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(57) **Abrégé/Abstract:**

Compositions and products based on the chemical crosslinking of hyaluronic acid with a polyfunctional polymer having a protein-like structure, bearing hydrazido pendent groups along the polymeric chain. The polymer is preferably, alpha-beta--polyaspartylhydrazide, a biocompatible macromolecule. The materials obtained after crosslinking, specifically hydrogels, undergo a reduced chemical and enzymatic degradation, unlike the starting hyaluronic acid, and they can be used to prepare systems for applications in the biomedical and pharmaceutical field.



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(57) Abstract: Compositions and products based on the chemical crosslinking of hyaluronic acid with a polyfunctional polymer having a protein-like structure, bearing hydrazido pendent groups along the polymeric chain. The polymer is preferably, alpha-beta-polyaspartylhydrazide, a biocompatible macromolecule. The materials obtained after crosslinking, specifically hydrogels, undergo a reduced chemical and enzymatic degradation, unlike the starting hyaluronic acid, and they can be used to prepare systems for applications in the biomedical and pharmaceutical field.

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**HYDROGELS OF HYALURONIC ACID AND ALPHA, BETA-
POLYASPARTYLHYDRAZIDE AND THEIR BIOMEDICAL AND
PHARMACEUTICAL USES**

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SPECIFICATION

The present invention relates to new hydrogels of hyaluronic acid and alpha,beta-polyaspartylhydrazide and their applications in the biomedical and pharmaceutical field. In particular, this invention concerns products and compositions based on chemical crosslinking of hyaluronic acid with a multifunctional biocompatible polymer with a protein-like structure bearing hydrazido pendent groups along the polymeric chain. Following this crosslinking it is possible to obtain materials characterized by a strong resistance to chemical and enzymatic degradation, unlike the starting hyaluronic acid, that can be utilized to prepare systems for biomedical and pharmaceutical applications.

As it is known, hydrogels consist of natural polymers or their derivatives, synthetic polymers or combinations of natural and synthetic polymers, whose molecules, interacting by electrostatic forces or chemical linkages, form hydrophilic crosslinked polymers, able to take up water in an amount ranging from 10-20% to several hundreds of times their dry weight. Due to their hydrophilic properties, together with their potential biocompatibility, hydrogels attract an increasing interest in the pharmaceutical and biomedical field.

In particular, hydrogels are ideal candidates in the preparation of tissue engineering matrices, with the aim to heal or reconstruct *ex novo* damaged, diseased or deteriorated human tissues or organs. Tissue engineering is actually a new science dealing with the development of technologies useful to obtain a regeneration of human damaged tissues or their complete reproduction. To allow growing and differentiation of repairing tissue cells (e.g. fibroblasts for cutaneous tissue, chondrocytes for cartilaginous tissue, osteoblasts for bone tissue, etc.) and subsequent deposition of extracellular matrix (ECM, that is the principal component of all tissues), three-dimensional structures are needed, specifically porous systems, wherein cells can find an envi-

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ronment similar as much as possible to the natural one and they can adhere, multiply and deposit new ECM. These three-dimensional structures are usually referred to as "scaffolds", and it is already known that hydrogels are particularly suitable to constitute similar matrices for tissue engineering, considering their several advantages, for an example, compared to such hydrophobic structures. In particular, these advantages include the ability of hydrogels to allow a good fluxing of nutrients to cells and refluxing of products outside the cells, their usual biocompatibility and progressive bio-reabsorption and their ability to easily incorporate peptide ligands for cellular adhesion by covalent or physical linkages, in order to stimulate adhesion, proliferation and growing of the cells inside the hydrogel matrix. The latter advantage makes hydrogels different, for example, from hydrophobic polymers applied for the same purpose, such as PLGA (polylactic-co-glycolic acid). On the other hand, hydrogels can suffer the disadvantage of a low mechanical resistance that can reduce handling, or they may be also be difficult to sterilize.

The tissue engineering applications include the opportunity to use biodegradable sponges or biodegradable films for articular cartilage regeneration, or to protect and support healing of wounds caused by trauma (i.e. burns) or diseases (diabetes, AIDS). In this case the application on wounds can support a faster regenerative activity of fibroblasts that, adhering on the scaffold, will synthesize more rapidly new ECM and heal the wound. As an alternative, the scaffold can be at first utilized *in vitro* to create a real artificial derma that can be subsequently used to cover the wound and to perform its function. Similar skin substitutes perform a temporary cover able to reduce the exudates loss from the wound and the infection risk. The scaffold, when opportunistically loaded with a drug, can also perform a drug delivery function, supporting as an example the prolonged release of antibiotics or growth factors, depending on type of wound. As an example, several products obtained from collagen based scaffolds are already marketed as skin substitutes (in particular a cellular bilayer obtained by growing of fibroblasts and keratinocytes on collagen scaffolds, marketed with the trade mark ApligrafTM).

Another biomedical application of hydrogels, having great interest for its

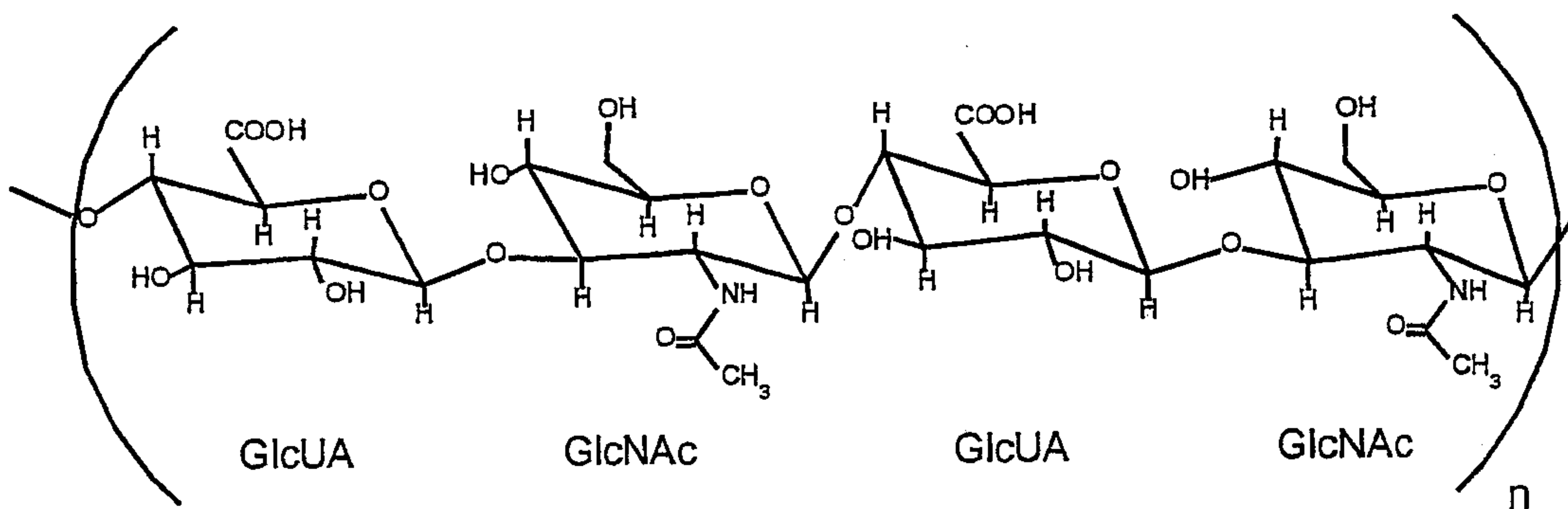
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potentialities, is the use in prevention of post-surgical adhesion. As it is known, the post-surgical adhesions consist in the formation of fibrous sutures between two opposite tissue surfaces, resulting from the trauma that tissues suffer during surgical activities. These post-surgical adhesions are a prominent problem not only in cardiovascular surgery but also in gastrointestinal and gynaecological surgery, where they can produce intestinal obstruction, infertility and pelvic pain. The most used method to prevent this bothersome problem is the positioning of biocompatible materials as physical barriers between tissues in touch, suitable to favour their complete separation and able to remain in place during the whole critical post-surgical period.

