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New hydrogel matrices based on chemical crosslinked α,β-polyasparthydrazide: synthesis, characterization and in vivo biocompatibility studies

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Abstract

New swellable micromatrices of α,β-polyasparthydrazide (PAHy) crosslinked with glutaraldehyde were prepared. The effect of crosslinking agent concentration was evaluated. In particular, crosslinking density affected aqueous dynamic swelling and glass-transition temperature of the material. The structure of prepared networks was also studied by scanning electron microscopy and X-ray analysis. Finally, biocompatibility of PAHy derivatives was investigated in vivo by subcutaneous implantation and in oral administration to laboratory animals.

Keywords: Crosslinked polyasparthydrazide; Hydrogels; Swellable micromatrices; Glutaraldehyde; Biocompatible polymers; Drug delivery systems

1. Introduction

In recent years, the study and development of drug delivery systems has become one of the most active fields of pharmaceutical research, not only for the possibility of obtaining prolonged release devices of therapeutic agents, but also for their ability to release them in a specific site thereby preventing accumulation in non-target tissues and increasing their bioavailability (Lee, 1985; Peppas, 1986; Anderson and Wan Kim, 1986). Some possible ways to achieve this object are both applications to body skin and mucous and subcutaneous implantations (Li et al., 1991).

Hydrogels are hydrophilic network polymers which are glassy in the dehydrated state. These systems brought into contact with water or biological medium can absorb a significant amount of water (usually > 20% of its dry weight), maintaining their structural integrity. Depending on the physical characteristics of the polymer, different thermodynamic transitions (e.g. glass-to-rubber transition) may occur during liquid penetration.

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On account of their inertness and biocompatibility, hydrogels have been examined for a wide range of biomedical applications such as soft contact lenses, wound dressing and implant materials and diagnostics (Corkhill et al., 1989). The biocompatibility of hydrogels is due to their ability to simulate natural tissues because of their high water content and their special surface properties. Furthermore, the ability to release entrapped solutes in aqueous medium makes hydrogels excellent carriers for a variety of pharmacological agents ranging from small molecular weight compounds to macromolecular and protein drugs (Jeyanthi and Panduranga Rao, 1990; Gutowska et al., 1992).

It has been shown that release rate of entrapped solute depends on many factors such as chain mobility, crosslinking density, shape and dimensions of the matrix, hydrophily of the polymer and its degree of swelling (Lee and Kim, 1991).

The drug release mechanism from dehydrated matrices involves water absorption, matrix swelling and simultaneous drug diffusion through the swollen region (Korsmeyer et al., 1983; Colombo et al., 1990).

In this paper we present the synthesis and characterization of new hydrogels originating from chemical reticulation of α,β-polyasparthydrazide (PAHy) by glutaraldehyde, at various degrees of crosslinking.

PAHy is a new water-soluble macromolecule synthesized from a polysuccinimide (PSI) by reaction with hydrazine (Giammona et al., 1989). Biological tests on laboratory animals have shown that this polymer is non-toxic and non-immunogenic and, because of its hemodynamic efficiency, it has been proposed as a plasma expander. Besides, the presence of reactive hydrazine groups makes PAHy particularly suitable as a carrier of drugs with carboxyl and aldehyde functions (Giammona et al., 1994).

This paper studies the ability of PAHy to be crosslinked in order to prepare three-dimensional networks for prolonged and controlled release of bioactive molecules. In this investigation we have prepared new hydrogel micromatrices, devised for subcutaneous implantation and oral administration. The characterization of these new materials was carried out by water swelling measurements, thermal, X-ray and SEM analysis. Finally we have evaluated the biocompatibility of PAHy hydrogels after subcutaneous implantation and oral administration in rats.

2. Materials and methods

2.1. Apparatus

PAHy weight — average molecular weight was measured by light scattering on a Dawn DSP-F Laser Spectra Physics Spectrometer.

Elemental analyses (C, H, N) were carried out on a Carlo Erba model 1106 analyzer; compounds were quantitatively dried before analysis under reduced pressure (10^{-3} mmHg) at room temperature for 48 h on P_2O_5.

Morphological investigation was made by a Philips 501 scanning electron microscope (SEM): the surface of samples was made conductive by a layer of gold in a vacuum chamber.

The dynamic swelling process was observed by an optical microscope (Wild Heerbrugg), according to the method proposed by Robert et al. (1987), in order to analyze the diameter changes of dry crosslinked microparticles.

Thermal analysis was performed by means of a Perkin Elmer DSC 7B calorimeter. Samples were heated from -10°C to 250°C; the heating rate was 2°C/min. Before each test the samples were carefully dried for 72 h under vacuum in the presence of P_2O_5 and then ground in a mortar in order to ensure a good contact with the aluminium pan. The glass transition temperature, T_g, was determined as the temperature corresponding to a change of the slope in the specific heat-temperature plot.

X-ray diffraction analysis was recorded using an X-ray powder diffractometer (PW 1050, Phillips). The experimental parameters were set as follows: Ni filtered Cu radiation ($\lambda = 1.5418$ Å); tube settings 40 kV, 30 mA; angular speed 2° (2Θ)/min; 1–0.1–1 slits.

