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ORIGINAL CONTRIBUTION

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Synthesis and characterization of water-swellable α, β-polyasparthydrazide derivatives.

II. Hydrogels at low crosslinking degree as potential systems for anticancer drug release

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Abstract α, β-polyasparthydrazide (PAHy) was crosslinked by glutaraldehyde to form water-swellable materials possessing a three-dimensional molecular network. Different crosslinking degrees were prepared varying glutaraldehyde/PAHy ratio and samples containing 5-fluorouracil were obtained by incorporating the drug into the polymer networks during the crosslinking reaction. All samples were characterized by swelling tests, thermal, x-ray and SEM analysis. Their microstructure was observed through scanning electron microscopy. Furthermore, for samples containing the anticancer drug, *in vitro* release studies were performed in pH 7.4 buffer solution.

Key words Crosslinked α, β-polyasparthydrazide – hydrogels – 5-fluorouracil – prolonged drug release

Introduction

The synthesis of water swellable polymers has received increased attention in recent years. In particular, their use has been suggested as drug-delivery systems (DDS) when a prolonged and/or a controlled release of drugs is desired; i.e., whenever it is necessary to increase their bioavailability and to prevent their accumulation in not-target tissues [1]. This approach is useful in the chemotherapy of several kinds of cancer, because a great number of antitumoral drugs presents relevant toxicity [2–4].

In a previous work [5], we presented the synthesis and characterization of new hydrogels, suggested as DDS, made of polymeric networks based on α, β-polyaparthydrazide (PAHy) chemically crosslinked by glutaraldehyde. Varying the ratio of crosslinking agent to polymer, we obtained networks with different crosslinking degrees and we characterized them through water swelling measurements, thermal analysis, and scanning electron microscopy.

In this work we present the synthesis of new hydrogels of PAHy and glutaraldehyde at lower crosslinking degrees in order to obtain systems able to promote the drug delivery. These matrices were tested as DDS using 5-fluorouracil (5-FU) as model drug.

5-FU is the antineoplastic of choice in the treatment of colorectal cancer and it is used in combination with other drugs for breast cancer, pancreatic, gastric, testicular and prostatic carcinomas [6]. Nevertheless, 5-FU is very toxic, because it produces leukopenia, gastrointestinal ulceration, alopecia, vomiting, and nausea. It is reasonable to suppose that these side-effects may effectively decrease if the drug is incorporated in a slow releasing polymer matrix [7, 8].

Unloaded and 5-Fu loaded PAHy networks were characterized through water swelling tests, thermal, x-ray and SEM analysis. We also studied *in vitro* release profiles of the entrapped drug.

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Experimental

Chemicals

DL-aspartic acid, hydrazine hydrate, N,N-dimethylformamide (DMF), glutaraldehyde (50% aqueous solution), acetic acid and 5-fluorouracil (5-FU) were from Fluka (Switzerland).

PAHy preparation

α, β-polyasparthydrazide (PAHy) was prepared and purified as already reported [5, 9]. PAHy weight-average molecular weight determined by light scattering, was 37 700.

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 at regular intervals of time (15 min) and at the suitable quantity in agreement with the crosslinking ratio (X) defined as [10]:

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

In particular, we prepared samples with $X = 0.12$ (a), $X = 0.16$ (b) and $X = 0.20$ (c), adding 82.5 μl, 110 μl and 138.5 μl of glutaraldehyde, respectively.

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.

We have also prepared hydrogel matrices containing an anticancer drug at the same crosslinking ratios (samples a', b', and c'). In particular, we added an acetic solution of 5-fluorouracil (100 mg in 8 ml of 10 vol% acid) to the polymer aqueous solution before the addition of glutaraldehyde.

In order to verify the absence of a chemical reaction between 5-FU and glutaraldehyde under crosslinking conditions, a preliminary test was performed without PAHy and the reaction mixture was analysed by HPLC.

Residue content

Unreacted glutaraldehyde and PAHy content was determined in the mother liquor of the reaction and in the water of washing.

A sensitive colorimetric micromethod [11] revealed the absence of glutaraldehyde in all prepared samples.