Among the barriers already marketed for this use, the barrier realized using expanded polytetrafluoroethylene (PrecludeTM, W. L. Gore, Flagstaff, AZ), proposed as pericardial substitute, has a good clinical efficacy but is not completely bioreabsorbable, and therefore a second surgical operation is necessary for its removal. Regenerated cellulose-based barriers (IntercedeTM, Johnson & Johnson Medical Inc., Arlington, TX), are also used but they have shown a good efficiency only if used avoiding blood contact. However at present the most widely marketed antiadhesion barrier is Seprafilm[®] (Genzyme, Cambridge, MA); it is a material based on hyaluronic acid modified with carboxymethylcellulose. Despite its favourable characteristics, this material shows a reduced ease of handling, it is brittle and not very elastic, and it is characterized by quite fast reabsorption times in spite of the chemical modification produced on hyaluronic acid.

As known, an excellent candidate for this and other biomedical and pharmaceutical applications reported below is hyaluronic acid, a linear polysaccharide with a high molecular weight composed of alternating units of D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc), whose chemical structure can be represented by the following formula:

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where two subsequent disaccharide units are shown, and where the number
 5 n of couples of repeating units is such that the polysaccharide molecular weight is between 50.000 and millions of dalton.

Hyaluronic acid (HA) is extensively diffused in animal tissues, being a
 fundamental component of the extracellular matrix, where it acts regulating the
 cellular proliferation and differentiation. It takes part in several important bio-
 10 logical processes, such as cellular mobility and tissue healing; it regulates the
 inflammatory response and acts as a "scavenger" of free radicals. It has been
 demonstrated that HA is involved in tumour growth by interacting with specific
 receptors placed on the cell surface: this may explain the recent interest that
 this polymer has caused for a possible application as a soluble carrier in the
 15 production of new macromolecular prodrugs with an antitumoral activity.

Generally, hyaluronic acid is applied in viscosupplementation – both
 as pharmaceutical agent and as surgical aid – in the ophthalmic field, and it is
 widely tested in viscosupplementation to relieve articular pains caused by
 osteoarthritis of different nature, as a lubricating agent administered by intra-
 20 articular injections, it is applied as a "drug delivery system", i.e. as a carrier
 for the prolonged or controlled release of drugs, and not least, as a cosmetic
 agent. Besides, in view of its biological functions as above mentioned, HA is
 reported to facilitate, by injection, the nerves regeneration, and when it is
 placed on wounds it facilitates tissue regeneration. Besides, its characteristics
 25 of fast cutaneous permeability and epidermic retention can extend the half-life
 of drugs administered with it, for example in pharmaceutical devices applied

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as transdermal administration.

It is evident from the foregoing that biomaterials based on hyaluronic acid, due to its biocompatibility property, are highly suitable as support materials for tissue engineering, useful to facilitate cellular growth processes both *in vivo* and *in vitro*, as well as barriers in the prevention of post-surgical adhesions. Nevertheless, the use of HA alone shows a disadvantage in that it results in scaffolds not very elastic and brittle. Besides, said scaffolds are provided with surfaces too hydrophilic to favour adhesion and cellular differentiation. Especially for this reason it has been several times proposed to modify HA by mixing or crosslinking it with biocompatible polymers as collagen or gelatine, or with synthetic polymers such as polylysine; or chemically modifying HA with hydrophobic groups.

The chemical modification of the polysaccharide molecule of HA by introducing pendent functional groups has as a further target, i.e. to obtain prolonged release pharmaceutical systems (drug delivery systems) where the drug can be carried to the action site while being chemically linked to the polysaccharide carrier chain, and can be released from it in a manner and with times capable to increase its bioavailability.

Another critical drawback of hyaluronic acid used as here considered is its low residence time *in vivo*, due to its fast chemical and enzymatic degradation. In fact, it is degraded by hyaluronidases (HAase), ubiquitous enzymes distributed in human cells and serum, as well as it undergoes a chemical hydrolysis even in the absence of enzymatic activity. For this reason, if on one hand for some applications here reported, the fact that this material could be reabsorbed after performing its function is a required and favourable characteristic, on the other hand it is important that the degradation is not so fast to excessively reduce the product half-life or permanence in the action site.

Therefore, there is a clear interest in developing hyaluronic acid based materials, capable to exploit the advantageous characteristics of this biocompatible material, specifically for the biomedical and pharmaceutical applications reported above, but that, at the same time, have better mechanic and elastic properties and, above all, a better *in vitro* and *in vivo* resistance to

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chemical and enzymatic hydrolysis, thus performing a prolonged action in the place of application. In addition, as the hydrogels until now considered, the studied materials must have the capability to entrap water and swell in contact with an aqueous medium.

5 In order to satisfy this demand it has been considered, according to this invention, the opportunity to chemically modify HA by reacting it with a suitable crosslinking agent having a polyaminoacid structure, that is substantially linear and with a protein-like structure, whose biocompatible characteristics have been already ascertained. In particular, the crosslinking agent proposed according to this invention has a polyhydrazide structure since it shows, 10 for each repeating unit, a pendent hydrazido group ($-\text{CO}-\text{NH}-\text{NH}_2$), potentially available to covalently link the carboxy group of the repeating disaccharide unit of hyaluronic acid.

The chemical modification of hyaluronic acid by functionalization with 15 bis-hydrazido groups has already been described, for example in the US patents US 5616568 and US 5652347, both to Pouyani et al. (assignee The Research Foundation of State University of New York) and in the corresponding scientific article (T. Pouyani, G. D. Prestwich, Functionalized Derivatives of Hyaluronic Acid Oligosaccharides: Drug Carriers and Novel Biomaterials, 20 *Bioconjugate Chem.*, 1994, **5**, 339-347). However, in this case it is not a crosslinking, rather a functionalization of HA, wherein the polysaccharide carboxylic groups reacted with bifunctional groups of general formula $\text{H}_2\text{N}-\text{NH}-\text{CO}-\text{A}-\text{CO}-\text{NH}-\text{NH}_2$, where A represents a generic spacer group, to produce a functionalized hyaluronic acid with hydrazido pendent groups: HA-CO-NH-NH- 25 CO-A-CO-NH-NH₂.

The mentioned documents also describe, as concerns the reaction, the known use of a carbodiimide (having general structure $\text{R}^1-\text{N}=\text{C}=\text{N}-\text{R}^2$) as an agent activating the reaction. For the possible subsequent crosslinking of functionalized HA, in such a manner that the resulting product could form 30 hydrogels, the documents suggest further reactions with a wide range of known crosslinking agents.