2.2. Materials

DL-Aspartic acid, hydrazine hydrate, *N,N*-dimethylformamide (DMF), glutaraldehyde (50% aqueous solution) and acetic acid were from Fluka (Switzerland).

α,β-Polyasparthydrazide (PAHy) was prepared via the polysuccinimide (PSI) by polycondensation of DL-aspartic acid in the presence of H_3PO_4 at 180°C followed by reaction with hydrazine in DMF solution. PAHy was isolated by filtration, washed several times with acetone and dried. An aqueous solution of PAHy was dialyzed for 3 days against several changes of distilled water using Visking Dialysis Tubing (18/32 inch) with a molecular cut-off of 12 000–14 000.

After dialysis, the PAHy was recovered by lyophilization in a yield of 97% w/w based on the starting PSI. Analytical data of PAHy were in agreement with literature values (Giammona et al., 1994).

PAHy weight — average molecular weight determined by light scattering, was 37 700.

2.3. PAHy crosslinking procedure

To a PAHy aqueous solution (500 mg in 11 ml of distilled water), continuously stirred and kept at 2°C by an ice bath, were added first 8 ml of 10 vol% acetic acid and then 50 vol% glutaraldehyde. The crosslinking agent was added gradually every 15 min and at a suitable quantity in agreement with the crosslinking ratio (X) defined as:

$$X = \text{mol glutaraldehyde/mol PAHy repeating unit} \quad (1)$$

Each reaction mixture was stirred for 2 h at 2°C and for 12 h at room temperature. Subsequently the samples were stirred at 50°C for 6 h. When the reaction was completed, the crosslinked hydrogels were isolated by filtration, purified by several washings with distilled water and then dried at 10^{-1} mmHg in the presence of P_2O_5 for 72 h at 25°C. Finally the samples were ground and the obtained particles, analyzed by sieving on a mechanical shaker, showed dimensions in the range 20–90 μm.

In particular the following samples were prepared.

Sample 1 ($X = 0.12$)
Quantity of glutaraldehyde used: 82.5 μl
Yield: 92.3% w/w
Analysis: Calculated for $C_{8.6}H_{14.48}N_6O_4$: C, 38.84; H, 5.49; N, 31.60
Found: C, 38.91; H, 5.57; N, 31.65

Sample 2 ($X = 0.2$)
Quantity of glutaraldehyde used: 277 μl
Yield: 96.0% w/w
Analysis: Calculated for $C_9H_{14.8}N_6O_4$: C, 39.88; H, 5.50; N, 31.01 Found: C, 39.93; H, 5.55; N, 31.07

Sample 3 ($X = 0.4$)
Quantity of glutaraldehyde used: 555 μl
Yield: 97.6% w/w
Analysis: Calculated for $C_{10}H_{15.6}N_6O_4$: C, 42.31; H, 5.54; N, 29.61 Found: C, 42.37; H, 5.57; N, 29.70

Sample 4 ($X = 0.6$)
Quantity of glutaraldehyde used: 831 μl
Yield: 98.2% w/w
Analysis: Calculated for $C_{11}H_{16.4}N_6O_4$: C, 44.53; H, 5.57; N, 28.33
Found: C, 44.61; H, 5.60, N, 28.42

2.4. Residue content

Unreacted glutaraldehyde and PAHy content was determined in the mother liquor of the reaction and in the washing water. A sensitive colorimetric micromethod (Boratynski and Zal, 1990) revealed the absence of glutaraldehyde in all prepared samples. Unreacted PAHy content was evaluated evaporating the mother and washing liquids under vacuum at 40°C. The obtained residue was washed with acetone, in which the polymer is insoluble, then it was filtered, dried and weighed. The amount of unreacted polymer was found to be about 8% and 4% w/w for the samples with $X = 0.12$ and $X = 0.2$, respectively, and absent for the other samples.

2.5. Stability of PAHy hydrogels at pH 1.1 and pH 7.4

In vitro stability of all prepared samples was investigated in pH 1.1 (HCl, NaCl and glycine) and pH 7.4 (NaCl, Na_2HPO_4, KH_2PO_4) aqueous buffer solutions.

Samples (25 mg) were dispersed in 10 ml of buffer solution, then kept in a water bath at 37 ± 0.1°C for 100 h. After this time, the liquid phase was separated by filtration and assayed colorimetrically for glutaraldehyde determination (Boratynski and Zal, 1990), revealing the absence of glutaraldehyde. The solid phase after drying showed the same weight as the starting sample.

2.6. Swelling studies

The dynamic swelling process of each prepared sample was carried out at room temperature (25°C) allowing dry crosslinked micromatrices to swell in distilled water. Six microparticles with initial dry diameter (d_o) between 20 and 90 μm were tested from each sample. The change of the diameter value (d_t) was observed until the microparticles achieved the full swollen equilibrium with a diameter d_∞.