We determined the unreacted content of PAHy 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%, 4.7%, and 1.6% w/w for samples with $X = 0.12$, $X = 0.16$ and $X = 0.20$, respectively.

Materials characterization

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

UV spectra were taken on a Perkin–Elmer 330 instrument equipped with a 3600 data station.

HPLC was carried out using a Varian 5000 Liquid Chromatograph equipped with a loop injector Rheodyne (fitted with a 10 μl loop), an Uvikon 722 LC detector, and a Hewlett Packard 3394 integrator. The samples of the preliminary compatibility test between 5-FU and glutaraldehyde were chromatographed on a reversed-phase C_{18} column (150 mm × 2 mm I.D.) with (10^{-4} M tetrabutylammonium phosphate plus 2×10^{-2} M potassium dihydrogen phosphate, pH 5.9) – methanol (95.5:4.5, v/v) as eluent at 0.3 ml/min. The eluate was monitored at 280 nm [12].

Swelling measurements were carried out by keeping the crosslinked samples in contact, through a Millipore 0.45 μm membrane, with bidistilled water or pH 7.4 buffer solution for 2 days at 37 ± 0.1 °C. Each experiment was performed in triplicate.

Thermal analysis was performed by means of a Perkin Elmer DSC 7B calorimeter. Samples were heated from 10° 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 aluminum 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.

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

X-ray diffraction pattern were recorded using an x-ray powder diffractometer (PW 1050, Philips). 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. In order to ensure valuable and significant patterns, we analyzed a sample with $X = 0.2$ containing 16% w/w of 5-FU. This sample was prepared following the same procedure as c' without washing with water.

Determination of drug entrapped in the hydrogels

25 mg of hydrogel loaded with 5-fluorouracil (samples a', b' and c') were extensively extracted at room temperature with 30 ml of water/methanol (1:1 v/v) mixture. The liquids of extraction were collected and evaporated under vacuum at 40 °C. The obtained residue dissolved in methanol was assayed using an UV spectrophotometer for the quantitative determination of the 5-FU (λ_{max} in methanol 264 nm, $E^{1\%}_{264\ nm} = 472.25$).

In vitro release at pH 7.4

100 mg of samples a', b' and c' were dispersed in 6 ml of 7.4 phosphate buffer (NaCl, Na_2HPO_4, KH_2PO_4) and introduced within dialysis tubes with a molecular cutoff of 3500. Each dialysis tube containing drug-loaded hydrogel powder was placed in a stoppered 150 ml Erlenmeyer flask containing 100 ml of the same phosphate buffer solution, equilibrated at 37 °C. Each flask was placed in a shaker bath which provided the temperature control and agitation (37 ± 0.1 °C, 100 r.p.m). All experiments were realized in sink conditions.

A preliminary test was performed in order to ensure the 5-FU concentration in the outer medium was equal, during the experiment, to the 5-FU concentration in the inner medium.

Then, at periodic intervals of time, aliquots of 3 ml were withdrawn from the receiver medium and assayed spectrophotometrically at 266 nm in order to determine the amount of drug released. The absorbance was converted to concentration using an extinction coefficient of 447 g/100 ml absorbance unit which was determined from calibration measurements. After each sampling, an equal volume of fresh buffer was added to the release medium. All measurements were carried out five times using five samples prepared under the same conditions. The results agreed with each other within 4% error.

Results and discussion

An outline of the steps involved in the synthesis of PAHy hydrogels was already reported [5] and it is also summarized in scheme 1.

In this work, we crosslinked PAHy molecules adding glutaraldehyde in the presence of acetic acid. We showed elsewhere [5] that both acetic and sulfuric acid act in the same way as catalysis agents in the PAHy crosslinking reaction, therefore we used only acetic acid because sulfuric acid is not desirable in pharmaceutical dosage forms [10]. Moreover, as already shown in the experimental section, in order to ensure a uniform distribution of the crosslinking agent in the reaction mixture, the initial temperature of the reaction was 2 °C. Then, to promote crosslinking the samples were taken to 50 °C for 6 h. All the prepared samples did not contain unreacted glutaraldehyde but they had small amounts (less than 8%) of unreacted polymer.