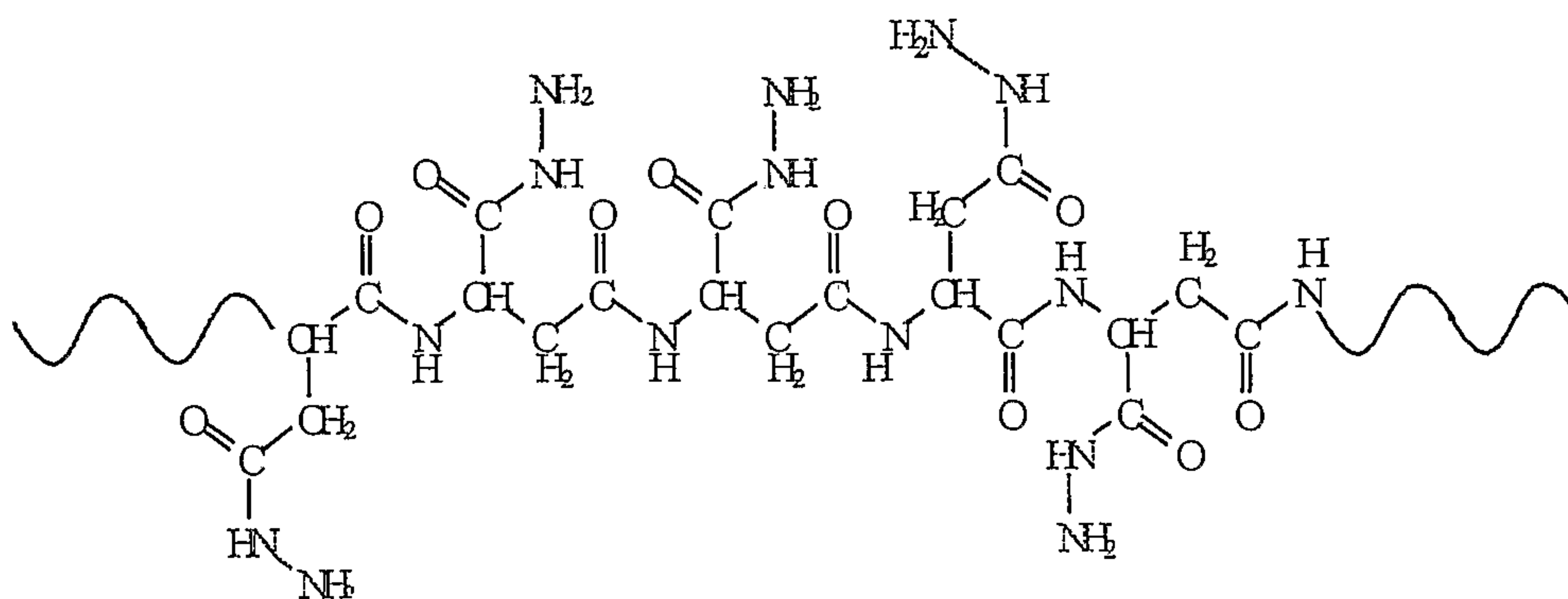
In the frame of the same research line, Vercruysse et al. have also

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proposed (Vercruysse et al., "Synthesis and *in Vitro* Degradation of New Polyvalent Hydrazide Cross-Linked Hydrogels of Hyaluronic Acid, *Bioconjugate Chem.*, 1997, **8**, 686-694) to modify HA by using agents that, having more than two terminal hydrazido groups, could result in a real crosslinking of the starting polysaccharide, to produce materials similar to hydrogels. Despite the work title refers to "polyvalent hydrazides", the reagents considered in the study are synthetic compounds containing from two to six hydrazido functions, and they are not polymeric chains. Apart from the absence of any consideration on the biocompatibility of the bis-, tri-, tetra-, penta- or hexahydrazides employed in the study, the document describes the production of materials having characteristics and structures different from those considered in this invention, firstly because it does not obtain the HA crosslinking by chemical bond with another linear polymer having a different nature, but by using multifunctional reagents with relatively small molecular size.

In view of the foregoing, the present invention proposes to employ as crosslinking agent for HA a polydrazide polymer having a polypeptide backbone, where each repeating unit contains one hydrazido pendent group. In particular, the preferred polymer of the type described is alpha,beta-polyaspartylhydrazide (PAHy), a water-soluble and biocompatible polymeric material, already synthesized and studied by the research group proposing this invention (G. Giammona, B. Carlisi, G. Cavallaro, G. Pitarresi, S. Spampinato, A new water-soluble synthetic polymer, α,β -polyasparthydrazide, *J. Control. Rel.*, 1994, **29**, 63-72). This material has been obtained, as reported in the mentioned literature, by aminolysis of a high molecular weight polysuccinimide with hydrazine. In particular, the polysuccinimide (PSI) has been obtained by polycondensation of D,L-aspartic acid, and it has been reacted subsequently with hydrazine (2HN-NH_2), to obtain a polymer represented by the following chemical formula:

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As it is evident from the previous formula, due to the cyclic structure of the starting polysuccinimide, the coupling of hydrazine can occur in such a way as to leave a methylene group either in the polymeric backbone or in the pendent functional group. Therefore, the repeating unit (the above formula shows five repeating units) can have a structure slightly different in the first or in the second case, but its molecular weight is the same.

In the mentioned literature, the synthesis and characterization of PAHy are reported as well as the proposal to use this protein-like polymer as a plasma substitute. For this aim, toxicity studies, immunogenic ability and platelet aggregation tests have been reported and they have demonstrated the total biocompatibility of this polyhydrazido polymer.

Therefore, the present invention specifically provides a composition comprising hyaluronic acid chemically crosslinked with a polyhydrazide polymer, wherein one or more carboxy groups of the disaccharide units of hyaluronic acid are chemically linked respectively to one or more hydrazido groups of the polyhydrazide polymer. Preferably, as pointed out before, said polyhydrazide polymer is alpha,beta-polyaspartylhydrazide (PAHy), that has been exhaustively investigated as concerns its water-solubility, biocompatibility and non-immunogenic properties. However, other polymers with a polyaminoacid structure having an essentially linear chain, with pendent hydrazido groups along the chain, could be employed in the same manner to crosslink hyaluronic acid so as to give compositions and materials with suitable properties of processability, mechanical resistance and resistance to degradation.

In particular, in the composition according to this invention, hyaluronic

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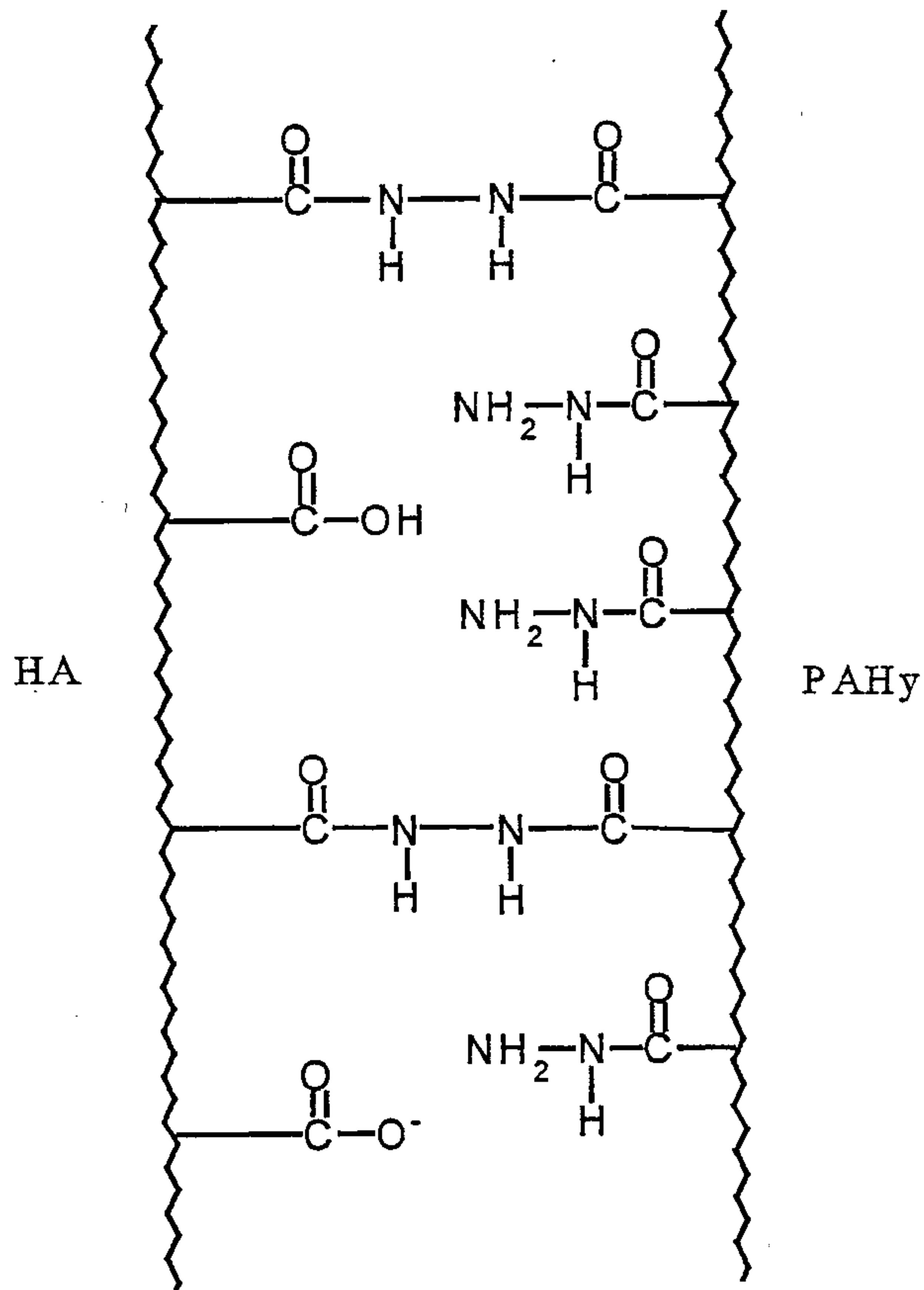
acid has a molecular weight from 50,000 to 1,500,000 dalton, whereas when the polyhydrazide polymer is PAHy, this has a molecular weight from 2,000 to 40,000 dalton.