Equilibrium weight swelling ratio, Q, was determined by keeping the crosslinked samples in contact, through a Millipore 0.45 μm membrane, with distilled water for 24 h at 25°C. Each experiment was performed in triplicate. The results agreed with each other within 5% error.

2.7. Biological trials

Tissue irritation and the accompanying damage and pain produced by the assayed compounds into the rat body was evaluated following subcutaneous or oral administration in male Sprague–Dawley rats weighing 150–200 g (Charles River Italia, Calco).

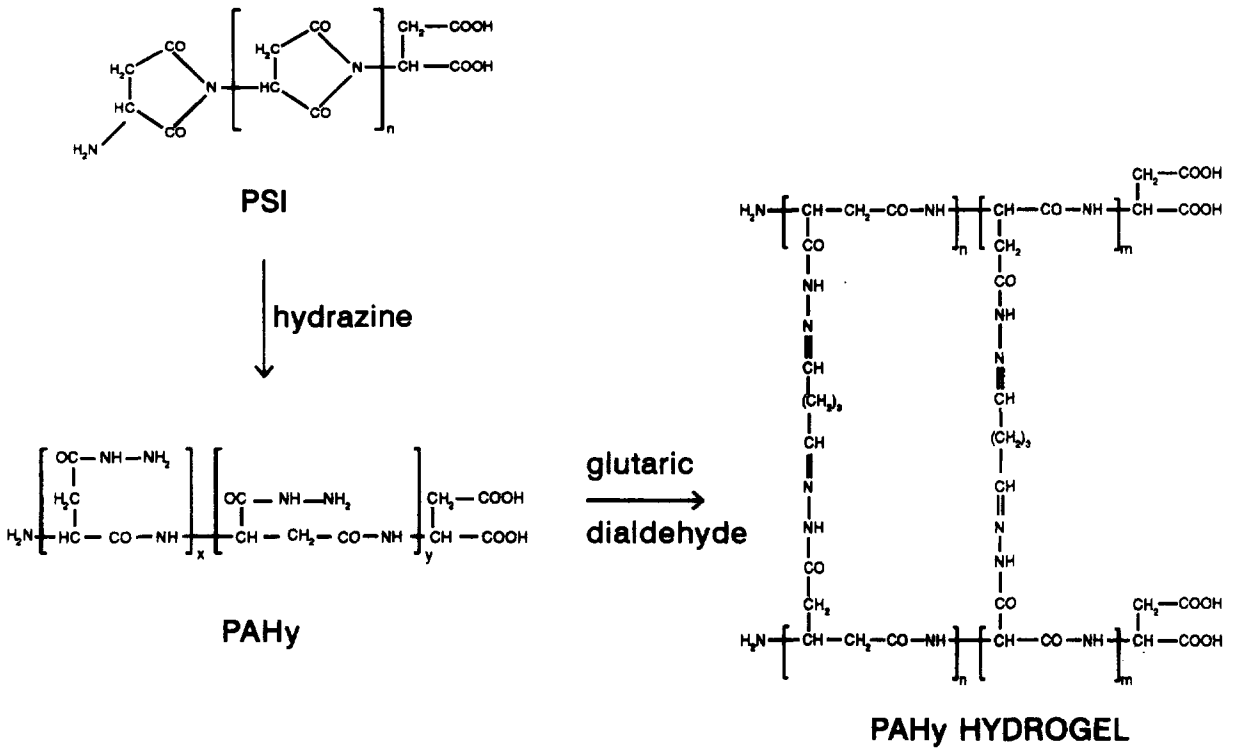
In the test system for parenteral irritation, the compounds were suspended in distilled water (1.2 g/kg in 20 ml of water) and given subcutaneously by injecting them into spaces between connective tissue of the intrascapular region (four rats/group). Control rats received distilled water (20 ml/kg). Rats were sacrificed by a lethal dose of sodium pentobarbitone (100 mg/kg, intraperitoneally) 1, 3 and 14 days after dosing. The subcutaneous injection sites were exposed by dissection and any reaction was scored for irritation on a scale of 0–5 as follows (Grad and Chengelis, 1988): 0, no discernable gross irritation; 1, slight hyperemia and discoloration; 2, moderate hyperemia and discoloration; 3, distinct discoloration in comparison with the surrounding area; 4, small area of necrosis; 5, widespread necrosis, possibly involving the underlying tissue. Moreover, a piece of the skin surrounding the injection site was collected for histological assessment by a standard technique (Luna, 1968).

In order to assess any potential damage of the assayed compounds in the gastrointestinal tract, these were suspended in distilled water (1.2 g/kg in 20 ml of water) and administered orally to groups of four rats which were fasted 24 h before treatment while water was withheld 6 h before. Control rats received distilled water (20 ml/kg). Rats were killed by an overdose of sodium pentobarbitone 24 h after treatment (rats did not receive any food or water during this period of time). The stomach, the duodenum and the ileum were removed, inverted, washed in ice-cold saline and any lesion on the mucosal layer counted by a visual examination under 5 × magnification. All lesions were counted regardless of size. Samples of each examined area were processed for histological analysis by a standard technique (Luna, 1968). Tissues were fixed in phosphate-buffered 10% formalin (4% formaldehyde) solution for at least 24 h before processing into 5 μm haematoxylin and eosin stained sections for morphologic evaluation. The prepared specimens were evaluated through a Leitz light microscope with a photomicrographic system.

