Photo-Cross-Linked Hydrogels with Polysaccharide−Poly(amino acid) Structure: New Biomaterials for Pharmaceutical Applications

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The aim of this work has been the preparation and characterization of novel hydrogels with polysaccharide−poly(amino acid) structure having suitable physicochemical properties for pharmaceutical applications. In the first step, hyaluronic acid (HA) and α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA) have been derivatized with methacrylic anhydride (AMA), thus obtaining HA−AMA and PHM derivatives, respectively. In the second step, aqueous solutions of both these derivatives have been irradiated at 313 nm to obtain chemical hydrogels. The hydrogel obtained by irradiating for 15 min an aqueous solution containing 4% w/v of HA−AMA and 4% w/v of PHM resulted in the highest yield. Its swelling ability was dependent on the pH and nature of the external medium. Besides, this hydrogel undergoes a partial hydrolysis, especially in the presence of enzymes, such as esterase or hyaluronidase, but the entity of this degradation is lower than that observed for a hydrogel based on HA−AMA alone. The ability of this hydrogel to entrap drug molecules has been evaluated by using thrombin as a model drug. In vitro release studies and a platelet aggregation test demonstrated that the HA−AMA/PHM hydrogel is able to release thrombin in the active form, thus suggesting its suitability for the treatment of hemorrhages.

Introduction

Hydrogels are a class of biomaterials used in various fields ranging from coating devices and drug delivery to tissue engineering.[1−5] The physicochemical characteristics of hydrogels depend on the choice of starting polymers (or monomers) and the synthesis procedure.

In this article, we report the preparation and characterization of new covalently cross-linked hydrogels obtained through UV irradiation of α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA) and hyaluronic acid (HA) derivatives, both biocompatible polymers.

PHEA is a synthetic poly(amino acid) proposed as a plasma expander and starting material to prepare various drug delivery systems, such as macromolecular prodrugs, polycations, polymeric micelles, nanoparticles, and chemical hydrogels.[6−11]

HA is the major carbohydrate component of the extracellular matrix of the connective tissue, and it is found in various locations such as synovial fluid, vitreous humor, skin, testes, and so forth. It is believed to be crucial in promoting cell motility and proliferation, as well as being involved in the processes of morphogenesis, wound repair, inflammation, and cancer metastases.[12] The unique physicochemical properties and biological functions suggest HA as an attractive material to prepare biocompatible devices with applications in drug delivery, tissue engineering, and viscosupplementation.[13−16] However, systems based on HA alone undergo in vivo a rapid degradation by hyaluronidase (HAase), then they are not useful for a lasting action. An effective approach to increase the resistance to degradation by HAaase is the preparation of hydrogels based on chemically cross-linked HA. For example, HA has been auto-cross-linked, or bisepoxides, formaldehyde, and divinylsulfone have been used as cross-linking agents.[17−20] Recently, a derivative of HA with adipic dihydrazide was cross-linked with a macromolecular agent, such as poly(ethylene glycol)−propiondialdehyde. [21]

Now, we are interested in preparing novel chemical hydrogels based on HA and PHEA in order to obtain materials that undergo, potentially, a reduced degradation by HAase. Considering that the cross-linking of polymers by UV irradiation offers considerable advantages over the conventional chemical methods, we have prepared methacrylate derivatives of HA and PHEA, named HA−AMA and PHM, respectively, that have been photo-cross-linked at 313 nm. The chemical and physicochemical characterization of the obtained hydrogels has been reported.

Finally, since HA plays an important role in tissue repair, e.g., in the wound healing process, we have entrapped thrombin into a hydrogel based on HA−AMA and PHM. In fact, it is well-known that thrombin, when concentrated, has a very potent hemostatic or clotting effect on blood. It is also useful for local application to cuts or injuries; in surgery and in emergency medicine, it is used for local application in the control of minor oozing and orally to arrest gastrointestinal bleeding. However, for extensive hemorrhages, a matrix must be applied to hold the thrombin in place. Commonly, such a matrix is provided by various products, including fibrin foam, gelatin sponge, collagen sponge, and so forth.[22,23] With the peculiar properties of hydrogels based on HA−AMA and PHM taken into consideration, they appear potentially useful as matrixes to allow the release of thrombin after an oral or topical administration.

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Experimental Section

Materials. All the reagents used were analytical grade, unless otherwise stated. Methacrylic anhydride (AMA) D,L-aspartic acid, ethanolamine, *N*,*N*-dimethylformamide (DMF), and triethylamine (TEA) were provided by Fluka (Milano, Italy). Anhydrous *N*,*N*-dimethylacetamide (DMA) and Sephadex G-25 (50−150 μm) were purchased from Aldrich (Milano, Italy). Hyaluronidase from bovine testes type IV-S (1320 units/mg protein), esterase from porcine liver (250 units/mg protein), thrombin from human plasma (1000 NIH units/mg protein), and collagen from human skin, type I, were purchased from Sigma (Milano, Italy). Acetone, ethanol, 2-propanol, and D_2O (isotopic purity 99.9%) were purchased from Merck.

Hyaluronic acid (HA) sodium salt, M_w 1500 kDa, was a generous gift from SIFI (Catania, Italy). HA with a low weight-average molecular weight (employed in our experiments) was prepared by acidic degradation as reported by X. Z. Shu et al.[24] Briefly, 1% w/v solution of hyaluronic acid sodium salt was degraded in HCl solution (pH 0.5) at 37 °C for 24 h. After this time, the pH was corrected to 7.0 and the solution subjected to extensive dialysis by using Spectrapor Tubing with a molecular cutoff of 3500. After dialysis, the solution was freeze-dried, then the weight-average molecular weight of HA was determined by size-exclusion chromatography (SEC) analysis and resulted to be 174 kDa ($M_w/M_n = 1.75$).

α,β-Poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA) was prepared by reaction of a polysuccinimide (PSI), obtained by thermal polycondensation of D,L-aspartic acid, with ethanolamine in DMF solution, purified and characterized according to a procedure reported elsewhere.[25] The batch of PHEA used in the present study had a weight-average molecular weight of 57 kDa ($M_w/M_n = 1.79$).

Apparatus. Weight-average molecular weights of PHEA and PHM were determined by a SEC system equipped with a Water 600 pump, two Phenogel columns from Phenomenex (5 μm particle size, 10^3 Å and 10^4 Å pore sizes), and a 410 differential refractometer (DRI) as a concentration detector, all from Waters (Mildford, MA). The following conditions have been employed to determine molecular weights of PHEA and PHM: DMF + 0.01 M LiBr as a mobile phase, flow rate 0.8 mL/min, 50 °C. The molecular weights were evaluated using PEO/PEG standards (range 4−318 kDa).

Weight-average molecular weights of HA and HA−AMA were determined by the same SEC system reported above, but using a Universal column from Waters (particle size 5 μm). The following conditions have been employed to determine molecular weights of HA and HA−AMA: 200 mM phosphate buffer (pH 6.5)/MeOH 90:10 (v/v), flow rate 0.6 mL/min, 35 °C. The molecular weights were evaluated by using HA standards (range 100−800 kDa) from Hyalose (U.S.A.).

