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Ionic hydrogel based on chitosan crosslinked with 6-Phosphogluconic Trisodium salt as a drug delivery system

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ABSTRACT

In this work, 6-phosphogluconic trisodium salt (6-PG$^-$Na$^+$) is introduced as a new aqueous and non-toxic crosslinking agent to obtain ionic hydrogels. Here, it is shown the formation of hydrogels based on chitosan crosslinked with 6-PG$^-$Na$^+$. This formulation is obtained by ionic interaction of cationic groups of polymer with anionic groups of the crosslinker. These hydrogels are non-toxic, do not cause dermal irritation, are easy to extend and have an adequate adhesion force to be applied as polymeric film over the skin. This formulation exhibits a first order release kinetic and can be applied as drug vehicle for topical administration or as wound dressing for wound healing. The primary goal of this communication is to report the identification and utility of 6-phosphogluconic trisodium salt (6-PG$^-$Na$^+$) as a non-toxic crosslinker applicable for cationic polymers.

Keywords: 6-phosphogluconic trisodium salt, ionic hydrogel, chitosan, wound healing, drug delivery, dermal irritation

1. INTRODUCTION

The skin is the most extensive organ of the body, it supposes 10-15% of the corporal weight, and it fulfills diverse physiological functions. A multitude of pharmaceutical forms of cutaneous application have been developed for different objectives: topical treatment of skin diseases or cosmetic application, transdermal administration of drugs for systemic treatment, etc.

Hydrogels have a special place in this field as they can act as local or transdermal drug delivery systems [1-4] such as patch´s matrices [5] for containing and controlling the release of the active ingredients and for forming compatible, adhesive and flexible polymer films on the skin with a variety of potential applications [6].

Another area of research in this field is the development of hydrogels applied to wound healing. There are many published works [7-12] on this topic and products marketed [13-15]. Also, multiple patents have contributed to increase this field of knowledge [16-18].

Due to the importance of these polymeric systems, the search for new molecules with crosslinking capacity allows the development of new formulations with different physicochemical characteristics that may lead to advances in various pharmaceutical applications.

In this work the 6-phosphogluconic acid trisodium salt (6-PG$^-$Na$^+$) has been investigated as a ionic crosslinking agent for cationic polymers (Fig. 1).

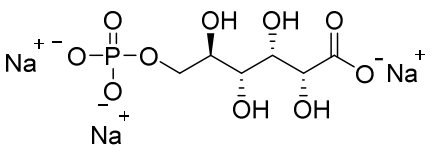

Fig 1. Chemical structure of 6-phosphogluconic acid trisodium salt (6-PG$^-$Na$^+$).

6-Phosphogluconic acid (6-PG) is a metabolite of the pentose phosphate pathway, in which glucose is degraded into pentose-phosphate for nucleic acid biosynthesis and into NADPH to obtain energy for said process. Degradation of glucose through this route is necessary in some specific tissues (adipose tissue, adrenal cortex, liver, etc.) in which the biosynthetic reactions are elevated [19].

In the early 70s a patent was issued in which it was demonstrated that 6-phosphogluconic acid (6-PG) and its salts can be used as regenerating agents of liver tissue. In this study, the liver of albino rats, previously excised, was regenerated after 2-8 days of intraperitoneal treatment with 150 mg/kg·day of 6-PG^-Na^+ [20].

To date, neither 6-PG nor its salts have been described as crosslinking agents. However, the literature describes several examples of molecules with phosphate groups capable of forming hydrogels by ionic crosslinking, such as β-glycerol phosphate [21], tripolyphosphate [22], pyrophosphate [23], glucose 1-phosphate [24], and ammonium hydrogen phosphate [25].

For all the above, this work aims to develop a modified drug delivery system based on a cationic polymer, chitosan, cross-linked with an anionic molecule, 6-phosphogluconic acid (6-PG^-Na^+) trisodium salt and to explore its applications as system for topical administration.

2. EXPERIMENTAL SECTION

2.1 Materials

The materials used in this research were: chitosan (deacetylation degree = 81%, MW = 190-310 kDa), piroxicam, 6-phosphogluconic acid trisodium salt (6-PG^-Na^+), dimethylsulfoxide (DMSO), KH_2PO_4, NaCl, HCl 35%, NaH_2PO_4 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazole (MTT) bromide were purchased from Sigma Aldrich.