According to some preferred embodiments thereof, this invention
5 provides a hydrogel composed of hyaluronic acid chemically crosslinked with a polyhydrazide polymer, as previously defined. Also in this case the polyhydrazido polymer is alpha,beta-polyaspartylhydrazide and, preferably, the hyaluronic acid employed for the hydrogel production has a molecular weight from 50,000 to 1,500,000 dalton, and the alpha,beta-polyaspartylhydrazide has a
10 molecular weight from 2,000 to 40,000 dalton, the most preferred range being from 10,000 to 30,000 dalton.

Preferably, in the production of the crosslinked polymeric material the ratio between moles of repeating unit of alpha, beta-polyaspartylhydrazide and moles of repeating unit of hyaluronic acid is from 0.01 to 5, the most preferred
15 range being from 0,5 to 3.

In view of the chemical structure of both polymers composing the hydrogel according to this invention, it is possible to hypothesise for the product resulting from the crosslinking reaction the following structure:

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According to some preferred embodiments of the invention, the proposed hydrogels can be obtained by reacting hyaluronic acid with the polyhydrazide polymer in the presence of a carbodiimide (having the general structure $\text{R}^1\text{-N=C=N-R}^2$) as an activating agent. The preferred activating product is N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide (EDC), but other similar agents could be employed in its place, such as, for instance, N,N'-dicyclohexylcarbodiimide, cyclohexyl- β -(N-methylmorpholine)ethyl carbodiimide p-toluensulphonate (CMC) or N-allyl-N'(β -hydroxyethyl)carbodiimide. Preferably, when EDC is employed the ratio between moles of N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide and moles of repeating unit of hyaluronic acid is from 0.01 to 10.

To activate the crosslinking reaction, it can be advantageous to add to the reaction medium another activating agent, such as a N-hydroxysuccinimide (NHS), specifically N-hydroxysulfosuccinimide (NHSS). In this case the

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NHSS is preferably present in the same molar amount as the said N-ethyl-N'-3-(3-dimethylaminopropyl)-carbodiimide.

According to another specific aspect thereof, the invention provides a manufacturing process for producing a hydrogel composed of hyaluronic acid
5 chemically crosslinked with a polyhydrazide polymer, wherein the hyaluronic acid, obtained from animal or vegetable sources or by biotechnological processes and having the molecular weight mentioned above, and the polyhydrazide polymer, preferably PAHy, with the molecular weight above reported, are dissolved in double-distilled water in a prefixed molar ratio and a fixed
10 amount of carbodiimide, preferably EDC, is added to them. The reaction mixture is kept from 0°C to 60°C for a time ranging from 1 hour to 5 days, and subsequently the product is recovered as a hydrogel. In this case, during the reaction the pH is maintained in the range between 3 and 8, in particular by using a 0.1N HCl solution or a solution of bis (2-hydroxyethyl) aminotris (hydroxymethyl)metane hydrochloride with a concentration ranging from 1 to 500
15 mM.

According to a further aspect thereof, the process according to this invention can include the addition, besides the said carbodiimide, of a prefixed amount of a N-hydroxysuccinimide, preferably NHSS, as a further agent activating the reaction. In this case, during the reaction the pH is maintained in
20 the range between 4 and 10.

After the reaction time, each product is purified by several washings with double-distilled water and then dried by lyophilization (to obtain nanoparticles, microparticles or sponges) or dried for some days at 25°C at a pressure
25 of 1 atm, inside suitable moulds, to obtain films, membranes or rods. As it will more evident below with reference to the reported experimental data, the systems prepared have a wide applicative versatility in the biomedical and pharmaceutical field, and are suitable, as an example, to heal wounds, to prevent post-surgical adhesion, to lubricate joints, to allow the *in vitro* and *in vivo* cell growth, to realize drug delivery systems.
30

It must be also considered that both the HA and the polyhydrazide polymer solutions could be singly sterilized. After their mixing, the gelation

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time, that is always greater than 10 minutes, could allow to apply the gel forming solution *in situ* so as to obtain, after a few minutes, the formation of a gel directly on the tissue. In this field there already exist on the market products proposed for the same application, for instance products consisting of a double syringe containing suitable reagents based on PEG (polyethyleneglycol) derivatives, able to crosslink *in situ* after their mixing. The solutions are sprayed on tissues to avoid the post-surgical adhesion.

Thus, the present invention further specifically provides a kit for the *in situ* production of a hydrogel composed of hyaluronic acid chemically crosslinked with a polyhydrazide polymer, preferably alpha,beta-polyaspartyl-hydrazide, comprising a container with a first hyaluronic acid-based component and a container with a second polyhydrazide polymer-based component, being said two components able to form the hydrogel after their mutual contact, directly on the application site.

Each product obtained according to this invention has been characterized by spectrophotometric techniques and swelling measurements in double-distilled water and in media that simulate some biological fluids (extracellular fluid, gastric fluid, intestinal fluid, synovial fluid, aqueous humour or vitreous humour) in a temperature range from 20°C to 40°C. The swelling values, as reported below, have shown a high affinity of the hydrogels prepared according to this invention towards an aqueous medium. The extent of this affinity resulted to be dependent on the crosslinking degree, and on the composition and pH of the swelling medium (investigated pH range from 1 to 9).

Each product of this invention has been also subjected to chemical hydrolysis studies at 37°C, by using media with various saline composition and pH values mimicking some biological fluids (range of investigated pH from 1 to 8) with incubation times from 1 to 30 days. The obtained results, partially reported below, have demonstrated that the proposed products are resistant to chemical hydrolysis as a function of the hydrolysis medium (composition and pH), of the incubation time and the crosslinking degree of the hydrogel.