^{1}H NMR spectra were obtained with a Bruker AC-250 instrument operating at 250.13 MHz. Samples were solubilized in D_2O. FT-IR spectra were recorded as pellets in KBr in the range 4000−400 cm^{-1} using a Perkin-Elmer 1720 Fourier Transform Spectrophotometer. The resolution was 1 cm^{-1}. The number of scans was 100. UV irradiation was performed by using a Rayonet reactor equipped with a Rayonet Carousel motor assembly and 16 mercury lamps of 8 W at medium pressure with an emission at 313 nm. Centrifugations were performed with an International Equipment Company Centra MP4R equipped with an 854 rotor and temperature control. UV spectra were recorded by using a Cary 50 Scan UV−vis spectrophotometer from Varian. Platelet aggregation was evaluated with a BCT Dade Behring apparatus.

HA−AMA Synthesis. Hyaluronic acid was derivatized with methacrylic anhydride by varying a procedure reported elsewhere.[26] In particular, in our experiment, we have employed a batch of HA with a low weight-average molecular weight (174 kDa) and a low polydispersity index ($M_w/M_n = 1.75$), obtained as reported in the Materials section, whereas other authors have employed HA with a pronounced polydispersity, with its molecular weight between 780 and 2400 kDa.[26] Briefly, the polymer (HA) was dissolved in twice-distilled water at 4 °C to form a 2% w/v solution that was kept at 4 °C under continuous stirring overnight. An excess of methacrylic anhydride (AMA) (20-fold excess with respect to the moles of repeating unit of hyaluronic acid) was added while maintaining the pH between 8 and 9 (by adding 5 N NaOH) for the entire reaction time. The reaction was kept at 4 °C under continuous stirring for 24 h. After this time, the reaction mixture was precipitated in ethanol and centrifuged for 15 min, at 11 800 rpm and 4 °C. The product was recovered and purified by various washes with ethanol, centrifuging, from time to time, at 18 100 rpm and 4 °C for 10 min, then dried under vacuum. The methacrylated polysaccharide, named HA−AMA, was further purified by gel permeation chromatography on Sephadex G-25, whereas in the literature, only purification by washing with ethanol has been performed.[26] The change in the purification procedure was necessary to obtain a product with a molecular weight distribution more narrow than that of the sample purified by washing with ethanol alone (see Figure 1).

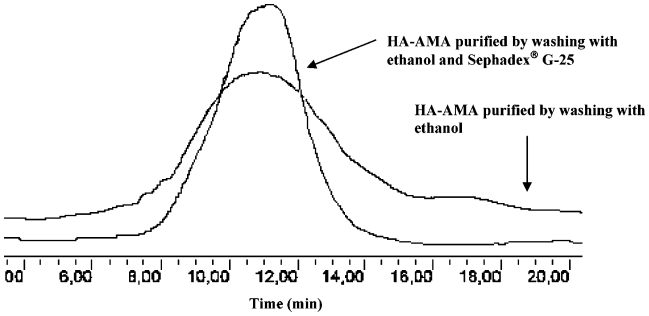


Figure 1. SEC profiles of the HA−AMA derivative purified by washing with ethanol alone or by washing with ethanol and gel permeation chromatography.

The purified product was recovered by freeze-drying with a yield of 99% (w/w) based on the starting HA and characterized by FT-IR and ^{1}H NMR analyses.

FT-IR spectrum (KBr) showed a broad band centered at 3400 cm^{-1} (ν_{as} OH + ν_{as} and ν_s NH) and bands at 1718 (ν_{as} COO), 1636 (amide I), 1408 (ν_s COO$^-$), 1302 (scissoring −C═C−H), 1164 and 1044 (C−O alcohol and ether stretching), and 950 (wagging −C═C−H + bending −OH) cm^{-1}.

^{1}H NMR spectrum (D_2O) showed: δ 1.96 (m, 3H, −CO−C(*CH*$_3$)═CH$_2$ belonging to AMA residues linked to HA and 3H, NH−CO−*CH*$_3$ belonging to HA), and 5.76 and 6.12 (2s, 2H, −CO−C(CH$_3$)═*CH*$_2$ belonging to AMA residues linked to HA).

The derivatization degree (DD%) was determined by H^1 NMR analysis, by comparing the integral related to protons at 5.76 and 6.12 (assigned to −CO−C(CH$_3$)═*CH*$_2$ belonging to AMA residues linked to HA) with the integral related to protons at δ 1.96 (considering only the contribution of NH−CO−*CH*$_3$ belonging to HA). The value of DD% resulted as 35 ± 3 mol %. The weight-average molecular weight of HA−AMA determined by SEC measurements was 180 kDa ($M_w/M_n = 1.81$).

PHM Synthesis. Derivatization of α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA) with methacrylic anhydride (MA) to obtain PHM copolymer was carried out in the organic phase (anhydrous DMA), using TEA as a catalyst, according to a procedure reported in a previous work.[27] Specifically, 2 g of PHEA were dissolved in 40 mL of anhydrous *N*,*N*-dimethylacetamide (DMA), then suitable amounts of triethylamine (TEA) and methacrylic anhydride were added, according to $X = 0.5$ and $Y = 0.5$, with X and Y defined as follows: X = moles of methacrylic anhydride/moles of PHEA repeating unit; Y = moles of TEA/moles of methacrylic anhydride

The reaction was kept at 40 °C under continuous stirring for 48 h. After this time, the reaction mixture was precipitated in 2-propanol and centrifuged for 10 min, at 11 800 rpm and 4 °C. The product was recovered, washed four times with 2-propanol and four times with acetone, and then dried under vacuum.

PHM copolymer thus obtained was dissolved in twice-distilled water and subjected to extensive dialysis using Visking Dialysis Tubing

Table 1. Yield (% w/w) Values of Hydrogels Obtained by Irradiating at 313 nm Various Aqueous Solutions Containing HA−AMA Alone or in the Presence of PHM as a Function of the Irradiation Time[a]

	yield % w/w			
irradiation time ($t = x'$, **min**)	hydrogels $A_{(t = x')}$ (from HA−AMA 4% w/v)	hydrogels $B_{(t = x')}$ (from HA-AMA2% w/v + PHM 2% w/v)	hydrogels $C_{(t = x')}$ (from HA-AMA 4% w/v + PHM 2% w/v)	hydrogels $D_{(t = x')}$ (from HA-AMA 4% w/v + PHM 4% w/v)
10	no gel	36.0 ± 0.9	60.5 ± 0.8	64.1 ± 0.4
15	27.5 ± 0.7	52.5 ± 1.1	65.0 ± 0.3	98.3 ± 0.5
30	31.3 ± 0.3	56.7 ± 0.7	70.7 ± 1.2	89.0 ± 0.4
60	75.0 ± 0.5	42.4 ± 0.5	63.5 ± 0.6	72.5 ± 0.7
90	70.3 ± 0.4	35.5 ± 0.4	52.5 ± 0.2	60.1 ± 0.6
120	67.2 ± 0.4	32.7 ± 0.5	48.0 ± 0.3	55.7 ± 1.2

[a] Values are means ± standard error ($n = 3$).

(18/32 in) with a molecular weight cutoff of 12 000−14 000. After dialysis, the solution was dried by freeze-drying. PHM was obtained with a yield of 97−99% (w/w), based on the starting PHEA, and characterized by FT-IR and ^{1}H NMR analyses. Spectral data agree with the values reported in a previous work.[27]

The derivatization degree (DD), determined by ^{1}H NMR analysis, resulted as 30 ± 1 mol %. The weight-average molecular weight of PHM determined by SEC measurements was 46.9 kDa ($M_w/M_n = 1.78$).