2.2 Hydrogel formation

In a standard preparation, to 0.77 g of chitosan 2% in HCl 0.1 N (0.05 mmol of chitosan repetitive units) were added different volumes of a stock solution of 6-PG$^-$Na$^+$ in water (100 mg/ml), which molar amounts were 0.02, 0.05, 0.10, 0.13 mmol, to achieve a crosslinking percentages (mol of 6-PG$^-$Na$^+$/mol of chitosan repetitive units) of 50%, 100%, 200%, 275%, respectively. This mixture was stirred for one minute until a homogeneous and whitish dispersion was obtained. After the stirring the gel formation was considered concluded because the gelation process is very fast.

Samples of the formulations were lyophilized for preservation. The product obtained can be easily ground and rehydrated in aqueous medium in the moment it has to be used. Swelling, release and wound healing tests were done on a disk shape dry sample (lyophilized hydrogel), the other tests shown were performed on re-hydrated samples [26].

2.3. Drug loading method

The loading of piroxicam, used as a model drug, was performed simultaneously to the formation of the hydrogel. A film of 2.5 mg of piroxicam was prepared by evaporating an adequate volume of stock solution of drug in acetone overnight. Then, 0.77 g of the polymer solution was added to the vial containing the drug and allowed to stir for about two hours until the drug was detached and dispersed among the polymer. The crosslinking agent was then added and stirred for one minute until the formation of the hydrogel.

Using this methodology, chitosan hydrogels were obtained with different percentages of crosslinking. Table 1 summarizes the nomenclatures used to name each of the formulations:

Crosslinking percentage (6-PG$^-$Na$^+$/chitosan)	Hydrogels	Hydrogels with piroxicam
50%	M60-50	M6P-50
100%	M60-100	M6P-100
200%	M60-200	M6P-200
275%	M60-275	M6P-275

Table 1. Scheme of hydrogels studied based on their percentage of crosslinking and presence or absence of drug.

2.4. Swelling

Dynamic swelling analysis was performed on lyophilized samples of M60-100 and M60-275, which had a disc shape. They were introduced into metal baskets, which allow the entry of fluid but avoid the exit of the formulation, in order to avoid that the possible disintegration of the system would hamper hydrogel weighing. The baskets were introduced into vessels with different media buffered at pH 1.2, 4.5, 6.8 and 7.5 for 48 hours at 37°C. At predetermined time intervals, the baskets were extracted from the medium, their surface was carefully dried with filter paper and weighed, finally being returned to the same medium. Before the beginning of the experiment, baskets and hydrogel samples were weighed individually on a balance (Acculab, Sartorious Group, Atilon, max 220 g, d = 0.1 mg). Each condition of pH and degree of crosslinking was studied in triplicate.

The weight of the hydrogels samples at each time was calculated by difference between the total weight and the weight of the basket used to keep the sample in. The swelling degree normalized at time t (Q_t) was calculated in terms of weight of water per weight of dry hydrogel using equation 1.

$$Q_t = \frac{m_t - m_0}{m_0} \quad (1)$$

Where m_0 is the initial weight of the dry hydrogel (xerogel) and m_t is the weight of the hydrogel at time t.

2.5 Release profiles

The test was carried out in vessels placed on a thermoregulated orbital incubator (Stuart orbital incubator SI50) at 37°C and 100 oscillating motions per minute for 48 hours. The hydrogels M6P-100 and M6P-275 were introduced into metal baskets, which allow the entry and exit of dissolved drug and fluid but avoid the exit of the hydrogel. Baskets were introduced into vessels with different media buffered at pH 1.2, 4.5, 6.8 and 7.5 for 48 hours at 37 ° C. Sampling was performed at predefined times and the removed volume was refilled with the corresponding medium. Amount of piroxicam in the samples was determined by HPLC as mentioned below. The experiment was performed in triplicate for each pH value and percent of cross-linking. The release profiles obtained were compared by the similarity factor, f2, using equation 2.

$$f_2 = 50 \cdot log\left\{\left[1 + \frac{1}{n} \cdot \sum_{t=1}^{n}(R_t - T_t)^2\right]^{-0.5} \cdot 100\right\} \quad (2)$$

Where n is the number of pairs of points compared, R_t is the release percentage for the reference formulation at each time and T_t is the percentage of release in the tested formulation at each time.