This modified procedure afforded hydrogel networks insoluble in water, common organic solvents (N,N-dimethylformamide, dimethylsulfoxide, methylene chloride, acetone, ethanol, methanol) and basic and acid aqueous solutions, like the systems described in the previous paper. This confirms that our prepared systems are made by individual chains of PAHy crosslinked by chemical bonds even when the ratio of crosslinking agent to polymer is significantly lower.

For the drug-loaded samples, the amount of 5-FU was 1.89, 2.20, and 1.75% w/w for the samples a', b', and c' respectively. We can observe that no significant differences in the loaded drug amount occurred by varying the X value.

The crosslinking density of unloaded and loaded matrices was investigated through swelling tests performed both in bidistilled water and in buffer solution (pH 7.4). Swelling effects were determined by the ratio:

$$Q = [(W_s - W_d)/W_s] \times 100 , \quad (2)$$

where W_s and W_d are the weight of the swollen and unswollen samples respectively. In Table 1, Q is reported as a function of X, i.e., the ratio of moles of glutaraldehyde to moles of PAHy repeating unit.

As a general consideration we observe that the swelling is lower for the samples treated in buffer solution than the corresponding samples treated in water. This effect can be related to the different osmotic pressure in these media. Furthermore, lower Q values were obtained for samples containing the drug with respect to the unloaded ones. Because the samples were kept in contact with aqueous media for 2 days before determining Q values and in these

Table 1 Swelling ratio for unloaded and loaded crosslinked samples.

Glutaraldehyde/PAHy ratio, X (mol/mol)		Swelling degree, Q (% w/w)	
		Bidistilled water	pH 7.4
0.12		74.70 ± 0.16	44.96 ± 0.62
0.16	unloaded	70.32 ± 0.34	33.17 ± 0.31
0.20		66.03 ± 0.25	29.57 ± 0.29
0.12		58.48 ± 0.09	31.45 ± 0.41
0.16	loaded	56.23 ± 0.43	28.38 ± 0.19
0.20		52.77 ± 0.39	26.71 ± 0.55

Table 2 Glass transition temperature for unloaded and loaded crosslinked samples.

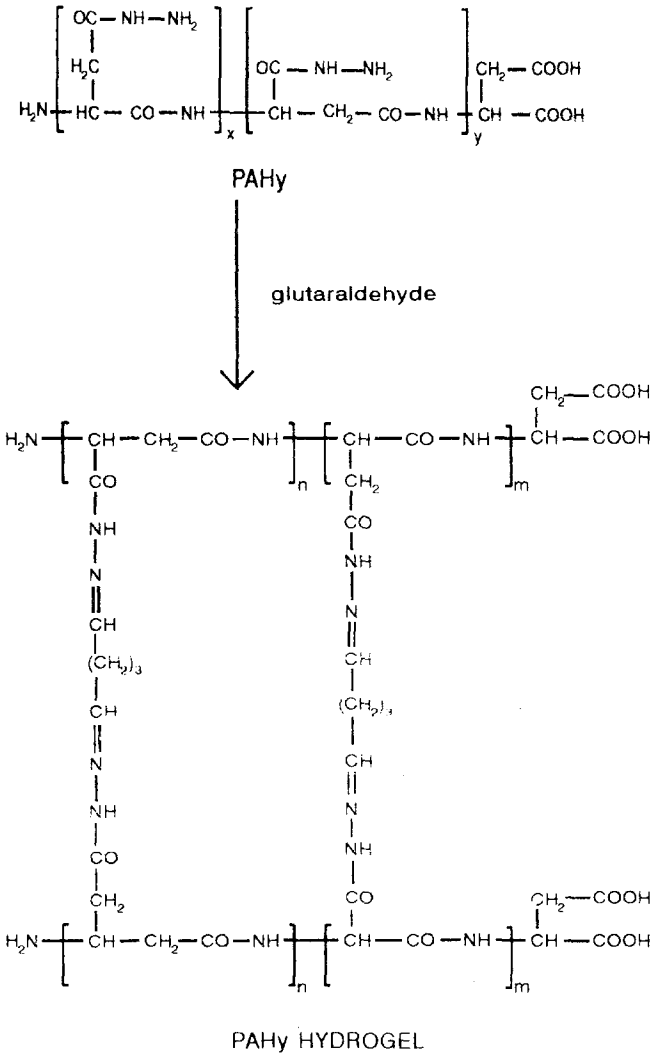
Glutaraldehyde/PAHy ratio, X (mol/mol)		Glass transition temperature T_g (°C)
0		71
0.12		61
0.16	unloaded	65
0.20		69
0.12		82
0.16	loaded	82
0.20		80