3. Results and discussion

The steps involved in the preparation of PAHy hydrogels are summarized in Scheme 1.

The crosslinking reaction takes place between the hydrazine residues of the PAHy and the aldehyde functionalities of the glutaraldehyde.



Scheme 1.

The obtained networks are insoluble in water, basic or acid aqueous solutions and common organic solvents. In addition all prepared samples have small amounts of unreacted polymer (less than 8%) and they do not contain unreacted glutaraldehyde; neither do they release this molecule in an aqueous medium (pH 1.1 and 7.4). This result is very important since free glutaraldehyde is not desirable in pharmaceutical dosage forms.

Samples 1–4 were analyzed by scanning electron microscopy. Any significant difference was observed in the shape and size of the microparticles varying the crosslinking degree. As an example, Fig. 1 shows a SEM micrograph of the sample with $X = 0.6$ where microparticles having a quasi-spherical shape are seen.

All prepared samples were characterized through swelling measurements. In particular, since the swelling process is reflected in the change of sample dimension as a function of time, we have observed, through an optical microscope, the variation of microparticle diameter.

Fig. 2 shows the normalized diameter values (d_t/d_0) as a function of time for all investigated

Fig. 1. Scanning electron micrograph of sample 4.

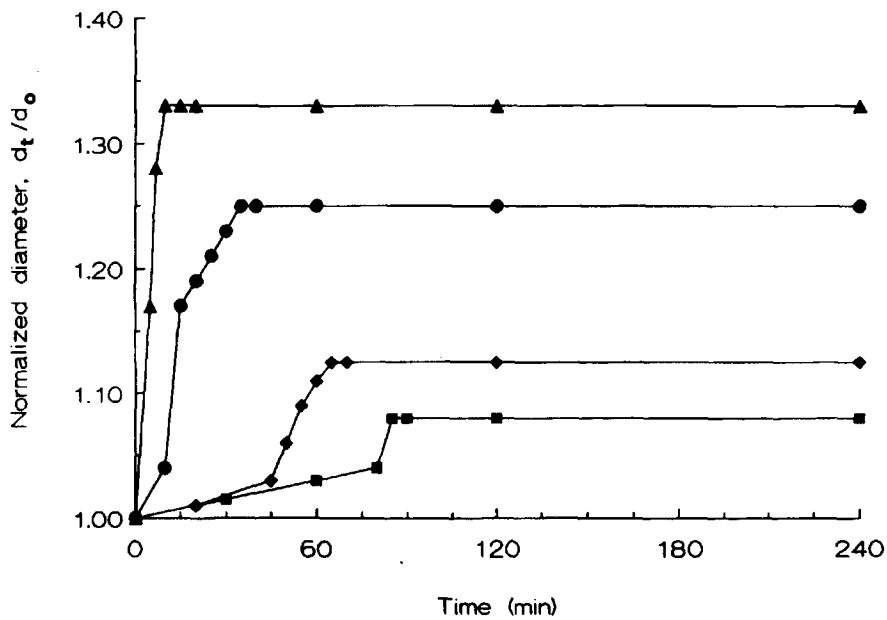


Fig. 2. Normalized diameter of PAHy crosslinked samples using different glutaraldehyde/PAHy ratios, X. (▲, $X = 0.12$; ●, $X = 0.2$; ◆, $X = 0.4$; ■, $X = 0.6$).

samples. As can be observed, the diameter of the microparticles increased monotonically towards the equilibrium swollen value (d_∞) according to the crosslinker concentration. In particular, the diameter of microparticles reached equilibrium values at different rates in the following order: $X = 0.12 > X = 0.2 > X = 0.4 > X = 0.6$. The d_∞ value does not modify during the entire experimental investigation (72 h). Moreover the value of the equilibrium normalized diameter (d_∞/d_0) decreased as the X ratio increased, as is seen in Table 1.

Table 1 also reports the values of the equilibrium weight swelling ratio Q, calculated as:

$$Q = W_s/W_d \quad (2)$$

where W_s and W_d are the weight of swollen and dry sample, respectively. According to the swelling dynamic data, Q continuously decreased with X, thus confirming a continuous increase of the crosslinking degree.

Table 1
Swelling parameters

Glutaraldehyde/PAHy ratio, X (mol/mol)	Equilibrium normalized diameter, d_∞/d_0	Equilibrium weight swelling ratio, Q
0.12	1.33	3.95
0.2	1.25	2.77
0.4	1.13	1.52
0.6	1.08	1.38

Fig. 3 shows, as an example, the photomicrographs of the sample with $X = 0.2$ before and during the swelling dynamic experiment. As can be observed, as a consequence of water penetration, the sample appears translucent and with greater dimensions.