Preparation of Polymeric Networks. Aqueous solutions of HA−AMA in the presence of PHM were placed in Pyrex tubes, each equipped with an internal Pyrex piston in order to have samples of about 2 mm in thickness, then irradiated with a UV source under argon at 313 nm. Four different solutions have been prepared by varying the concentration of both polymers. In particular, the following aqueous solutions have been irradiated: HA−AMA 2% w/v + PHM 2% w/v (solution B), HA−AMA 4% w/v + PHM 2% w/v (solution C), and HA−AMA 4% w/v + PHM 4% w/v (solution D). For each sample, the reaction times were 10, 15, 30, 60, 90, and 120 min. Therefore, by irradiating the solutions B, C, or D, cross-linked samples named as hydrogels $B_{(t = x')}$, $C_{(t = x')}$, or $D_{(t = x')}$ have been obtained, respectively, where x' is the irradiation time, i.e., 10, 15, 30, 60, 90, or 120 min (see Table 1).

As a comparison, an aqueous solution of HA−AMA with a 4% w/v concentration (solution A) was irradiated at 313 nm for the same irradiation times, thus obtaining samples named hydrogels $A_{(t = x')}$, with x' being the irradiation time, in the range reported above (see Table 1).

After irradiation, hydrogels of HA−AMA/PHM or HA−AMA alone were purified by several washings with twice-distilled water, centrifuging, from time to time, at 11 800 rpm and 4 °C for 15 min and then recovered after freeze-drying. The values of yield % (w/w), reported in the Table 1, are based on the starting amounts of HA−AMA and PHM.

Swelling Studies. Aliquots, exactly weighed, of the hydrogel $D_{(t = 15')}$ were placed in a tared 5 mL sintered glass filter (∅, 10 mm; porosity, G3) and left to swell at 37 ± 0.1 °C by immersing the filter plus support in a beaker containing the swelling media, i.e., twice-distilled water, HCl 0.1 N (pH 1.0), and phosphate buffer solution pH 7.0 (NaCl 0.4 M, Na_2HPO_4 0.127 M, NaH_2PO_4 0.065 M). After a fixed time (range 5 min to 24 h), the excess of liquid was removed by percolation at atmospheric pressure. The filter was placed in a properly sized centrifuge test tube and then centrifuged at 3000 rpm for 5 min and weighed. The filter tare was determined after centrifugation with water alone. The weight swelling ratio (q) was calculated as follows:

$$q = W_s/W_d$$

where W_s and W_d are the weights of the swollen and dry samples, respectively.

Each experiment was carried out in triplicate, and the results were in agreement within ±3% standard error.

Chemical Degradation. Aliquots (30 mg/mL) of the hydrogel $D_{(t = 15')}$ were dispersed in 5 mL of degradation media, i.e., twice-distilled water, HCl 0.1 N (pH 1.0), citric acid buffer solution pH 6.3 (citric acid 0.030 M, Na_2HPO_4 0.150 M, NaCl 0.150 M), or phosphate buffer solution pH 7.0 (NaCl 0.4 M, Na_2HPO_4 0.127 M, NaH_2PO_4 0.065 M), and incubated at 37 ± 0.1 °C under continuous stirring (100 rpm). After 24 h, the samples were neutralized (when pH 1.0 solution has been employed) and centrifuged at 11 800 rpm at 4 °C for 10 min, and the supernatant was separated. For each sample, the remaining hydrogel was washed five times with twice-distilled water under continuous stirring for 1 h to extract soluble polymer degradation products and electrolytes entrapped in the network. Finally, the recovered hydrogel was dried by freeze-drying and weighed. The value of degradation percent has been determined as

$$\text{degradation \% (w/w)} = W_r/W_s \times 100$$

where W_r and W_s are the weights of the recovered (after the chemical degradation) and starting hydrogel, respectively.

In addition, each sample recovered after the chemical degradation has been characterized by swelling measurements in twice-distilled water, to evaluate the increase in the swelling as a consequence of the degradation. Each experiment was performed in triplicate, and the results were in agreement within ±2% standard error.

Enzymatic Degradation with Esterase or Hyaluronidase. Aliquots (30 mg/mL) of hydrogel $A_{(t = 15')}$ or hydrogel $D_{(t = 15')}$ were incubated with 2 mL of phosphate buffer solution pH 7.0 (NaCl 0.4 M, Na_2HPO_4 0.127 M, NaH_2PO_4 0.065 M) containing esterase (final enzyme concentration 100 U/mL) or 2 mL of citric acid buffer solution pH 6.3 (citric acid 0.030 M, Na_2HPO_4 0.150 M, NaCl 0.150 M) containing hyaluronidase (final enzyme concentration 100 U/mL), under continuous stirring (100 rpm) at 37 ± 0.1 °C for 24 h. Enzyme solutions were prepared immediately before the experiments. After 24 h, each sample was purified by using the same procedure used for the samples recovered after the chemical degradation. The value of degradation percent has been determined as

$$\text{degradation \% (w/w)} = W_r/W_s \times 100$$

where W_r and W_s are the weights of the recovered (after the enzymatic degradation) and starting hydrogels, respectively.

In addition, each sample recovered after the enzymatic degradation has been characterized by swelling measurements in twice-distilled water, to evaluate the increase in the swelling as a consequence of the degradation. Each experiment was performed in triplicate, and the results were in agreement within ±3% standard error.

Chemical Stability of Thrombin. Solutions of thrombin (200 U/mL) in twice-distilled water, HCl 0.1 N (pH 1.0) or phosphate buffer solution pH 7.0 (NaCl 0.4 M, Na_2HPO_4 0.127 M, NaH_2PO_4 0.065 M) were incubated at 37 ± 0.1 °C under continuous stirring (100 rpm) for various times (range 5 min to 6 h). Then, each solution was analyzed by UV spectrophotometry at 280 nm to determine the amount of thrombin recovered after each incubation time in the investigated media.

Effect of UV Irradiation on Thrombin. Solutions of thrombin in twice-distilled water (200 U/mL) were placed in Pyrex tubes equipped with an internal Pyrex piston in order to have samples of about 2 mm in thickness, then irradiated with a UV source under argon at 313 nm for 10, 15, 30, 60, 90, or 120 min. Then, each solution was analyzed by UV spectrophotometry at 280 nm to determine the amount of thrombin recovered after each irradiation time.

Preparation of Thrombin Containing HA−AMA/PHM Hydrogel. The preparation of the thrombin containing hydrogel (named hydrogel $D'_{(t=15')}$) was performed by using the same procedure employed to prepare the hydrogel $D_{(t\ =\ 15')}$ but dissolving, in this case, thrombin (200 U/mL) in the aqueous solution containing HA−AMA 4% w/w and PHM 4% w/w before irradiation. The amount of loaded thrombin resulted as 25 U/mg of hydrogel, as determined by an extensive extraction with twice-distilled water and subsequent evaluation by UV spectrophotometry at 280 nm.