Finally, the products according to this invention have been subjected to enzymatic hydrolysis studies by using aqueous solutions containing HAase

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at various concentrations (ranging from 1 to 1000 U/ml), at 37°C and for incubation times ranging from 30 minutes to 30 days. In this case, as reported below, the obtained results have demonstrated that the products of this invention are also resistant to hydrolysis by hyaluronidase, as a function of enzyme
5 concentration, incubation time and crosslinking degree of the hydrogel.

The specific features of the invention, as well as its advantages and the corresponding operating conditions, will be more evident in the detailed description reported below, by way of example only, together with the results of the experiments performed on the invention and the data for comparison
10 with the prior art. Some experimental results are also reported in the attached figures wherein:

Figure 1 shows the swelling behaviour, in aqueous solution with citrate buffer pH 6.3, of a series of sponges based on HA-PAHy hydrogels according to the invention;

15 Figure 2 shows the swelling behaviour, in aqueous solution with Dulbecco phosphate buffer solution (DPBS) pH 7.4, of a series of sponges similar to those of Figure 1;

Figure 3 shows the results of degradation studies, in the absence of HAase, of a series of sponges based on HA-PAHy hydrogels according to the
20 invention;

Figure 4 shows the results of degradation studies by enzymatic hydrolysis, of a similar series of sponges, wherein the concentration of the employed HAase enzyme is 75U/ml; and

Figure 5 shows the results of degradation studies by enzymatic hydrolysis of a similar series of sponges, wherein the concentration of the employed HAase enzyme is 150 U/ml.
25

EXAMPLE 1

An aqueous solution containing hyaluronic acid (0.5 % w/v) has been added to an amount of alpha,beta-polyaspartylhydrazide (PAHy) such as to
30 have a ratio between the moles of repeating unit of PAHy and the moles of repeating unit of hyaluronic acid (ratio indicated as "X") equal to 2.

To activate the reaction between hyaluronic acid and PAHy, N-ethyl-

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N'-(3-dimethylaminopropyl)-carbodiimide (EDC) alone was employed, in an amount such as to have a ratio between moles of EDC and moles of repeating unit of hyaluronic acid (ratio indicated as "Y") equal to 1.8.

5 The reaction mixture was kept at 37°C for 4 hours. During the reaction, the pH value was maintained at a constant value of 4.75 by using a solution of bis (2-hydroxyethyl)aminotris(hydroxymethyl)methane hydrochloride with a concentration of 300mM.

10 After the reaction time, the obtained product was purified by several washings in double-distilled water and then dried by lyophilization to obtain microparticles.

The obtained product has been weighed (yield 94 % $\pm$ 1.9) and characterized by spectrophotometric techniques.

By using the same procedure it is also possible to obtain nanoparticles, films, rods, sponges and gels.

15 EXAMPLE 2

The same procedure reported in the Example 1 was repeated with the only difference that the crosslinking reaction was carried out in the presence of EDC as a activant and in the presence of the same molar amount of N-hydroxysulfosuccinimide (NHSS).

20 In this case the pH value was maintained at 7.5.

By performing the reaction as in Example 1, similar yields were obtained for the purified product, that was characterized by spectrophotometric techniques.

Swelling studies

25 The product obtained in various sizes and shapes such as nanoparticles, microparticles, film, rods, sponges and gels, has a high swelling degree in double-distilled water. As reported above, this property confers on the hydrogels of this invention a potential biocompatibility that, in addition, is confirmed by the biocompatibility of both starting polymers, thus giving a wide possibility for the use of these products in the biomedical and pharmaceutical field.

30 By using a series of HA-PAHy sponges obtained according to the

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schemes of the previous examples, by varying the molar ratio X and Y defined above, tests of swelling in DPBS buffer pH 7.4 and citrate buffer pH 6.3 were performed. By using suitable procedures, the swelling behaviour was expressed by the ratio (Q) between the equilibrium weight of swollen sponge
5 and the weight of the same sponge dry.

Some results of these experiments are shown in Figures 1 and 2, performed by using two different buffer solutions as aqueous media. It is evident, by examining these figures, that the swelling values in phosphate buffer are twice the corresponding Q values in citrate buffer. Moreover, in both media it is observed that, by increasing the amount of PAHy in the sponges (X
10 ratio between 1 and 2), a slight increase in the swelling ability occurs, this being more evident for smaller Y values.

Chemical and enzymatic degradation studies

The product obtained according to the schemes of the previous Examples 1 and 2 was subjected to chemical hydrolysis studies at 37°C for 10
15 days in phosphate buffer solution pH 7.4 (which simulates the extracellular fluid) and pH 5.5 (which simulates the skin pH). After 10 days, the product was recovered, purified by washing with double-distilled water, lyophilized and weighed to determine the percentage of degradation, that resulted to be less
20 than 4%.

For another set of experiments, the series of hydrogels above reported, obtained with various molar ratios X and Y, have been extensively washed and lyophilized, then kept in citrate buffer pH 6.3, in the presence or in the absence of hyaluronidase. The latter was used in two different tests at a
25 concentration of 75 and 150 U/ml, respectively. After incubation at 37°C under stirring for fixed times, the extent of hydrogel degradation was evaluated by using suitable analytical procedures.

The results of some tests of chemical degradation (in the absence of HAase) and enzymatic degradation with two different concentrations of responsible enzymes, are shown in Figures 3, 4 and 5 respectively. In this case,
30 the tests are also assembled in series as a function of the molar ratios X and Y employed in the hydrogel preparation. By examining these figures, it is evi-

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dent that for each medium chosen for this experiment, a progressive degradation of the sponges occurs. For each series, with an equal value of X, the degradation decreases by increasing the value of Y. This means, as expected, that the efficiency of crosslinking increases by increasing the amounts of EDC
5 and NHSS.

In addition, with an equal value of Y, it is observed an evident decrease in the percentage of degradation by increasing the amount of crosslinking agent (PAHy), i.e. the value of X, thus demonstrating that the rate of degradation increases by decreasing the crosslinking degree. Finally, it can
10 be observed that the sponge showing the best resistance to degradation is the one obtained with X=1 and Y=1; in fact, after 2 weeks, it shows only a degradation of 10% in the absence of HAase.

Taking into consideration the above results, it is evident that the hydrogels according to this invention possess the advantage to undergo a hydro-
15 lytic or enzymatic degradation dependent on time and that can be fixed in advance, as a function of the desired application, by changing the conditions of preparation of the product of this invention. The prepared materials, besides having an excellent compactness and elasticity, are resistant to chemical and enzymatic hydrolysis for several days, but they are totally degradable and
20 reabsorbable after long periods of time.

These advantages are obtained, according to this invention, without penalizing the costs and the ease of production. The latter, on the contrary, is very simple, inexpensive and easily reproducible with high yields. Finally, it must be evidenced that the biomaterials proposed according to this invention
25 represent an excellent combination between the advantages due to biocompatibility of hyaluronic acid and the peculiar properties of a synthetic (artificial) polymer, such as chemical versatility, easy processability and low-cost production.

The present invention has been described with particular reference to
30 specific embodiments thereof, but it should be understood that modifications and changes may be made by the persons skilled in the art and the scope of the invention is as defined in the appended claims.