The adjustment of the profiles to establish the kinetic release model was performed by applying Korsmeyer-Peppas equation (equation 3). Experimental values were used up to a release percentage of 60% of the total amount present in the hydrogel. To better characterize the release process, we also used the study of the first order model (equation 4) and Peppas-Sahlin model [27] (equation 5):

$$M_t = M_0\, e^{k_1 t} \quad (4)$$

Where k_1 is the first order kinetic constant and M_t is the amount of drug released at time t.

$$\frac{M_t}{M_\infty} = k_D t^m + k_R t^{2m} \quad (5)$$

Where the term $k_D t^m$ corresponds to the contribution of the Fickian diffusion mechanism (k_D is the diffusional constant), and the term $k_R t^{2m}$, to the Case II transport mechanism (k_R is the relaxation/erosion constant). The coefficient m is the purely Fickian diffusion exponent for a matrix of any geometric form that has controlled release. The adjustment of the kinetic models and the calculation of the parameters was done using Solver to minimize the Residual Sum of Squares (RSS) between experimental and theoretical concentrations.

Quantification method

Determination of piroxicam was carried out at 30°C on a Waters stainless steel column model Nova Pak C-18 150mm length, 3.9 mm in diameter and particle size of 4µm preceded by a Phenomenex® KJ0-4282 precolumn (Phenomenex AJO-4287, C18 4 x 3.0 mm). The solvent delivery system (Alliance System (Waters 2695)) was used to deliver a mobile phase 50:50 trifluoroacetic acid aqueous solution at pH 3 and acetonitrile. The flow rate was 1 mL/min. Detection was performed using a Waters® 2487 double λ absorbance detector with a wavelength of 356 nm.

2.6. Toxicity

The ability of the hydrogels to interfere with the growth of Caco-2 cells was determined *in vitro* using the 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay based on the metabolic reduction reaction in mitochondria of the yellow-colored compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazole (MTT) into the blue-colored formazan. The formation of the blue compound can be followed spectrofotometrically at 490 nm and the absorbance value is proportional to the number of living cells.

Assays were carried out in 96-well microliter plates with flat-bottomed wells at a density of 25000 cells per well. Cultures were grown at standard conditions. 24 h later, the medium was replaced with a fresh medium and 50 µL of the dispersed gel samples, 6-PG^{-}Na^{+} and chitosan (5 mg/mL in PBS) were added. Plates were incubated for 24, 48, or 72 h and then, 20 µL of MTT solution (5 mg/mL) were added to each well. The samples were incubated at 37°C for 3 h to allow reaction for the formazan formation. The content of each well was removed and the dark blue crystals were redissolved in DMSO. Absorbance was measured at 490 nm with a Labsystems Multiskan EX plate reader. Assays were carried out in quadruplicate for each treatment. Data were expressed as a percentage of the absorbance of the untreated control.

Differences between cellular survival values were studied by the ANOVA statistical test with a level of significance $p = 0.05$.

2.7. Rheological characterization

Rheological characterization was performed using a controlled stress rheometer, RheoStress RS 1 (Thermo Haake, Germany) equipped with control and data logging software (RheoWin 4.0.1) and a Haake K10 thermostat bath. After loading the samples, they were kept at rest for at 600 seconds in order to assure sample relaxation and temperature equilibrium. To avoid drying during measurement the free edges of the sample were covered with silicone oil (Dimethicone RFE/Ph. Eur.). All measurements were performed in triplicate at 25 ^{o}C using a titanium cone-plate sensor (35 mm, 2 degree).

Flow curves, viscosity as a function of shear rate, $\eta = f(\dot{\gamma})$, were performed both in control stress mode and control rate mode. In order to obtain zero shear viscosity, η_0, stepped flow curves in controlled stress mode were carried out (30 s each step in logarithmic stress values distribution). Shear stress range was chosen in order to obtain viscosities for shear rates ranging from 0.001 to 100 s^{-1} approximately. This interval was chosen in order to avoid centrifugation of the sample occurring at higher shear rates. Due to the abrupt viscosity drop,

the flow curve was complemented with a step flow curve in control rate mode (10 s each step in logarithmic distribution).