Samples 1–4 have been also characterized by calorimetric analysis. Table 2 reports the value of the glass transition temperature, T_g, as a function of X. As expected, the T_g value increases with X. This effect can be related to the rigidity of the network structure which become higher by increasing the crosslinking degree (Korsmeyer and Peppas, 1981). At low crosslinking density ($X \leq 0.2$), the value of T_g is smaller than that of the starting uncrosslinked polymer. This can be as-

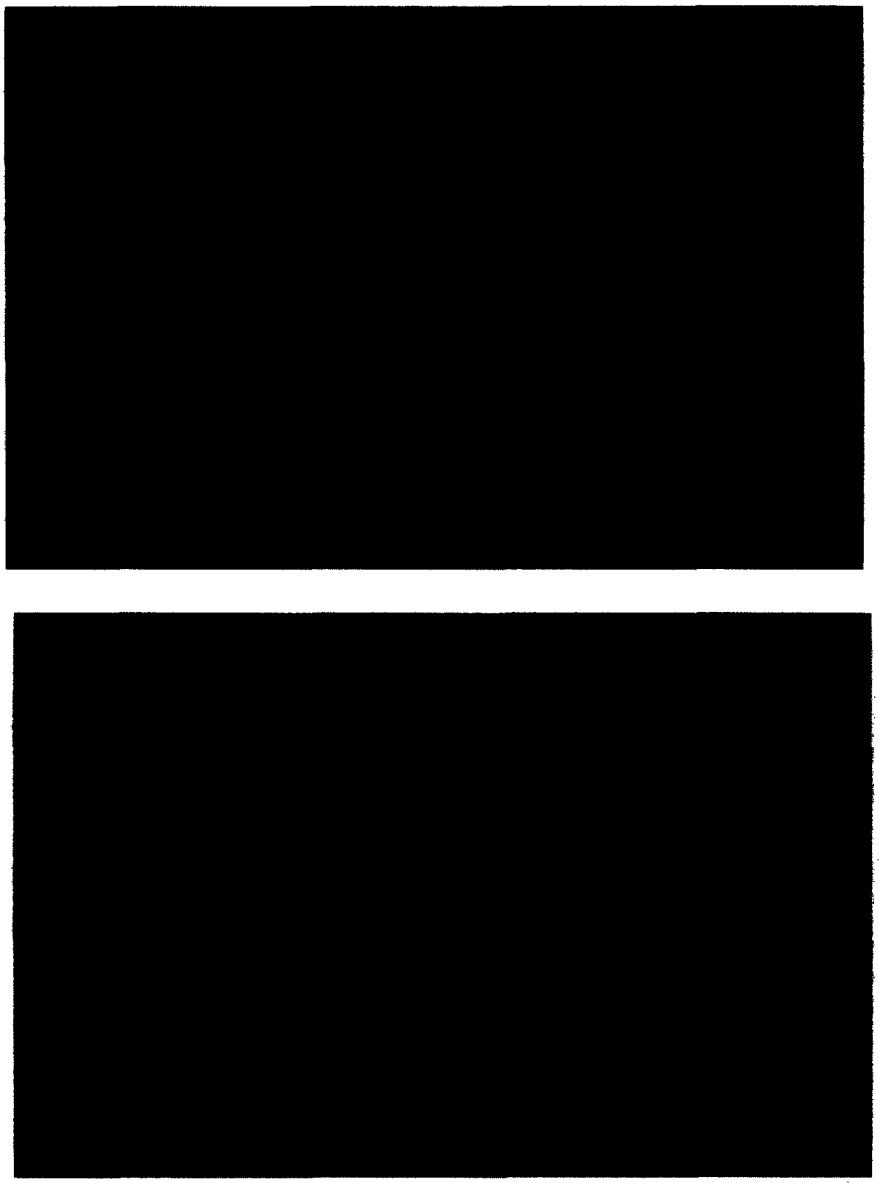


Fig. 3. Photomicrographs of the sample with $X = 0.2$ at time zero (A) and after 15 min (B) during the dynamic swelling experiment.

cribed to the presence of glutaraldehyde which reduces the attraction forces between neighbouring chains of the polymer. It is well known that this phenomenon causes a decrease of the glass transition temperature. On the contrary, at crosslinking density > 0.2 the prevailing effect is the increase of rigidity of the network structure, therefore the T_g value is greater than that of uncrosslinked PAHy.

In addition, the absence of melting peaks in the DSC thermograms indicates that all prepared samples are in the amorphous state like uncrosslinked polymer. This has been also confirmed by X-ray analysis (data not shown).

Histological analysis revealed that the assayed compounds did not cause any discernable gross

Table 2
Glass transition temperature of samples 1–4

Glutaraldehyde/PAHy ratio, X (mol/mol)	Glass transition temperature, T_g (°C)
0.0	71.0
0.12	61.0
0.2	69.0
0.4	73.7

irritation when injected subcutaneously. In all treated rats sacrificed 1, 3 or 14 days after treatment, as well as in the controls, the assigned score was 0. As illustrated in Fig. 4, histological analysis of the skin taken from the injection area of a rat treated 14 days before with sample 2 did not show any significant alteration. No significant alterations were observed in the other groups of treated rats (data not shown).