Thrombin Release from HA−AMA/PHM Hydrogel. Aliquots (10 mg) of the thrombin-containing hydrogel $D'_{(t=15')}$ were dispersed in flasks containing HCl 0.1 N (pH 1.0) or phosphate buffer solution pH 7.0 (NaCl 0.4 M, Na_2HPO_4 0.127 M, NaH_2PO_4 0.065 M) and maintained at 37 ± 0.1 °C under continuous stirring (100 rpm). Sink conditions were maintained throughout the experiment. At suitable time intervals, samples were centrifuged for 2 min at 4 °C and 3000 rpm, then the supernatant was analyzed by UV spectrophotometry at 280 nm in order to determine the amount of thrombin released from the hydrogel.

Each experiment was carried out in triplicate, and the results were in agreement within ±3% standard error.

In Vitro Platelet Aggregation Test. Platelet aggregation experiments were performed on platelet-rich human plasma (PRP). Blood was obtained from three healthy human donors. PRP was prepared as follows: Venous samples were collected in tubes containing sodium citrate to prevent clotting, and they were centrifuged for 10 min at 750 rpm. PRP was mixed with phosphate buffer solution pH 7.0 containing thrombin released from hydrogel $D'_{(t=15')}$ as a function of time. For each sample, the aggregation was evaluated by measuring the decrease in light absorbance due to the formation of platelet aggregates and calculated as

$$\text{platelet aggregation \%} = (\text{Abs}_i - \text{Abs}_f/\text{Abs}_i) \times 100$$

where Abs_i and Abs_f are the values of initial (no platelet aggregation) and final (platelet aggregation) light absorbance, respectively.

The normal platelet aggregation function was verified by using collagen (2 mg/mL in phosphate buffer pH 7.0) as a positive control. Each experiment was carried out in triplicate, and the results were in agreement within ±3% standard error.

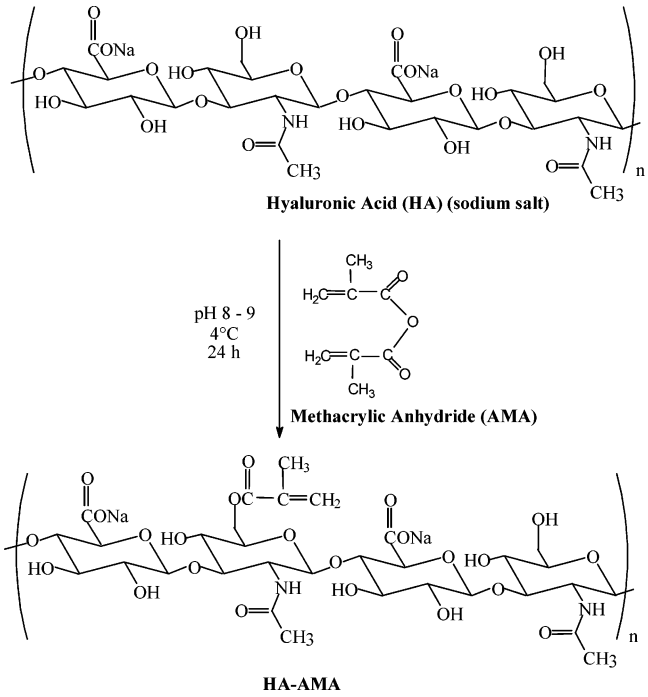
Results and Discussion

To obtain novel biomaterials useful for pharmaceutical purposes, hyaluronic acid (HA) and α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA) have been derivatized with methacrylic anhydride (AMA) to obtain photo-cross-linkable derivatives, named HA−AMA and PHM, respectively.

The derivatization of HA with AMA has been performed in aqueous solution (pH 8−9) in the presence of an excess of methacrylic anhydride for 24 h at 4 °C (see Experimental Section) (Scheme 1).

Since the reaction of derivatization of HA with AMA has been performed in alkaline medium (pH 8−9), this condition could cause degradation on the HA backbone. To prove that the alkaline medium did not cause alteration on the HA structure, SEC analysis has been performed on the starting HA and HA treated under the same conditions employed for the derivatization reaction but in the absence of AMA. The chromatograms

Scheme 1. Reaction between Hyaluronic Acid (HA) and Methacrylic Anhydride (AMA) to Give the HA−AMA Derivative



obtained for both samples confirmed that no degradation occurred in the HA backbone.

On the other hand, the low molecular weight and low polydispersity index of HA used in our experiment proved important parameters to obtain an HA−AMA derivative with a high derivatization degree (35 mol %) and a narrow molecular weight distribution (weight-average molecular weight of HA−AMA was 180 kDa and $M_w/M_n = 1.81$). On the contrary, the reaction reported by Smeds et al., using HA with a large molecular weight distribution (from 780 to 2400 kDa), allowed preparation of a methacrylated HA with a lower derivatization degree (3, 8, or 17 mol %) and a greater molecular weight distribution.[26]

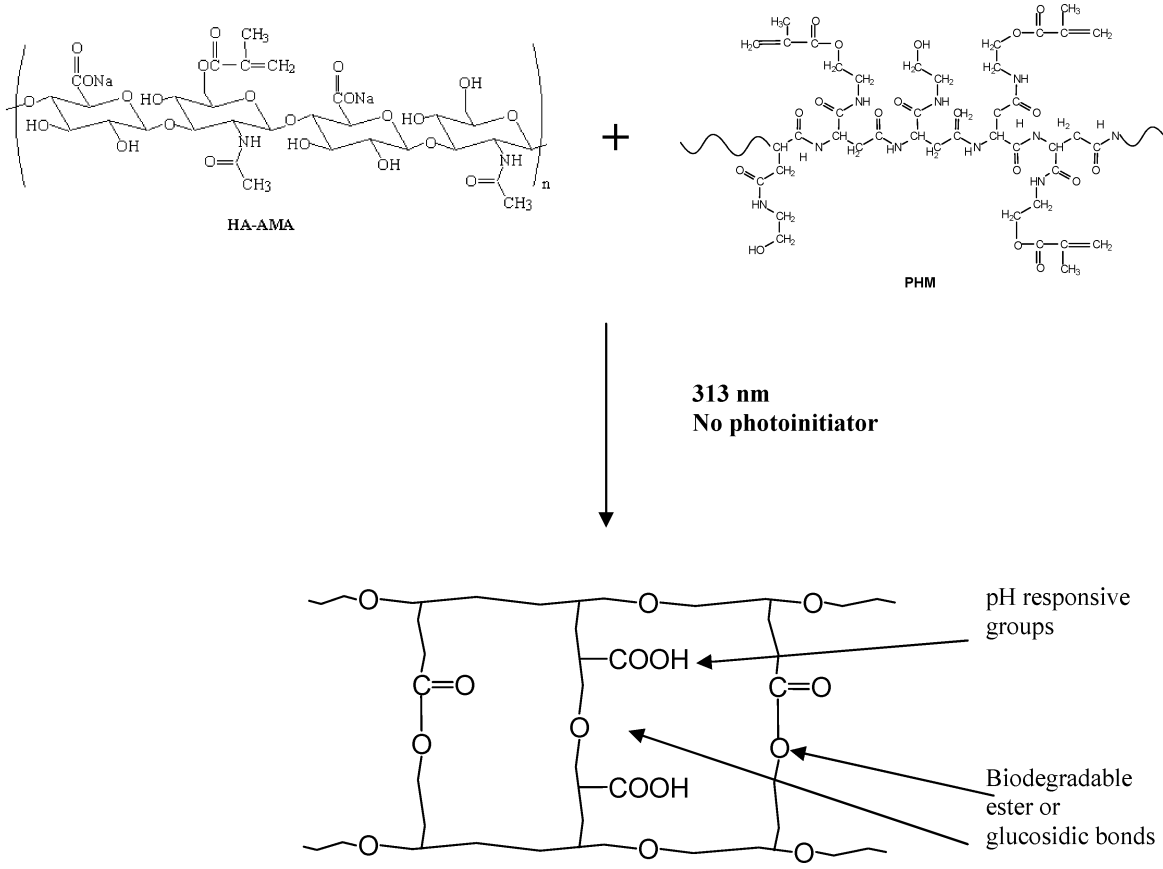
The success of the derivatization reaction has been confirmed by FT-IR and ^{1}H NMR analyses. In fact, FT-IR spectrum of HA−AMA, compared to that of HA, showed new bands at 1718 (ν_{as} COO), 1302 (scissoring −C═C−H), and 949 (wagging −C═C−) cm^{-1} due to the introduction of methacrylate residues. In effect, the band at 949 cm^{-1} is also present in the spectrum of starting HA due to the bending of the hydroxylic group, but its intensity increases when methacrylate residues are linked to HA.