Small amplitude oscillatory sweeps (SAOS) were carried out in order to analyse viscoelastic behaviour of the gel. The linear viscoelastic region (LVR) was previously determined by a stress sweep performed at 1 Hz. A stress amplitude of 5 Pa, within the LVR was chosen for SAOS. Both, storage modulus (elastic modulus), G', and loss modulus (viscous modulus), G", at different frequencies from 0.01 to 10 Hz in logarithmic distribution (9 points per decade), were obtained.

2.8. Extensibility

The extensibility test was carried out according to the standard procedure PN/L/CP/003/00 of extensibility determination in the second edition of the Spanish National Formulary (Order SSI/23/2015).

In this case, 0.5 g of sample was deposited between two glass plates and it was compressed by applying increasing weights for 30 seconds. After each time interval the area of extension was calculated with the aid of a millimeter paper located below the bottom plate.

The assays were performed in quadruplicate for the rehydrated M60-275 hydrogel (120 mg dry hydrogel/ml water, equivalent to 30 mg chitosan/ml water). In order to compare, commercial carbomer gel (Salvacam®) and rehydrated chitosan were also tested. The latter formulation was prepared by lyophilizing chitosan 2% in 0.1 N HCl and adding water to obtain the same polymer concentration as that of the hydrogel, i.e., 30 mg / ml.

2.9. Adhesion to the skin

Patch adhesion to the skin was measured in terms of force required to peel it off the skin, using a modification of the Loop Tack test [28].

This measurement was carried out in a dynamometer constituted by a lower support in which the skin is placed and an upper device with a clamp function that is able to elevate registering the force exerted for that purpose. Pig ear skin was firmly attached to plates which were placed on the bottom of the dynamometer. 0.4 g of the rehydrated hydrogel (120 mg dry hydrogel/ml water) were spread over the skin forming a film of 1 mm of thickness and 1.9 × 4.5 cm of area. The hydrogel was allowed to dry for 30 minutes and an adhesive tape of 1.9 x 4.5 cm was attached to it. The ends of the tape were fastened to the top of the instrument by a clamp-type grip. Measurement of patch adhesiveness to the skin was performed by raising said top device at a speed of 500 mm/s and recording the maximum force taken to peel it off. Five measurements were made for the M60-275 hydrogel.

2.10. Dermal irritation

Acute dermal irritation was assessed according to the procedure described in OECD guide 404 [29]. The protocol was approved by the Ethics Committee (2016/VSC/PEA/00137). The test was realized as follows: First, the dorsal area of the animal is shaved avoiding to cause abrasion. 24 hours later, 0.3 grams of rehydrated hydrogel (120 mg dry formulation/ml water) is applied over an area of 6 cm^2. The patch is left for 15 minutes, then it is removed and the skin is observed. If there are no signs of irritation, the formulation is applied again on another skin area and left for 1 hour. In case of no irritant effects when removing the hydrogel, the procedure is repeated allowing the patch to contact with skin for 4 hours. When removing each patch, the area has to be washed with moistened gauze to eliminate any traces of the formulation. If skin irritation is not observed after removal of the formulation at 4 h, the animal is observed for 14 days. This is a sequential test, so this procedure is done first in a rabbit; a second rabbit serves as a confirmatory test if in the initial test the formulation has been observed to be non-irritant. The procedure in the second animal is the same, removing the

formulation at 15 minutes, 1 hour and 4 hours. If the result in the two rabbits is the same it is not necessary to test a third one; otherwise, a third animal is tested with the same procedure.

During the trial, images of treated areas and control area (healthy skin) were taken at different times to assess the presence of signs of irritation. The assay was performed on two rabbits.

2.11. Franz cell permeability

Skin permeation studies were performed using excised porcine ear skin on diffusion cells called Franz cells. Excised porcine ear skin was equilibrated for 30 min in 0.9% NaCl before being mounted in vertical Franz-type diffusion cells (area of 2 cm^2). The receptor compartment (10 mL) was filled with PBS (pH 7.4). The receptor fluid was stirred at a constant speed of 100 rpm. Temperature was adjusted to 32 ± 0.1 °C using a circulating water bath. Rehydrated hydrogel (0.5 g, containing 2.5 mg piroxicam) was applied on the donor compartment. Aliquots of the receptor fluid were withdrawn at different time points (14, 16, 18, 20, 22 and 24 h) and were immediately replaced with an equal volume of fresh PBS. The drug concentration in each sample was quantified using the HPLC method mentioned above in order to calculate the flux of piroxicam across the skin.