conditions more than 40% of the initial entrapped drug is still present in the matrix (see *in vitro* drug release profiles), we can attribute this effect to the interactions of the drug with the polymer polar groups which reduce the hydrophilicity of the material. Finally, for all experimental conditions Q decreases with X according to a continuous increase of the crosslinking degree.

Calorimetric analysis is in good agreement with swelling tests. In Table 2, we report the glass transition temperature, T_g, as a function of X.

For unloaded systems, T_g increases with X; on the contrary, drug-loaded samples present glass transition temperature significantly higher and almost constant with the crosslinking degree. This effect can be related, as discussed before to the presence of interactions among 5-FU and polar groups of the matrix, which hinders the mobility of the macromolecular chains, thus increasing the T_g value. Furthermore, this effect is marked enough to overcome the influence of the crosslinking degree; all the investigated samples show almost constant T_g values.

Polymer networks were analyzed by scanning electron microscopy. We did not observe significant differences in the structure between unloaded and loaded samples at the various crosslinking densities. As an example, we show in Fig. 1 an SEM micrograph for unloaded crosslinked PAHy, relative to $X = 0.16$, where microparticles of about 100 μm are seen.



Scheme 1

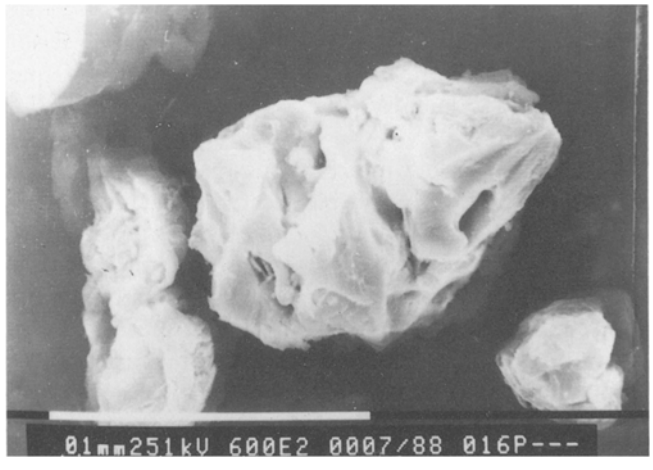


Fig. 1 Scanning electron micrograph of unloaded crosslinked PAHy ($X = 0.16$)

The morphological structure of the drug loaded in the polymer matrix was investigated through x-ray analysis. In Fig. 2, we report x-ray diffraction patterns of pure 5-FU and unloaded and loaded crosslinked PAHy.

We can see that the pure 5-FU is in the crystalline state; on the contrary, when dispersed in the PAHy network it is in the amorphous state like the unloaded matrix.

Finally, we carried out studies of the *in vitro* release of 5-fluorouracil from the prepared matrices in phosphate buffer solution at pH 7.4 and 37 °C.

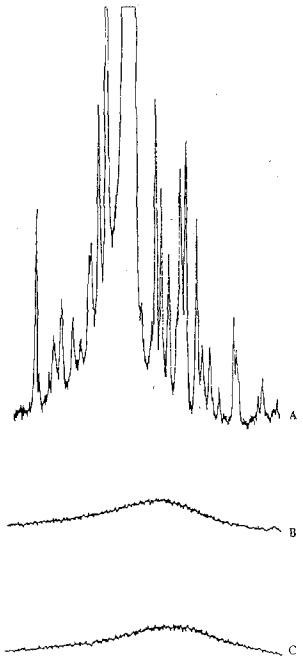


Fig. 2 X-ray diffraction patterns of: A) pure 5-fluorouracil; B) unloaded crosslinked PAHy; C) loaded crosslinked PAHy

Figure 3 depicts the drug release, expressed as the relative amount of drug (related to the entrapped total dose) released in a given time period.