^{1}H NMR analysis of HA−AMA has confirmed the presence of the methacrylate residues, and it allowed us to calculate the derivatization degree (see Experimental Section) that resulted as 35 ± 3 mol %.

The derivatization of PHEA with AMA to obtain a PHM copolymer with a derivatization degree of 30 ± 1 mol % has been performed as reported in a previous work (see Experimental Section).[27]

Preparation of Polymeric Networks. UV irradition is a convenient method to prepare chemical hydrogels for biomedical purposes, since it provides several advantages in comparison with conventional chemical cross-linking, such as simplicity (the synthesis is often carried out in a single step and without the presence of initiators), safety, and low cost.[28,29] In addition, UV irradiation is more selective than irradiation with other sources (e.g., by γ rays), and it involves only the chromophore groups

Scheme 2. Structure of the Network HA−AMA/PHM Obtained after UV Irradiation of Aqueous Solutions Containing HA−AMA and PHM



able to absorb radiation with suitable energy. For these reasons, aqueous solutions containing HA−AMA alone (as a comparison) or in combination with PHM have been irradiated at 313 nm, in the absence of photoinitiators. In particular, an aqueous solution containing HA−AMA alone (solution A, with a concentration of 4% w/v) has been irradiated at 313 nm for various times ranging from 10 to 120 min. Cross-linked samples named hydrogels $A_{(t = x')}$ have been obtained where x' is the irradiation time, i.e., 10, 15, 30, 60, 90, or 120 min.

Table 1 reports the yield of hydrogels $A_{(t = x')}$ recovered after each irradiation time.

It is evident that the shortest time of irradiation which produced an insoluble sample (gel) was 15 min; then, the yield of the gel phase increases by increasing the irradiation time until 60 min, but for longer times, it slightly decreases probably because of a partial photodegradation that occurs as a consequence of a prolonged exposure to UV light. Similar results have been obtained for other polymers when irradiated with UV rays.[30,31]

To investigate if the presence of another polymer, like PHM, combined with HA−AMA, affects the efficiency of the photocross-linking process as well as the physicochemical properties of the resulting hydrogels, aqueous solutions containing both these polymers have been irradiated at 313 nm in the absence of photoinitiators. In particular, the following aqueous solutions have been irradiated: HA−AMA 2% w/v + PHM 2% w/v (solution B), HA−AMA 4% w/v + PHM 2% w/v (solution C), and HA−AMA 4% w/v + PHM 4% w/v (solution D). For each sample, the reaction times were 10, 15, 30, 60, 90, and 120 min. Therefore, by irradiating solution B, C, or D, cross-linked samples named hydrogels $B_{(t = x')}$, $C_{(t = x')}$, or $D_{(t = x')}$ have been obtained, respectively, where x' is the irradiation time, ranging from 10 to 120 min.

Scheme 2 reports a schematic structure of the network produced after UV irradiation of HA−AMA and PHM.

HA-AMA/PHM networks are obtained without using photoinitiators; this could be explained by taking into consideration that both PHM and HA−AMA contain methacrylate residues (chromophore groups) very reactive toward UV rays, and then, after UV irradiation the formation of a biradical (through a $\pi \rightarrow \pi^*$ transition) in the vinyl group could give rise to a radical cross-linking in accordance with the radical polymerization of vinyl molecules.[32] Since in PHM and HA−AMA chains there are several chromophore groups in the excited state, it is possible to obtain a chemical cross-linking without the presence of photoinitiators. This is an important result, since it is well-known that photoinitiators are reactive molecules (such as benzophenone, acetophenone, and 2,2-dimethoxy-2-phenylacetophenone) whose traces can cause toxic effects in humans. As a consequence, the opportunity to obtain hydrogels of HA−AMA/PHM in the absence of initiators gives a potential biocompatibility to these systems.

FT-IR analysis has revealed that the cross-linking reaction happened. In fact, the FT-IR spectrum of a typical HA−AMA/PHM hydrogel (Figure 2 reports as an example the FT-IR spectrum of the hydrogel $D_{(t = x')}$ compared with those of un-cross-linked HA−AMA and PHM) shows the following principal features: a broad band centered at 3360 cm^{-1} (ν_{as} OH + ν_{as} NH and ν_s NH of HA−AMA and ν_{as} OH + ν_{as} NH of PHM); bands at 1718 (ν_{as} COO ester), 1655 (amide I of HA−AMA + amide I of PHM), 1542 (amide II of PHM), 1410 (ν_s COO^- of HA−AMA), 1169 and 1043 (C−O alcohol and ether stretching of HA−AMA), and 3360 cm^{-1}. The complete disappearance of the peak at 1302 cm^{-1} (scissoring −C═C−H) and a decrease in the peak at 950 cm^{-1} (the wagging −C═C−H disappears but the