At the end of the transdermal permeation experiments (24 h), skin samples were immersed in methanol for 24 h under constant stirring in order to extract the drug. The hydrogel residues obtained from the donor compartment were treated with 35% HCl, sonication and methanol to dissolve the remaining piroxicam therein. Subsequently, they were neutralized with the addition of a diluted NaOH solution. Samples were filtered and analysed using the validated HPLC method to determine the amount of piroxicam retained in the skin.

The rehydrated M6P-275 hydrogel (120 mg dry preparation/ml solution) was studied in two different media: formulation 1 (10% propylene glycol) and formulation 2 (10% glycerol, 10% isopropyl alcohol and 10% propylene glycol).

Assays were performed septuplicate (n=7) for each tested formulation. The results were evaluated using the t-student statistical test for independent samples with a 95% confidence interval.

2.12. Wound healing

The aim of this section was to determine the healing capacity of the chitosan hydrogels based on 6-PG^{-}Na^{+} by evaluating the healing rate of full thickness wounds.

The *in vivo* animal study was approved by the Ethics Committee on Experimental Research (protocol UMH-DI-MBS-03-14), at Miguel Hernandez University, Spain. Female Wistar rats, weighing 250−290 g and 4−6 weeks of age, were used in this study. The rats were divided into four groups and each group contained four rats (n = 4); the rats of group 1 were treated with chitosan dressing, those of group 2 with 0.25 ml of 6-PG-Na + 180 mg / ml solution in water, group 3 with the hydrogel M60-275 and those of group 4 were not treated. The chitosan dressing consisted in 0.77 g of 2% polymer; dressings for group 3 contained 0.77 g of 2% polymer crosslinked with 275% 6-PG^{-}Na^{+}, both prepared as disk shaped lyophilized samples. Animals were allowed to take normal rat feed and water without restriction.

On the day of wounding, the rats were anaesthetized by intraperitoneal injection of 40.0 mg/kg pentobarbital and were also injected subcutaneous buprenorphine 0.3 mg/kg as analgesic. The dorsal area of the rats was depilated and the operative area of skin cleaned with alcohol. A full thickness skin wound of 3 cm diameter was prepared by excising with surgical scissors; the dorsum of the rat was photographed and covered with the corresponding formulation studied, a non-stick gauze and a plaster. In the case of the untreated group, the wound was covered only with the nonstick gauze and the plaster. After applying the dressing materials, the rats were housed individually in cages under normal room temperature. During the two weeks following the surgical creation of the wound, 1 g/L acetaminophen was administered in water.

The dressing materials were changed at days 7, 14 and 21 after skin excision. During the changing of dressings, photographs were taken at the wound area. After day 21, animals were sacrificed by intravenous injection of sodium pentobarbital 90 mg/kg and the skin wound tissue was excised, fixed with 4% formalin, processed and paraffin embedded. The cross-sections prepared were stained with hematoxylin–eosin (H&E) reagent for histological observations. These histological samples were observed using a vertical optical microscope (Leica model DMR) recording the digital images with a camera (Leica model DFC450C) to study the state of the skin in the different treatment groups.

Photographs taken at days 0, 7, 14 and 21 were analyzed with ImageJ® software to quantify the wound area and to calculate the healing rate in different groups. The percentage of wound closure for each time was determined by equation 6:

$$\%\, wound\ closure = \frac{A_0 - A_t}{A_0} \cdot 100 \qquad (6)$$

Where A_0 is the initial wound area and A_t is the wound area at time t.

The differences between the percentages of wound closure were studied using the statistical ANOVA test of a factor with a significance level p = 0.05. A parametric or non-parametric *post hoc* test was selected based on the homogeneity of the variances determined by the Levene test.

3. RESULTS AND DISCUSSION

3.1 Mechanism of reaction chitosan-6-PG$^-$Na$^+$

The hydrogel is prepared by the ionic interaction between the positive charges of the amino groups of the polymer and the anions of the 6-PG$^-$Na$^+$. The mechanism of formation is based on the fact that chitosan is a weak basic polymer (pKa 6.5) and therefore, at acidic pH chitosan

is ionized. In this same sense, the pKa of the phosphoric group of 6-PG are 1.5 and 6.6, and the pKa of the acid group is 3.4, so at neutral pH the molecule is ionized (Fig 2).