CLAIMS

1. A composition comprising hyaluronic acid chemically crosslinked with a polyhydrazide polymer, wherein the polyhydrazide polymer has a polypeptide backbone having an essentially linear chain, where each repeating unit contains one hydrazide pendant group and one or more carboxy groups of the disaccharide units of hyaluronic acid are chemically linked, respectively, to one or more hydrazido groups of the polyhydrazide polymer.
2. A composition according to claim 1, wherein said polyhydrazide polymer is alpha,beta-polyaspartylhydrazide.
3. A composition according to claims 1 or 2, wherein the hyaluronic acid has a molecular weight from 50,000 to 1,500,000 dalton.
4. A composition according to claim 2, wherein the alpha,beta-polyaspartylhydrazide has a molecular weight from 2,000 to 40,000 dalton.
5. A hydrogel composed of hyaluronic acid chemically crosslinked with a polyhydrazide polymer, wherein the polyhydrazide polymer has a polypeptide backbone having an essentially linear chain, where each repeating unit contains one hydrazide pendant group and one or more carboxy groups of the disaccharide units of hyaluronic acid are chemically linked, respectively, to one or more hydrazido groups of the polyhydrazide polymer.
6. A hydrogel claimed in claim 5, wherein said polyhydrazido polymer is alpha,beta-polyaspartylhydrazide.
7. A hydrogel according to claim 6, wherein the hyaluronic acid has a molecular weight from 50,000 to 1,500,000 dalton and the alpha,beta-polyaspartylhydrazide has a molecular weight from 2,000 to 40,000 dalton.
8. A hydrogel according to claims 6 or 7, wherein the ratio between moles of repeating unit of alpha,beta-polyaspartylhydrazide and moles of repeating unit of hyaluronic acid is from 0.01 to 5.
9. A hydrogel according to any one of claims 5-8, obtained by reacting hyaluronic acid and a polyhydrazide polymer in the presence of a car-bodiimide as an activating agent.

10. A hydrogel according to claim 9, wherein said carbodiimide is N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide.
11. A hydrogel according to claim 10 wherein the ratio between moles of N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide and moles of repeating unit of hyaluronic acid is from 0.01 to 10.
12. A hydrogel according to any one of claims 9-11, further obtainable in the presence of a N-hydroxysuccinimide as a further activating agent.
13. A hydrogel according to claim 12 wherein said N-hydroxysuccinimide is N-hydroxysulfosuccinimide.
14. A hydrogel according to claim 13 wherein said N-hydroxysulfosuccinimide is present in the same molar amount as the said N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide.
15. A manufacturing process for producing a hydrogel as defined in claims 5 or 6, wherein said hyaluronic acid and said polyhydrazide polymer are dissolved in double-distilled water in a prefixed molar ratio and a fixed amount of carbodiimide is added thereto, the resulting reaction mixture is kept at a temperature from 0°C to 60°C for a period of time from 1 hour to 5 days, and subsequently the product is recovered in hydrogel form.
16. A process according to claim 15 wherein said polyhydrazide polymer is alpha,beta-polyaspartylhydrazide and said carbodiimide is N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide.
17. A process according to claims 15 or 16 wherein during the reaction the pH is maintained in the range from 3 to 8.
18. A process according to claims 15 or 16, wherein in addition to said carbodiimide, a prefixed in advance amount of a N-hydroxysuccinimide is added.
19. A process according to claim 18 wherein said N-hydroxysuccinimide is N-hydroxysulfosuccinimide.

20. A process according to claims 18 or 19 wherein during the reaction the pH is maintained in a range from 4 to 10.
21. Compositions according to any one of claims 1 to 4 and hydrogels according to any one of claims 5 to 14, in the form of nanoparticles, microparticles, film, membranes, rods, sponges or gels.
22. A kit for the in situ preparation of a hydrogel composed of hyaluronic acid chemically crosslinked with a polyhydrazide polymer, comprising a container with a first hyaluronic acid-based component and a container with a second polyhydrazido polymer-based component, wherein the polyhydrazide polymer has a polypeptide backbone having an essentially linear chain, where each repeating unit contains one hydrazide pendant group and one or more carboxy groups of the disaccharide units of hyaluronic acid are chemically linked, respectively, to one or more hydrazido groups of the polyhydrazide polymer.
23. A kit according to claim 22, wherein said polyhydrazide polymer is alpha,beta-polyaspartylhydrazide.