Twenty-four hours following oral administration, the assayed compounds did not cause any gross lesion of the gastrointestinal tract and their safety was confirmed by histological analysis as shown in Fig. 5 for sample 2.

In fact, the histological findings in the control rats were similar to those found in the treated animals. In particular, no atrophic or disorderly arranged and vacuolated cells were detected. Superimposable findings were observed for the other compounds (data not shown).

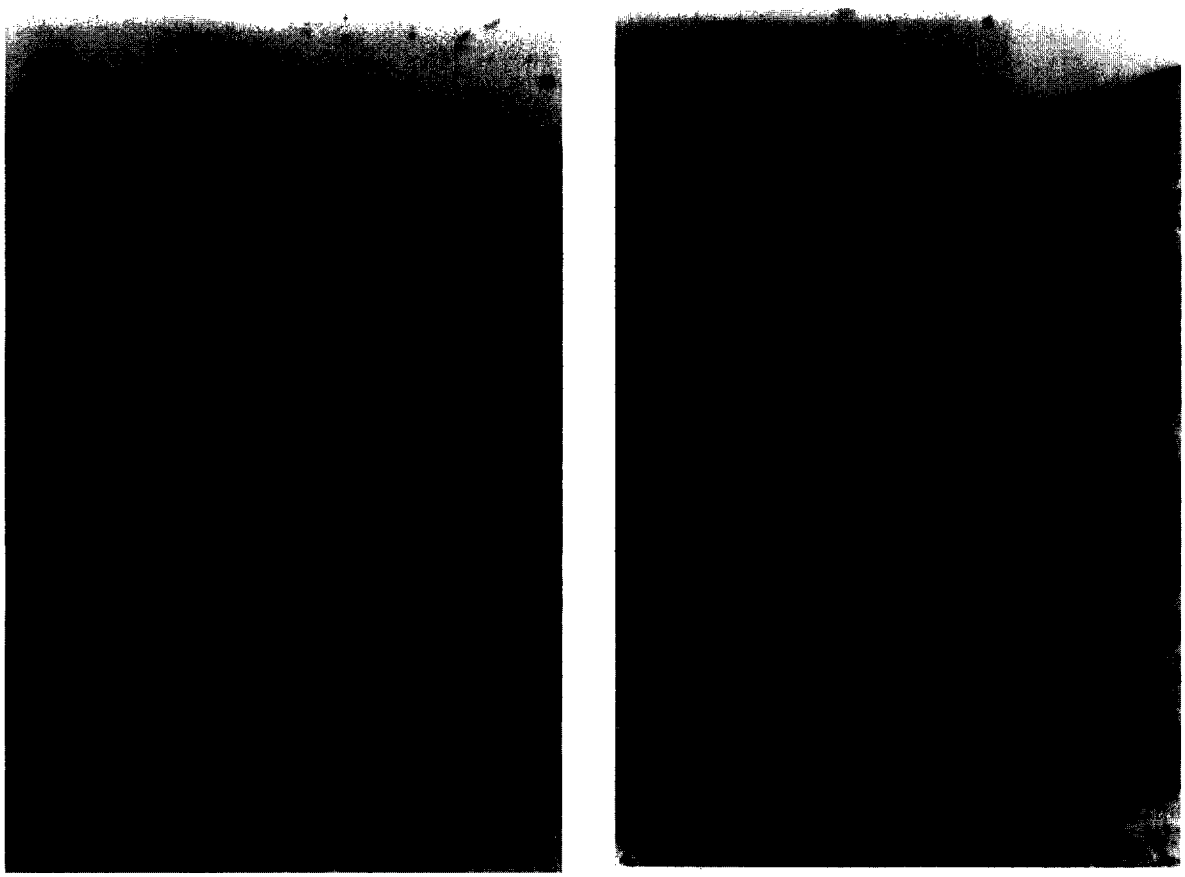


Fig. 4. Histological analysis of skin samples obtained from rats treated 14 days before with the vehicle (panel A) or the PAHy hydrogel with $X = 0.2$ (panel B). The skin samples were taken from the injection area.