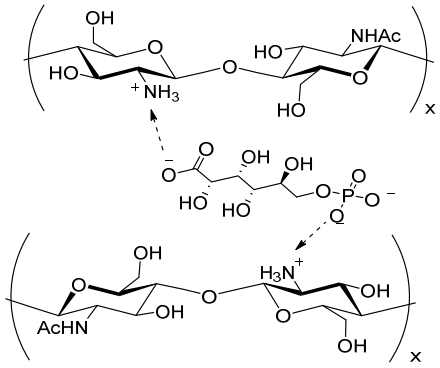

Fig. 2. General mechanism of hydrogel formation based on chitosan crosslinked with 6-PG^{-}Na^{+}

3.2. Swelling

The swelling study reveals the ionic character of the polymer network, since at pH≤4.5 the hydrogel loses its integrity in a few hours (from 2 to 4 hours) (Fig. 3).

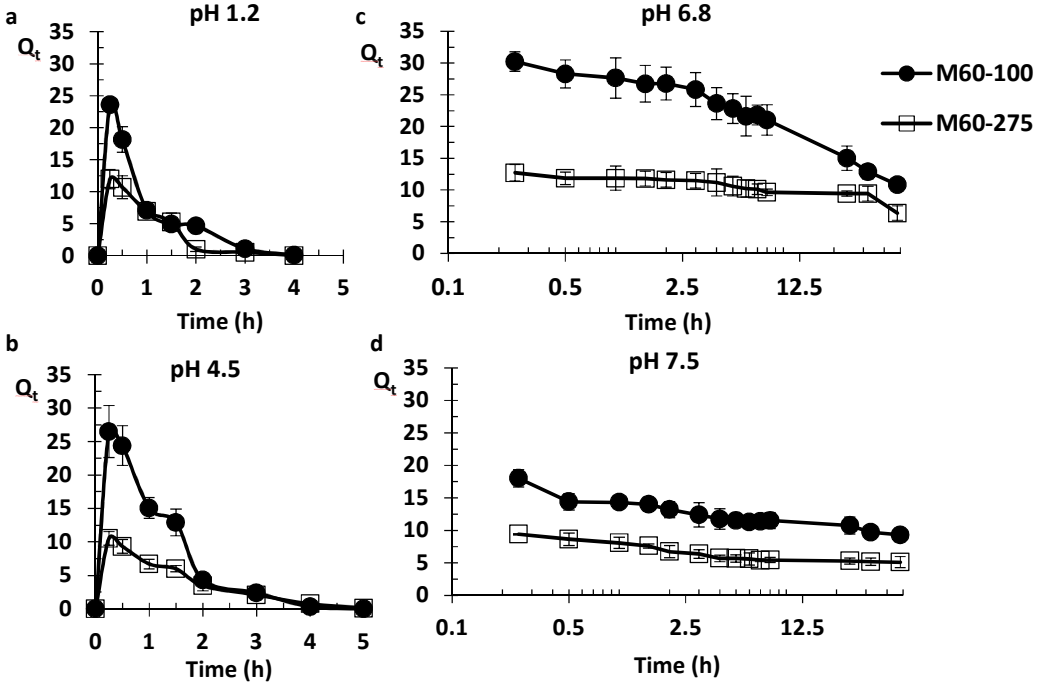

Fig.3. Swelling profiles of samples M60-100 and M60-275 in buffered aqueous medium at: a) pH 1.2, b) pH 6.8, c) pH 4.5 and d) pH 7.5

On the other hand, at pH values close to neutrality, that is, pH 6.8 and 7.5, an overshooting effect is observed, that is, the swelling curves show a maximum following with a gradual decrease until the swelling equilibrium. The difference in the results obtained as a function of pH is due to the different degree of ionization of the functional groups that form part of the crosslinking ionic bond. When the hydrogel is in a relatively neutral pH medium, the 6-$PG^{-}Na^{+}$ molecules have a greater number of negative charges, which favors a greater number of crosslinking interactions. However, at acidic pH 6-$PG^{-}Na^{+}$ does not have sufficient negative charges to maintain polymer cross-linking.