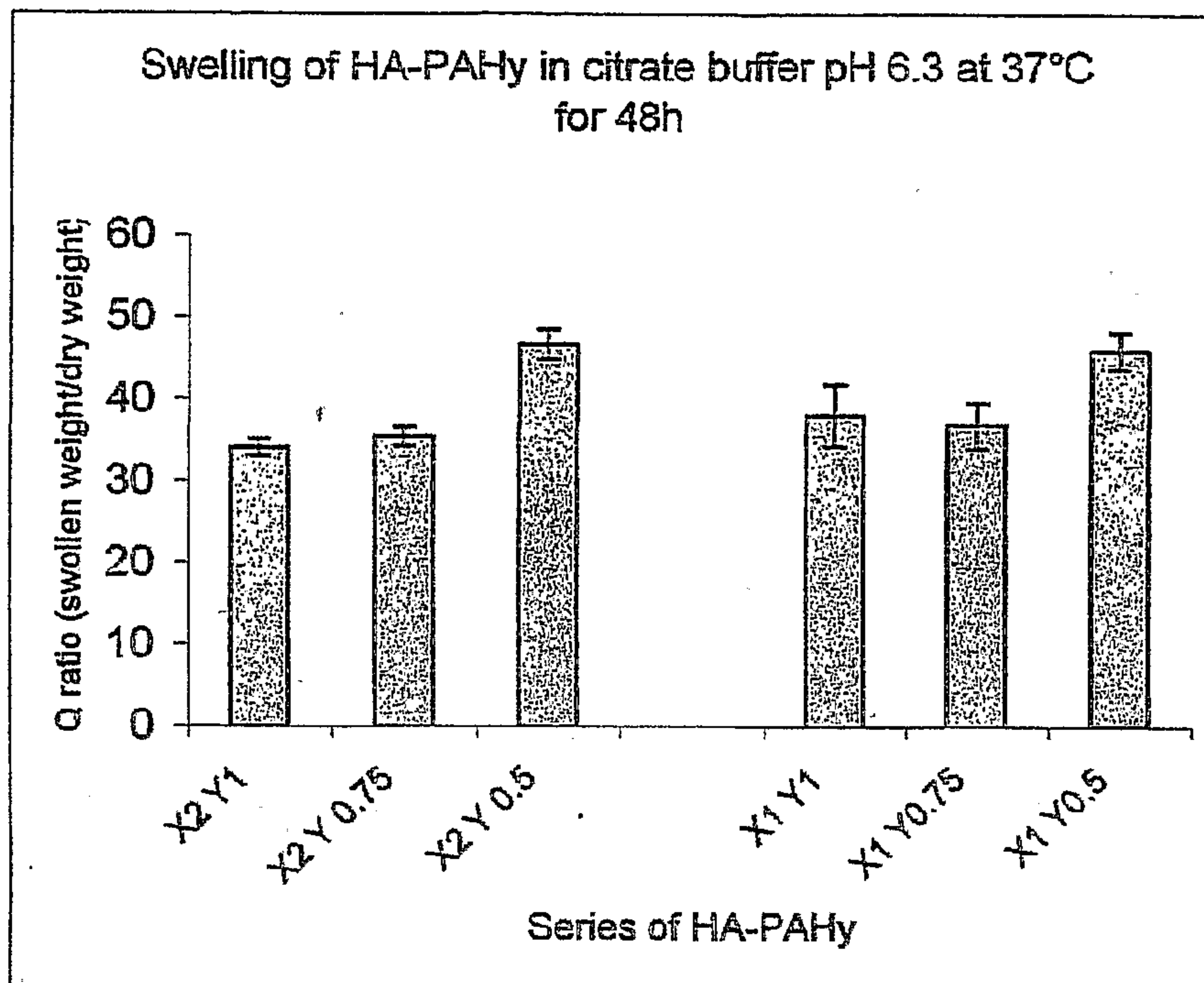


FIG. 1

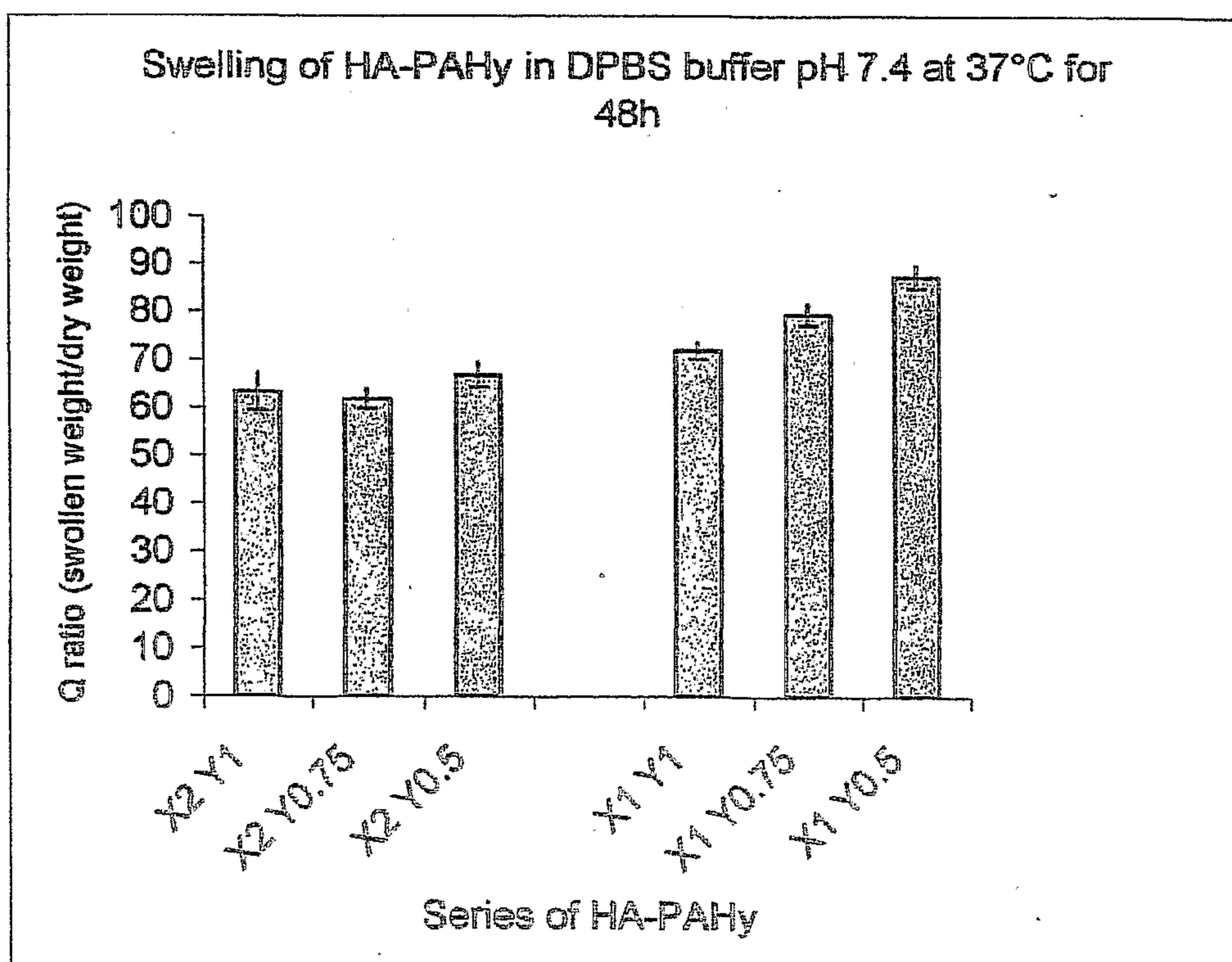


FIG. 2

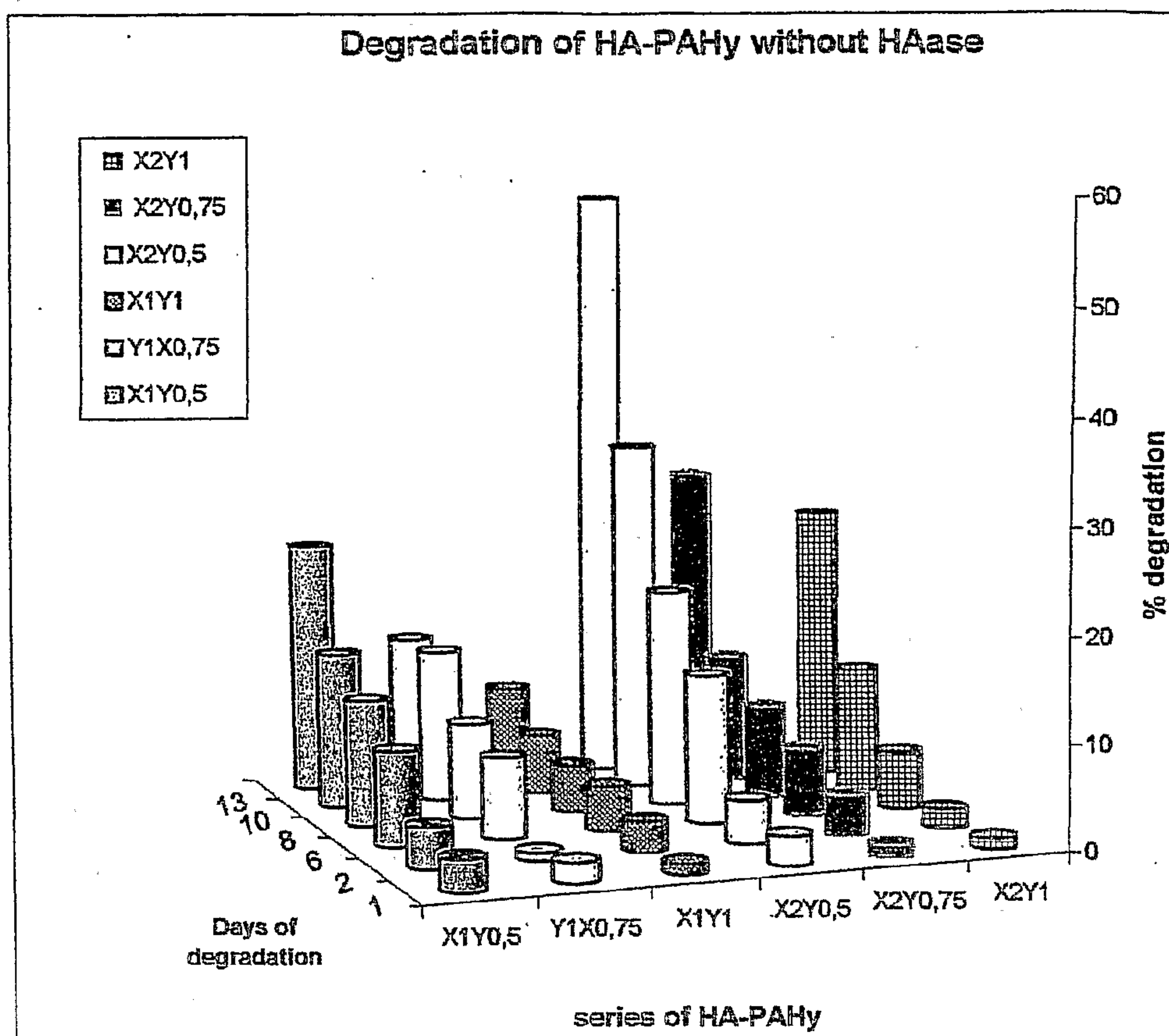