Since the microparticles are not monodispersed and their geometry is not well-defined, we did not determine the mechanism of drug release (Fickian or non-Fickian) [4], but we performed only a phenomenological analysis of drug release. As we can see in Fig. 3, a bimodal release pattern occurs with an initial fast process followed by a slower one.

The initial burst effect is due probably to drug molecules adsorbed on the surface of particles, which present a large surface area of contact with the release medium due to their small dimensions.

The slower release process derives from the crosslinked structure of the matrix which decreases drug mobility. Values of $t_{50\%}$ and $t_{75\%}$ provide better evidence of the prolongation of drug delivery from the prepared hydrogels (see Table 3).

Because the period of the second process is longer than the initial burst effect, the prepared hydrogels offer a good potential as systems for the sustained release of bioactive

Table 3 Values of drug loading, $t_{50\%}$ and $t_{75\%}$ as a function of glutaraldehyde/PAHy ratio, X.

Glutaraldehyde/ PAHy ratio, X (mol/mol)	Drug loading (wt%)	$t_{50\%}$ (hours)	$t_{75\%}$ (hours)
0.12	1.89	6	102
0.16	2.20	17	168
0.20	1.75	122	211

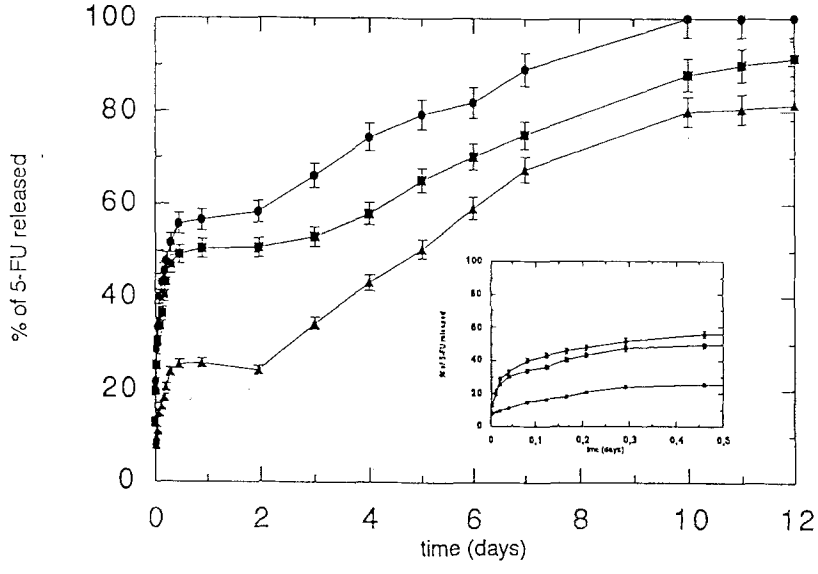


Fig. 3 Release of 5-fluorouracil from PAHy hydrogels at various crosslinking ratio X (● $X = 0.12$; ■ $X = 0.16$; ▲ $X = 0.2$) at 37 ± 0.1 °C in pH 7.4 buffer solution. The small inserted figure shows early times of the release, 0 to 0.5 days

molecules. In addition, the examination of release profiles shows that the amount of crosslinking agent influences the rate of drug release. In particular, as the crosslinking ratio X increases, the release profiles show a decrease of both the burst effect and the drug release rate. These results indicate that the increased number of crosslinks in the PAHy hydrogels depresses the mobility of 5-fluorouracil molecules [8]; infact, on increasing the crosslinking degree the mesh size of three-dimensional network is smaller with a reduced free space for drug mobility [13].

Concluding remarks

In this work, we present network systems prepared by reaction between α,β-polyasparthydrazide and glutaraldehyde at various crosslinking degrees. We show that these systems are able to swell in an aqueous medium and to incorporate bioactive agents, such as 5-fluorouracil. Swelling tests and thermal analysis evidence a peculiar effect of the crosslinking degree and x-ray analysis indicates that these matrices contain 5-fluorouracil in the amorphous state. Finally, *in vitro* release tests show that these systems are potentially useful to give a prolonged drug delivery.

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