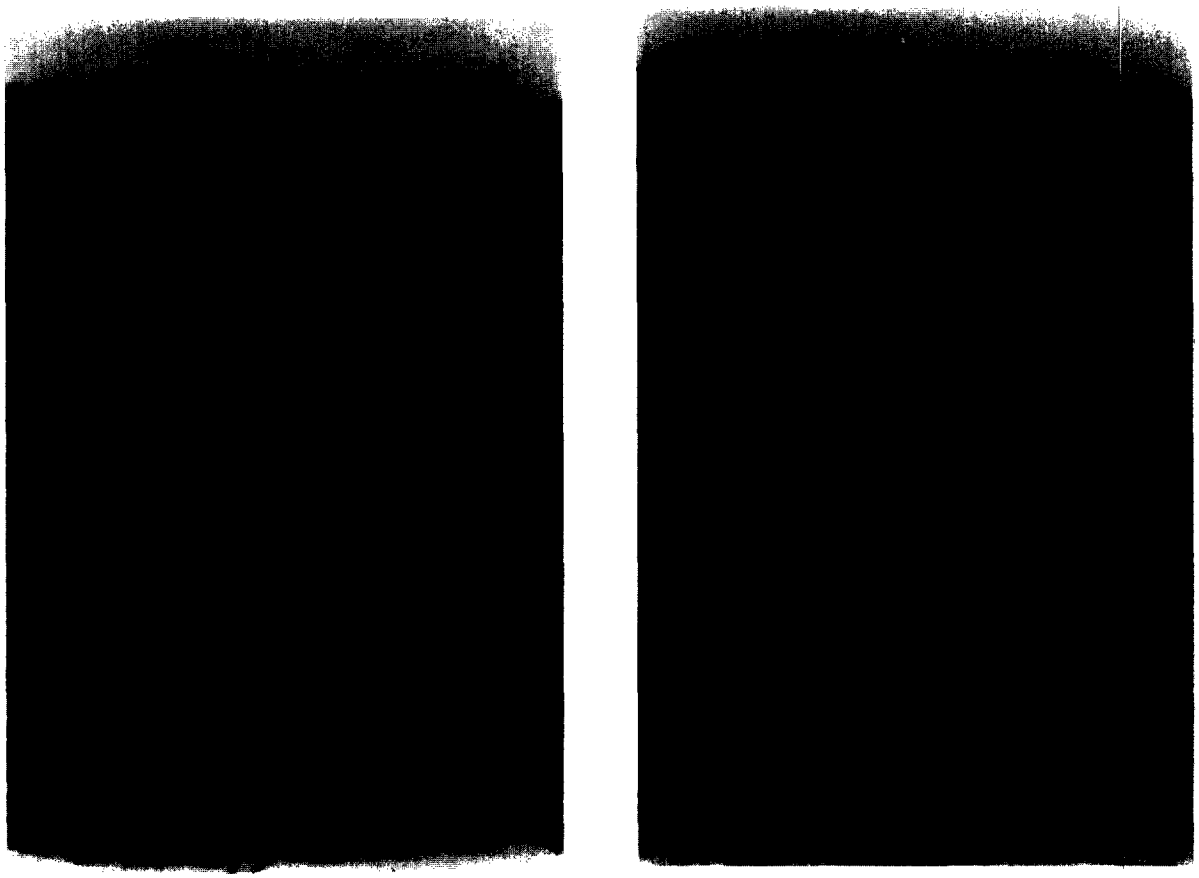


Fig. 5. Histological analysis of gastrointestinal samples taken from rats treated with the compound $X = 0.2$ or water alone, administered orally 21 h before. Panel A: stomach of water-treated rat; panel B: stomach of $X = 0.2$-treated rat; panel C: duodenum of water-treated rat; panel D: duodenum of $X = 0.2$-treated rat; panel E: ileum of water-treated rat; panel F: ileum of $X = 0.2$-treated rat.

4. Conclusions

New hydrogel matrices have been prepared by crosslinking of α,β-polyasparthydrazide with glutaraldehyde at various degrees of reticulation. These systems, formed by microparticles, have outstanding swelling properties in aqueous medium. Calorimetric analysis performed in all prepared samples indicates a peculiar effect of crosslinker concentration. Finally, biological tests on laboratory animals established that PAHy hydrogels are inert, thus suggesting their potential use for oral and implantable drug delivery.

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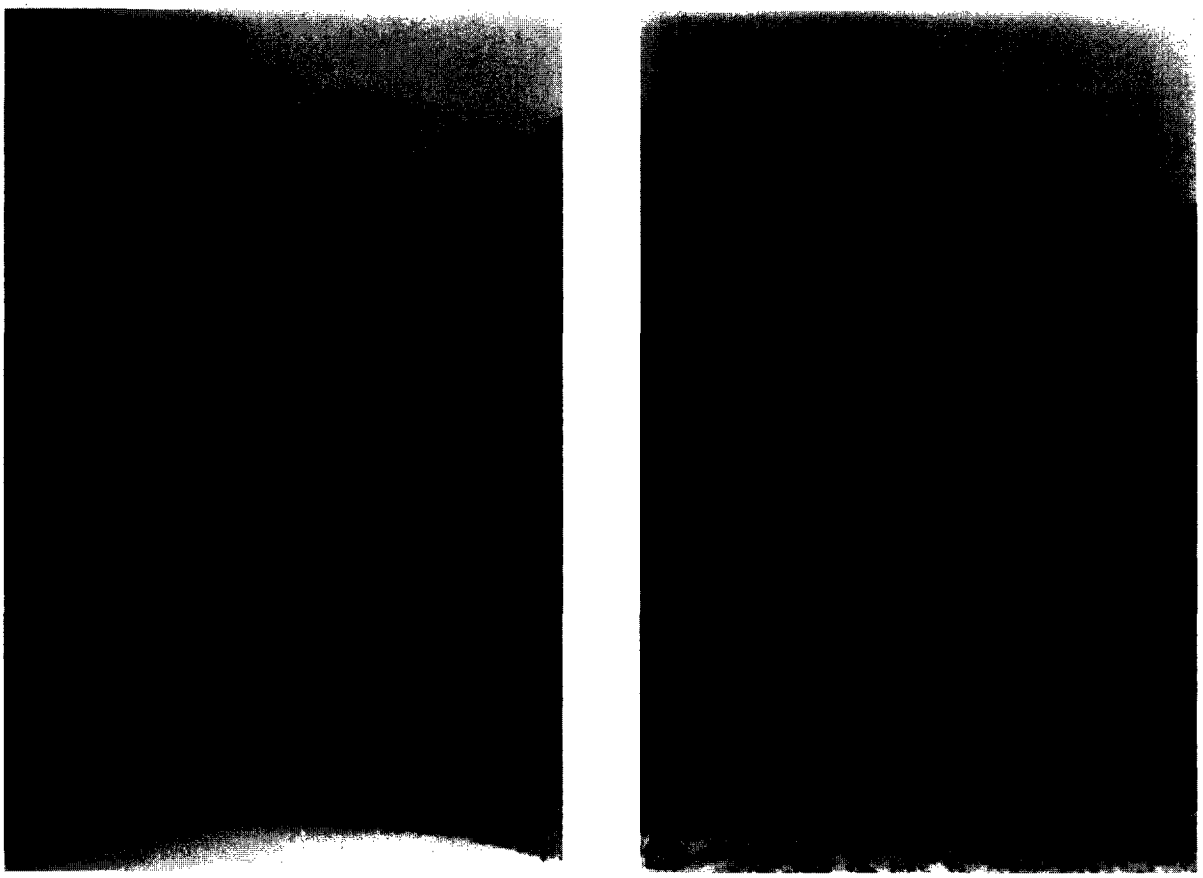