FIG. 3

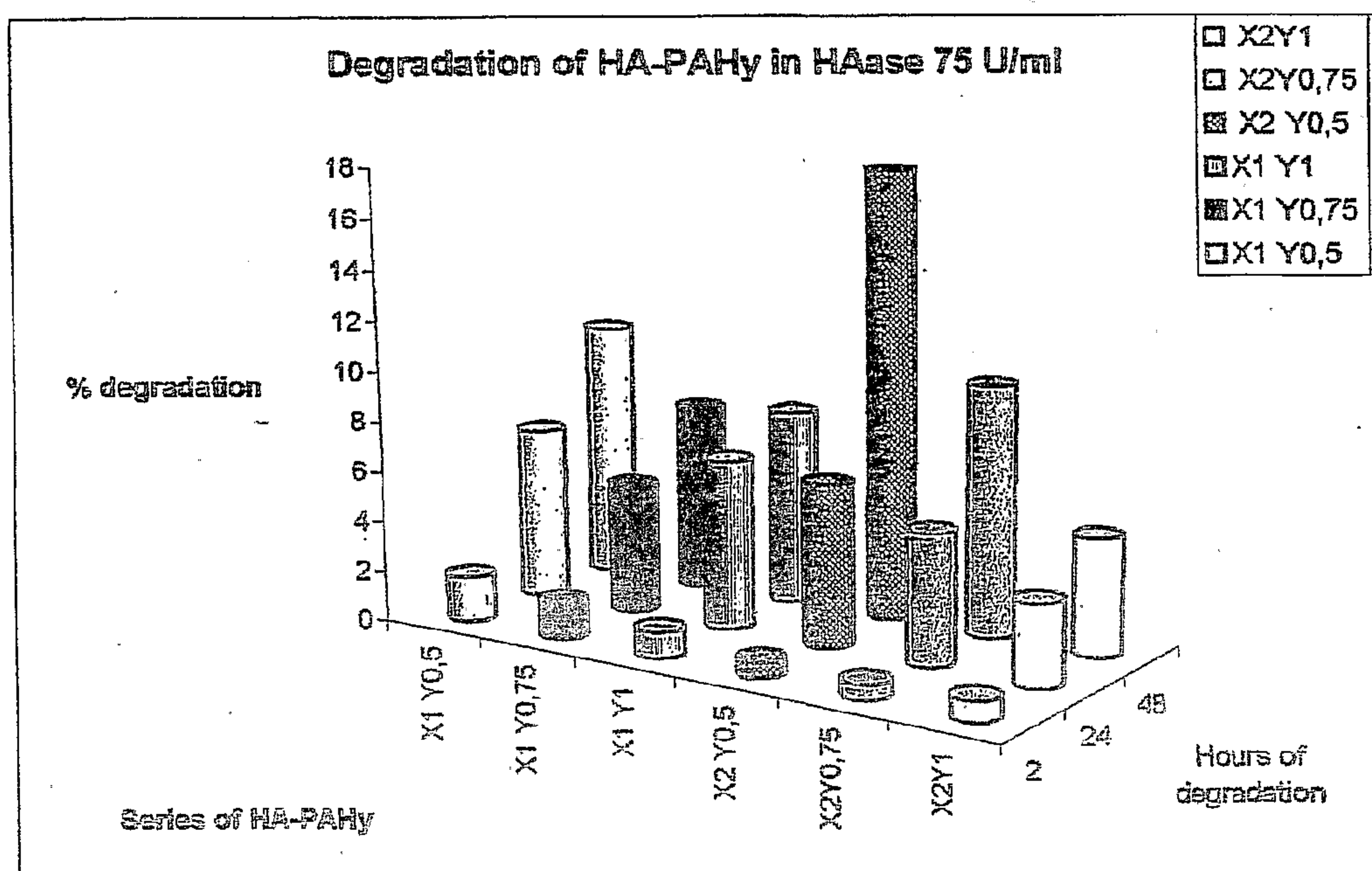


FIG. 4

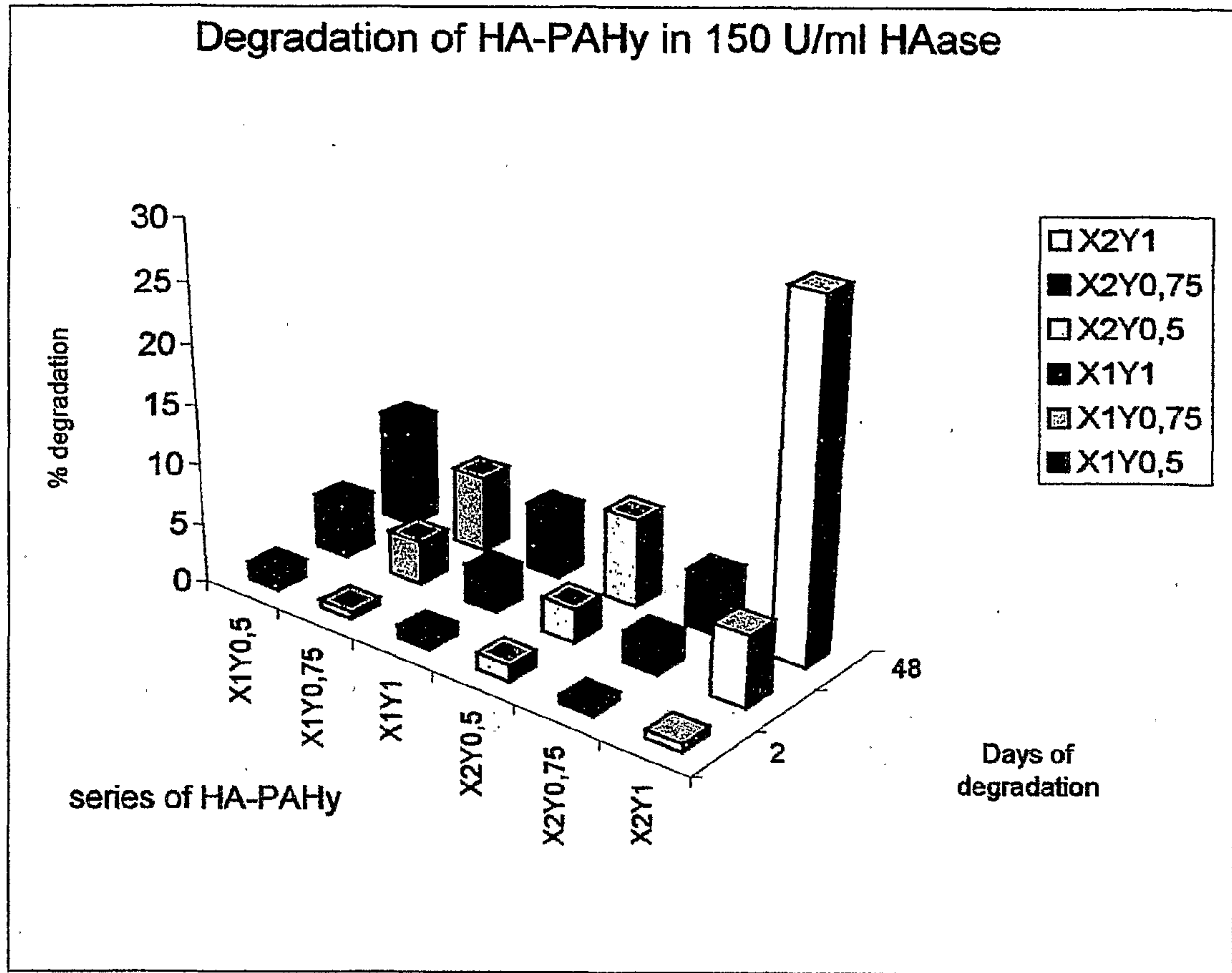


FIG. 5