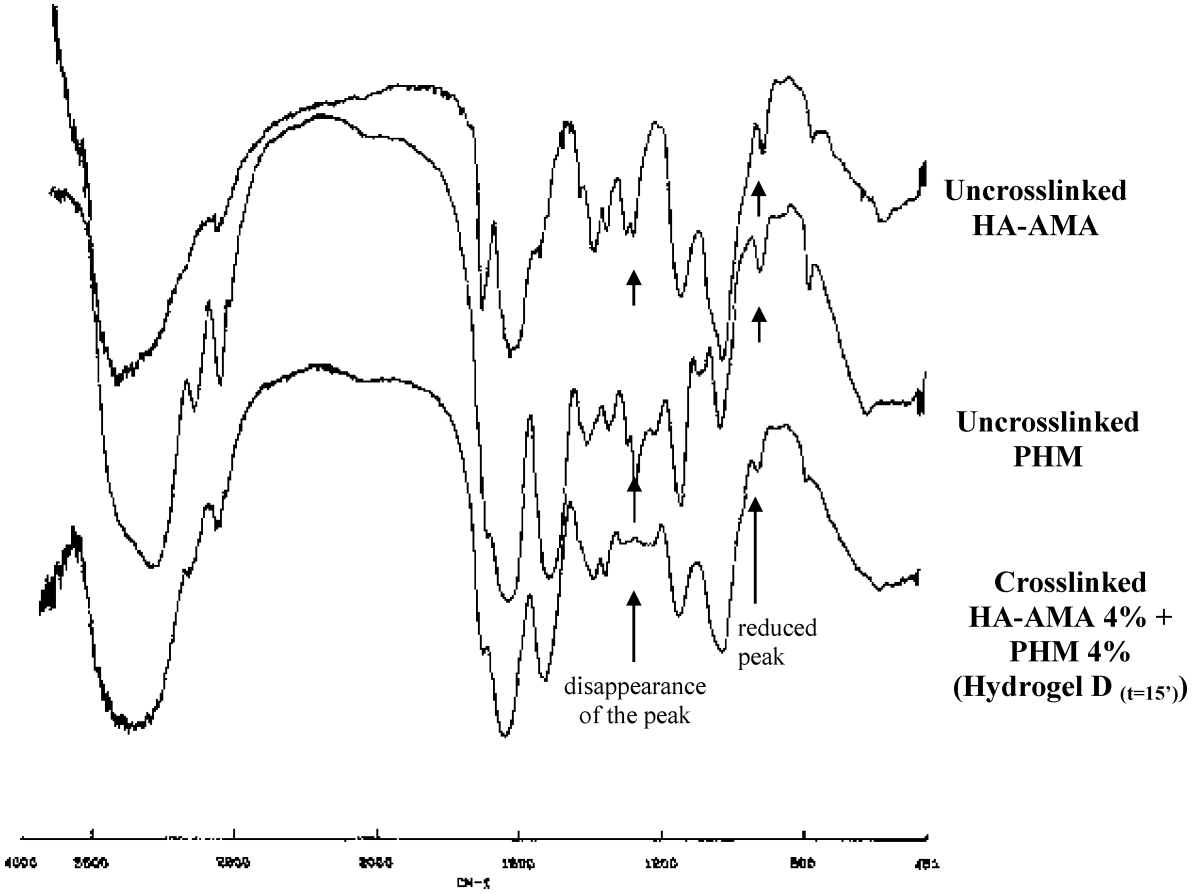


Figure 2. FT-IR spectra of the hydrogel $D_{(t = 15')}$, un-cross-linked HA−AMA and un-cross-linked PHM.

bending of −OH of hyaluronic acid remains) confirm that the cross-linking reaction induced by UV rays involves the opening of double bonds present in both HA−AMA and PHM, probably through the formation of free radicals which give rise to inter- and intrapolymeric cross-linked bonds.

The yields of the hydrogels $B_{(t = x')}$, $C_{(t = x')}$, and $D_{(t = x')}$ as a function of the irradiation time are reported in the Table 1.

It is evident that, when HA−AMA is irradiated in the presence of PHM, the gelation occurs just after 10 min of irradiation, even for solution B that contains a halved concentration of HA−AMA, thus indicating that the presence of the poly(amino acid) facilitates the cross-linking process. However, for each irradiated solution containing HA−AMA and PHM, like for HA−AMA alone, a bimodal trend of the yield versus irradiation time has been observed. In particular, when solutions B (HA−AMA 2% w/v + PHM 2% w/v) or C (HA−AMA 4% w/v + PHM 2% w/v) are irradiated, the maximum yield of the hydrogel is obtained after 30 min of irradiation, and then, this value decreases as the irradiation time increases, probably because of a partial photodegradation which occurs also when HA−AMA is combined with PHM. Instead, when solution D (HA−AMA 4% w/v + PHM 4% w/v) is irradiated, the maximum yield (98.3% w/w) is obtained just after 15 min of irradiation.

The obtained data indicate that the combination of HA−AMA 4% w/v and PHM 4% w/v is useful to obtain an interesting hydrogel for biomedical purposes, since after a short irradiation time (15 min), a transparent, soft, elastic, and homogeneous hydrogel is obtained (hydrogel $D_{(t = 15')}$) with an almost quantitative yield (about 98% w/w).

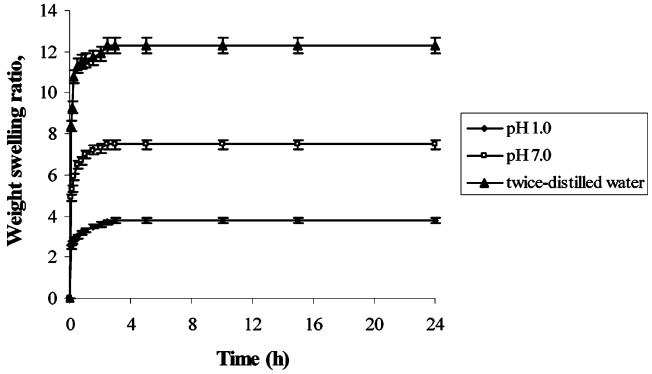


Figure 3. Dynamic swelling profiles for the hydrogel $D_{(t = 15')}$ in various media.

This hydrogel ($D_{(t = 15')}$) has been characterized with regard to the swelling behavior, degradability, and ability to entrap and release drug molecules.

The evaluation of the swelling ability is very important, since this property influences the absorption and diffusion of solutes, surface characteritics, and mechanical properties. Therefore, the weight swelling ratio, q, of the hydrogel $D_{(t = 15')}$, has been determined in various aqueous media (twice-distilled water, HCl 0.1 N solution, or phosphate buffer solution pH 7.0 (NaCl 0.4 M, Na_2HPO_4 0.127 M, NaH_2PO_4 0.065 M)) as a function of the time ranging from 5 min to 24 h.

As shown in Figure 3, the investigated hydrogel swells rapidly, but the entity of swelling depends on the interaction that occurs between the external medium and the polymeric network. In particular, the lowest swelling is observed in acidic medium (equilibrium q value = 3.8) where the undissociated

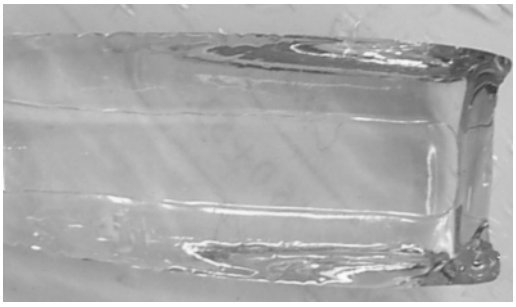


Figure 4. Photograph of the hydrogel $D_{(t = 15')}$ in the swollen state.

form of carboxyl groups of HA prevails, thus causing a lower affinity toward the aqueous medium. The highest swelling has been observed in twice-distilled water (equilibrium q value = 12.3), since in this medium, the dissociate form of carboxyl groups of HA prevails with a higher affinity toward the aqueous external medium. At pH 7.0, the dissociated form of the carboxyl group is also predominant, but due to the osmotic pressure of the medium, the swelling degree is lower (equilibrium q value = 7.5) than that observed in twice-distilled water. On the other hand, it is known that the presence of electrolytes, such as NaCl, reduces the electrostatic repulsions between HA chains, thus causing a decrease in the swelling ability.[33]

However, in all the investigated media, the swollen state of the hydrogel $D_{(t = 15')}$ appears transparent, soft, and rubbery (see Figure 4); this property allows one to minimize, potentially, the mechanical irritation to the surrounding tissues that will come into contact with this material.