Fig. 5 (C and D).

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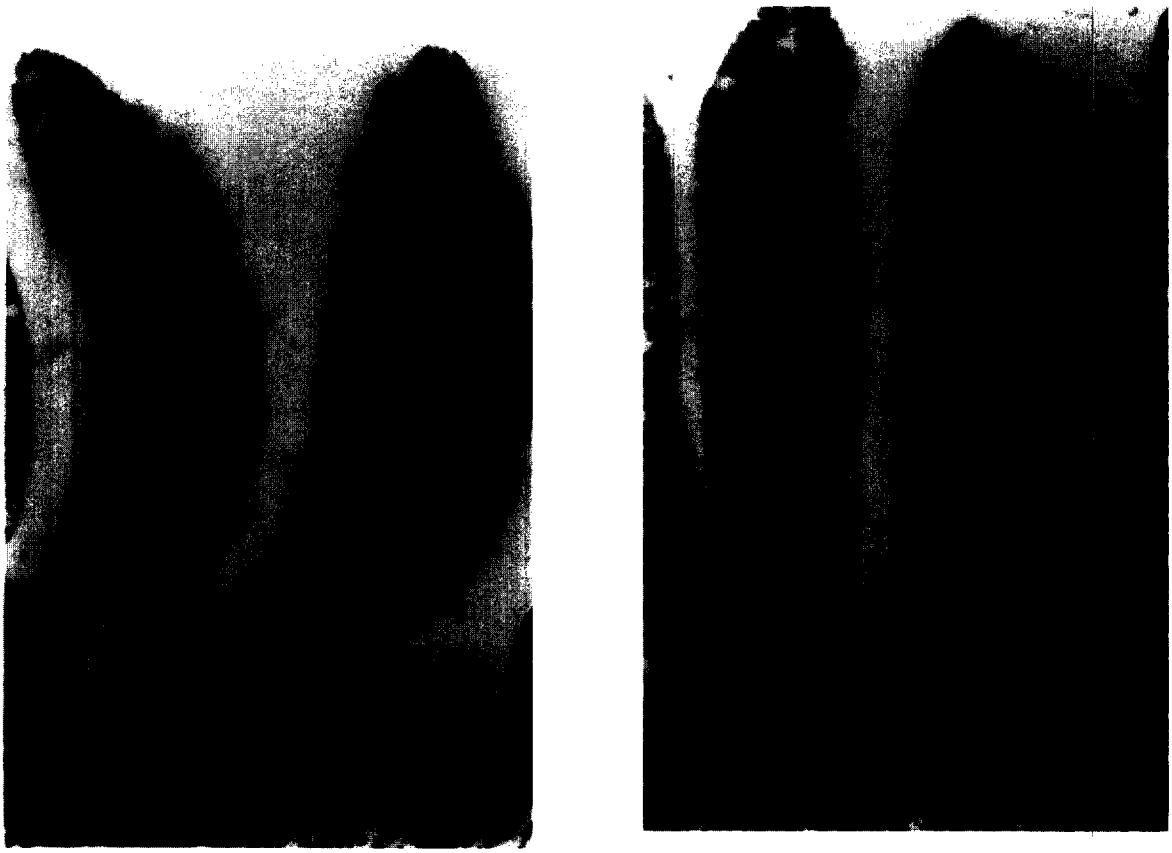

Fig. 5. (E and F).

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