It is also observed that the swelling value is 1.5 to 2.5 times greater for the less crosslinked hydrogel. Therefore, it is concluded that the higher percentage of crosslinking, the lower the swelling is, because the crosslinks between the polymer chains limit the relaxation and expansion of the matrix.

3.3. Release profiles

The release profiles are plotted to compare the influence of pH the percentage of cross-linking (Fig. 4) on drug release. Values of the similarity factor for each of these comparisons are shown in table 2.

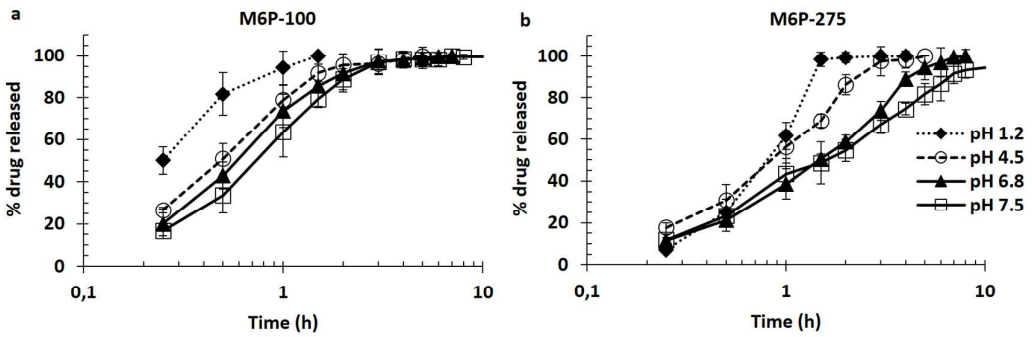

Fig. 4. Piroxicam release profiles as a function of the pH medium for the sample: a) M6P-100 and b) M6P-275

		pH 1.2		pH 4.5		pH 6.8		pH 7.5	
		100	**275**	**100**	**275**	**100**	**275**	**100**	**275**
pH 1.2	**100**		17.24						
	275								
pH 4.5	**100**	30.87			35.45				
	275		39.47						
pH 6.8	**100**	25.57		58.81			28.06		
	275		28.60		37.71				
pH 7.5	**100**	20.85		42.53		55.37			31.89
	275		28.70		36.89		58.95		

Table 2. Values of f2 for the comparisons of profiles as a function of pH and degree of crosslinking

Based on the f2 values it is concluded that there are significant differences between the release from hydrogels with different degree of crosslinking at any pH. Also, differences between the release profiles of the same hydrogel at different pH values were observed, except for M6P-100 at pH 4.5-6.8, and at pH 6.8-7.5, and for M6P-275 at pH 6.8-7.5.

Korsmeyer-Peppas equation was applied to determine values of the diffusional exponent (n) and the kinetic constant (K) (table 3). In general, the diffusional exponent value is between 0.5 and 1, which is consistent with a release influenced by both the diffusion and the relaxation of the polymer chains, called "anomalous transport". Results obtained with Korsmeyer-Peppas suggested the study of other kinetic models, as order one and Peppas-Sahlin to better characterize the release. First order is the kinetic release model that best fits the experimental data, as shown in table 3, which collects the RSS values of these models. This kinetics is representative of the pharmaceutical forms which combined release by Fick diffusion and relaxation/erosion of the polymer matrix, which is in agreement with the experimental observations of matrix disintegration at acidic pH (pH 1.2-4.5). The higher the pH of the medium and higher the crosslinking percentage, the smaller the order one constant values, and, therefore, the slower and longer the release is.

		Korsmeyer-Peppas			**First order**		**Peppas-Sahlin**
		n	**k (h^{-1})**	**RSS**	**k_1 (h^{-1})**	**RSS**	**RSS**
M6P-100	**pH 1.2**	0.71	1.34	$5.08 \cdot 10^{-12}$	3.04	$2.53 \cdot 10^{-3}$	$1.71 \cdot 10^{-2}$
	pH 4.5	0.73	0.80	$1.40 \cdot 10^{-3}$	1.47	$3.91 \cdot 10^{-3}$	$9.67 \cdot 10^{-2}$
	pH 6.8	0.87	0.74	$1.40 \cdot 10^{-3}$	1.20	$6.23 \cdot 10^{-3}$	$1.57 \cdot 10^{-1}$
	pH 7.5	0.95	0.64	$6.10 \cdot 10^{-5}$	0.98	$7.67 \cdot 10^{-3}$	$4.08 \cdot 10^{-1}$
M6P-275	**pH 1.2**	1.39	0.62	$5.79 \cdot 10^{-4}$	1.06	$1.04 \cdot 10^{-1}$	$2.05 \cdot 10^{-1}$
	pH 4.5	0.73	0.53	$2.01 \cdot 10^{-3}$	0.85	$8.31 \cdot 10^{-3}$	$6.00 \cdot 10^{-2}$
	pH 6.8	0.67	0.36	$2.98 \cdot 10^{-3}$	0.48	$6.24 \cdot 10^{-3}$	$4.45 \cdot 10^{-2}$
	pH 7.5	0.57	0.37	$7.67 \cdot 10^{-3}$	0.39	$2.46 \cdot 10^{-2}$	$2.18 \cdot 10^{-1}$