With the consideration that in the hydrogel $D_{(t = 15')}$ there are ester and glucosidic bonds, both potentially degradable, chemical and enzymatic hydrolysis studies have been also performed. In particular, this sample has been treated at 37 °C for 24 h with various media, such as twice-distilled water, HCl 0.1 N (pH 1.0), phosphate buffer solution pH 7.0 (NaCl 0.4 M, Na_2HPO_4 0.127 M, NaH_2PO_4 0.065 M) alone or in the presence of esterase (100 U/mL), or citric acid buffer solution pH 6.3 (citric acid 0.030 M, Na_2HPO_4 0.150 M, NaCl 0.150 M) alone or in the presence of hyaluronidase (100 U/mL). The values of degradation percent (w/w) have been evaluated as described in the Experimental Section and reported in the Table 2. It is evident that the hydrogel $D_{(t = 15')}$ undergoes a negligible chemical degradation in twice-distilled water (3.5% w/w) and a partial chemical degradation in the presence of phosphate buffer solution pH 7.0 (9.6% w/w), citric acid buffer solution pH 6.3 (10.9% w/w), or HCl 0.1 N (18.2% w/w). This trend has also been confirmed by the values of weight swelling ratio, q, determined in twice-distilled water for the samples recovered after the hydrolysis (see values reported in Table 2). In fact, the increase in the value of q, in comparison with the value determined before hydrolysis (q = 12.3), is further evidence of the partial chemical degradation that occurs. These data are in accordance with the hydrolysis that poly(amino acid)s and HA undergo even in the absence of enzymes.[30,31,34] Obviously, a more evident degradation has been found in the presence of hyaluronidase or esterase. However, the degradation that HA−AMA/PHM hydrogel undergoes in the presence of hyaluronidase resulted as almost half of that occurring for the hydrogel $A_{(t = 15')}$ (40.9% w/w vs 80.1% w/w). This result is very important, since it confirms that the combination of HA−AMA with PHM is a useful approach to reduce the rapid degradation that hyaluronic acid undergoes in vivo, thus allowing one to prolonge its action. Also, the hydrolysis caused by esterase in hydrogel $D_{(t = 15')}$ is lower than that occurring for hydrogel $A_{(t = 15')}$ (38.2% w/w vs 65.0% w/w).

Table 2. Degradation (% w/w) and Weight Swelling Ratio (q) Values after Chemical or Enzymatic Hydrolysis of the Hydrogel $D_{(t = 15')}$ and Hydrogel $A_{(t = 15')}$[a]

medium of hydrolysis	degradation % (w/w)	weight swelling ratio, q (determined after 24 h in twice-distilled water)
	Hydrogel $D_{(t = 15')}$	
twice-distilled water	3.5 ± 0.5	13.0 ± 0.3
HCl 0.1 N (pH 1.0)	18.2 ± 0.2	17.6 ± 0. 4
citric acid buffer pH 6.3	10.9 ± 0.2	15.3 ± 0.5
phosphate buffer pH 7.0	9.6 ± 0.3	14.2 ± 0.4
citric acid buffer pH 6.3 + hyaluronidase (100 U/mL)	40.9 ± 0.4	27.5 ± 0.2
phosphate buffer pH 7.0 + esterase (100 U/mL)	38.2 ± 0.5	24.8 ± 0.1
	Hydrogel $A_{(t = 15')}$	
citric acid buffer pH 6.3 + hyaluronidase (100 U/mL)	80.1 ± 0.2	55.7 ± 0.2
phosphate buffer pH 7.0 + esterase (100 U/mL)	65.0 ± 0.3	43.6 ± 0.2

[a] Values are means ± standard error (n = 3).

The greater resistance to the enzymatic hydrolysis showed by hydrogel $D_{(t = 15')}$ could be due to the formation of a more compact network with a lower permeability to the enzymes.

Finally, the excellent swelling ability that hydrogel $D_{(t = 15')}$ showed in the investigated media suggests its ability to entrap water-soluble molecules and afterward to release them in physiological fluids. To confirm this hypothesis, this sample has been loaded with thrombin, chosen as a model of a protein drug. On the other hand, the preparation of a thrombin containing HA−AMA/PHM hydrogel could have a potential application in wound healing, since it is well-known that thrombin is a physiological protein involved in the coagulation process and HA has a multifaceted role in the mediation of the tissue repair.[35−37]

To prepare this sample, thrombin has been dissolved in the aqueous solution containing HA−AMA 4% w/w and PHM 4% w/w before irradiation. After irradiation at 313 nm for 15 min and purification of the obtained network (named hydrogel $D'_{(t=15')}$), the amount of loaded thrombin resulted as 25 U/mg of hydrogel.

Obviously, a preliminary test has been performed to verify the absence of alteration on the thrombin caused by UV irradiation (see Experimental Section). Experimental data revealed that, until 30 min of irradiation, no difference in the UV spectrum and no decrease in the thrombin concentration have been found in comparison with the starting thrombin. This result suggests that it is possible to incorporate thrombin in HA−AMA/PHM hydrogel during the irradiation process for 15 min.

Besides, preliminary studies have also been performed to evaluate the chemical stability of the thrombin in the media employed for the release experiments, i.e., HCl 0.1 N (pH 1.0, simulated gastric fluid) or phosphate buffer solution pH 7.0 (NaCl 0.4 M, Na_2HPO_4 0.127 M, NaH_2PO_4 0.065 M, simulated intestinal or physiological fluid). As a comparison, the chemical stability of the thrombin has also been evaluated in twice-distilled water. Experimental data showed that no degradation occurs in twice-distilled water and in phosphate buffer solution pH 7.0 in the investigated time, whereas in HCl 0.1 N solution, a 10% w/w of degradation occurs after 2 h, considered the mean

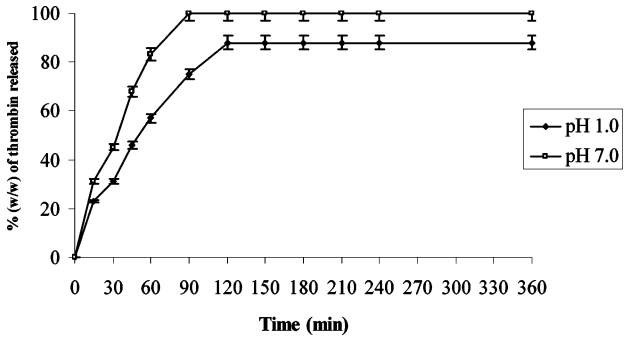


Figure 5. Thrombin release at 37 °C from the hydrogel $D'_{(t\,=\,15')}$ in HCl 0.1 N (pH 1.0) or phosphate buffer solution pH 7.0.

time of transit in the stomach. These results have been considered to explain the release profiles reported in Figure 5 as the percent of drug (related to the entrapped total dose) delivered as a function of the time in HCl 0.1 N solution and in phosphate buffer solution pH 7.0.

It is possible to observe that the release of thrombin is complete after about 90 min in phosphate buffer solution pH 7.0, whereas in acidic medium, about 88% of the drug is released within 120 min. However, the percentage of drug detected at pH 1.0 agrees with the small degradation (about 10% w/w) that thrombin undergoes in this medium; therefore, the release of thrombin in acidic medium can be also considered complete.

