide is positively charged in both release media.

Therefore, in PBS (pH 6.8), the lower molecular mass and electrostatic repulsions between GSH and carboxylate groups in the network, together with the pH-sensitive swelling of the hydrogel, could cause the release of an amount greater than that found for OT.

However, for both investigated peptides, taking into account the pronounced degradation by inulinase that INUPAHy/A(t=24 h) hydrogel undergoes (see Table 2), it is reasonable to suppose that, in vivo, in the presence of these enzymes, a colon-specific peptide release could occur.

4. Conclusions

New chemical hydrogels potentially suitable for oral delivery of glutathione and oxytocin were produced by cross-linking between succinic derivatives of inulin (INU–SA) and α,β-polyaspartylhydrazide (PAHy). The physicochemical and biological characterizations of these hydrogels were carried out by swelling, mucoadhesion, enzymatic degradation studies, as well as by cell compatibility assays using Caco-2 cells. The obtained results have evidenced the cell compatibility, the pH-sensitive swelling, a potential mucoadhesion and degradability by inulinase, thus suggesting the use of these hydrogels for the intestinal delivery of chosen peptides.

In vitro studies showed that the release of glutathione and oxytocin occurs essentially in simulated intestinal fluid and it depends on the properties of the polymeric network and on the molecular mass and electric charge of the peptide. All obtained results suggest a potential use of the hybrid hydrogels based on INU–SA and PAHy for the treatment of inflammatory bowel disease.

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References