Obviously, it was necessary to confirm that thrombin released from the hydrogel $D'_{(t=15')}$ maintains its activity. This evaluation is very important, since even if during the UV irradiation for 15 min thrombin does not undergo a chemical modification, conformational changes could occur during the preparation of the sample or in the release phase that could alter its activity. To evaluate the activity of thrombin released from the hydrogel, an in vitro platelet aggregation test has been performed. In particular, platelet aggregation experiments have been performed on platelet rich human plasma (PRP) kept in contact with thrombin released from hydrogel $D'_{(t\,=\,15')}$ as a function of the release time (see Experimental Section). For each sample, we have evaluated the decrease in light absorbance due to the clumping of platelets caused by thrombin. The obtained results have been compared with those obtained by using collagen (2 mg/mL in phosphate buffer pH 7.0) chosen as a positive control. In fact, it is known that platelets adhere naturally to collagen and they are stimulated to release substances that promote further aggregation.[22] For this reason, collagen is commonly used as a reagent to evaluate the platelet aggregation function.[23]

Table 3. Platelet Aggregation Values (%) for Thrombin Released from the Hydrogel $D'_{(t=15')}$ as a Function of the Release Time in Phosphate Buffer Solution pH 7.0 and for Collagen (2 mg/mL in PBS pH 7.0) Chosen as a Positive Control[a]

time of release of thrombin from the hydrogel $D'_{(t=15')}$ (min)	platelet aggregation %
5	28.4 ± 2.4
10	54.3 ± 1.8
15	72.6 ± 1.5
30	84.6 ± 1.1
60	85.3 ± 0.8
90	85.9 ± 1.3
control: collagen (2 mg/mL)	83.5 ± 1.3

[a] Values are means ± standard error ($n = 3$).

Figure 6 shows, as an example, the platelet aggregation pattern for the collagen (plot A) and thrombin released from hydrogel $D'_{(t=15')}$ (plot B) in phosphate buffer solution pH 7.0 after 90 min of incubation. It is evident that the released thrombin, like the collagen, causes a decrease in light absorbance due to the formation of platelet aggregates.

Table 3 reports the values of platelet aggregation percent calculated for each sample as a function of the release time in phosphate buffer solution pH 7.0 and compared with the value obtained by using collagen (2 mg/mL in phosphate buffer pH 7.0).

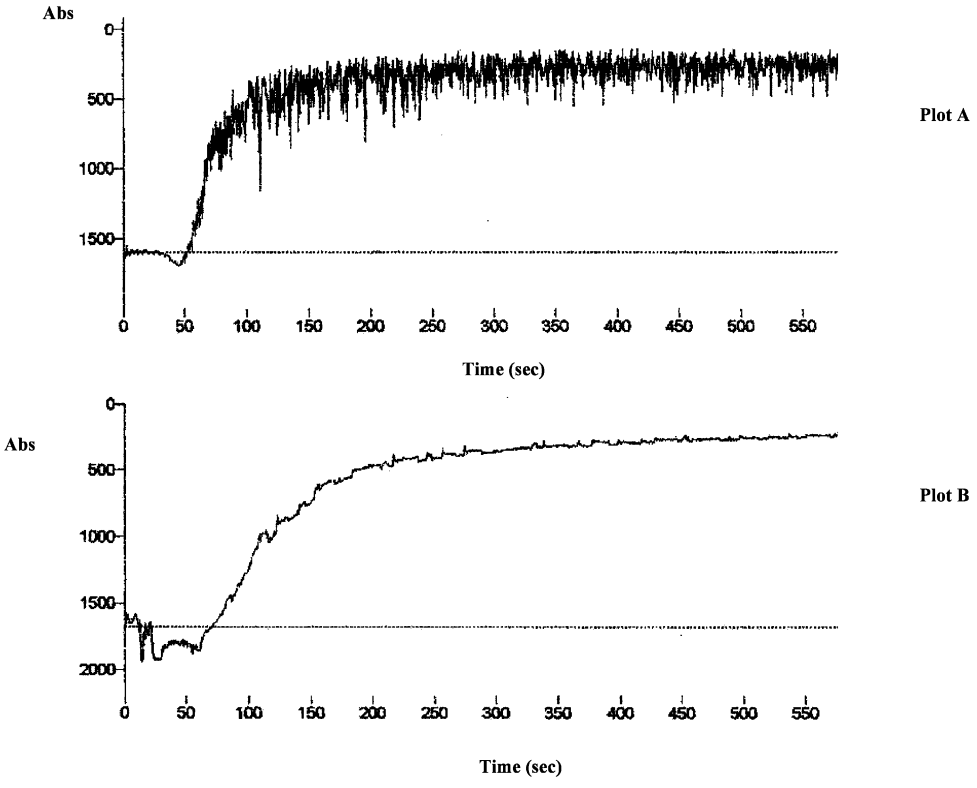


Figure 6. Platelet aggregation pattern for collagen (plot A) and thrombin released from the hydrogel $D'_{(t\,=\,15')}$ (plot B).

It is evident that the activity of thrombin released from the hydrogel $D'_{(t=15')}$ is similar to that of the control, particularly after a release time of 30 min. Analogous results have been obtained for thrombin released from the hydrogel $D'_{(t=15')}$ in HCl 0.1 N; obviously in this case, before the aggregation test, solutions have been neutralized (data not reported).

The obtained results suggest that the HA−AMA/PHM hydrogel loaded with thrombin can be a potential candidate to staunch nasal, vaginal, or gastrointestinal hemorrhages caused by various pathologies or as a consequence of surgical operation, as well as to heal bleeding wounds on skin. On the other hand, the cross-linked structure of the HA−AMA/PHM hydrogel could promote clot formation, and the presence of the hyaluronic acid in this network could facilitate tissue repair, with it being implicated in the wound healing process.[35−37]

Conclusions

New hydrogels with polysaccharide−poly(amino acid) structure have been prepared by UV irradiation of aqueous solutions containing methacrylated derivatives of hyaluronic acid (HA) and α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA), named HA−AMA and PHM, respectively. Photo-cross-linking has been performed at 313 nm, in the absence of photoinitiators, by varying the polymer concentration and the irradiation time. The hydrogel obtained by irradiating for 15 min an aqueous solution containing 4% w/v of HA−AMA and 4% w/v of PHM resulted in the highest yield (about 98% w/w) and a remarkable swelling ability, dependent on the pH and the nature of the external medium. In vitro studies suggested that this hydrogel undergoes a negligible hydrolysis in twice-distilled water, a partial hydrolysis in the presence of HCl 0.1 N, phosphate buffer solution pH 7.0, or citric acid buffer solution pH 6.3, and a more evident hydrolysis in the presence of enzymes, such as esterase or hyaluronidase. However, the percentage of enzymatic degradation of HA−AMA/PHM hydrogel is lower than what occurs for a hydrogel based on HA−AMA alone, thus allowing explanation of a prolonged action in the application site. The potential suitability of the prepared sample for the treatment of external or internal hemorrhages has been evaluated by loading thrombin during the cross-linking process. In vitro release studies performed in simulated gastrointestinal and physiological fluids, as well as platelet aggregation tests, have demonstrated the ability of the investigated HA−AMA/PHM hydrogel to release thrombin in the active form, with its activity being similar to that of collagen, chosen as a positive control.

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