Table 3.RSS values of model fit: Korsmeyer-Peppas, first order and Peppas-Sahlin, and model parameters: Korsmeyer-Peppas (n y k) and first order (k_1)

Our results concerning swelling and drug release agree with those found by other authors [30, 31] that is, as polymer crosslinking increases swelling and drug release decrease. These facts that can be attributed to increased microstructural tortuosity and decreased space between macromolecular chains, as porosity is reduced water entrance is reduced and release slowed.

3.4. Caco-2 toxicity

Despite chitosan are widely described as non toxic and biocompatible molecule [26] it is necessary to perform toxicity assays of the hydrogels in order to verify the toxicity after the gelation process with the linker molecule. There are no statistically significant differences in percentage of cell survival between the different groups, so it is concluded that both the starting components (polymer and crosslinking agent) and the hydrogels lack of toxicity (Fig. 5).

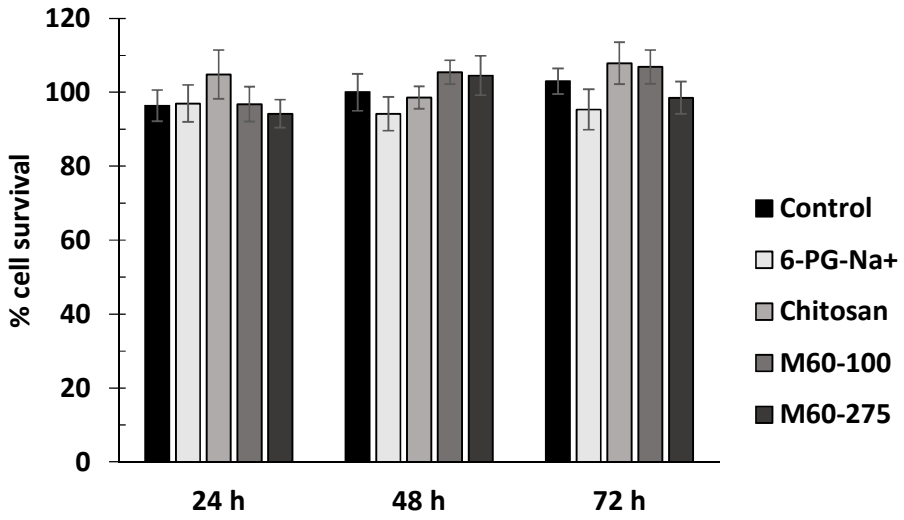

Fig. 5. Percentages of cell survival of Caco-2 cultures at 24, 48 and 72 h in absence (control) and presence of: 6-PG$^-$Na$^+$, chitosan M60-100 and M60-275

3.5. Rheological characterization

It is well known that chitosan forms aqueous solutions with a flow behaviour that is almost Newtonian, not dependent on shear rate, and a fluid-like viscoelastic behaviour [32, 33]. Therefore, this polymer needs a crosslinking agent in order to form rheological gels [34, 35].

The gels studied have a strong shear thinning behaviour, since the viscosity decreases more than four order of magnitude when increasing shear rate. A representative flow curve is shown in Figure 6. The experimental data obtained fit well to Carreau simplified model

$$\eta = \frac{\eta_0}{\left(1 + \left(\frac{\dot{\gamma}}{\dot{\gamma}_c}\right)^2\right)^s} \qquad (7)$$

Where the zero shear viscosity, η_0 corresponds to the plateau of constant viscosity observed for very low shear rates, $\dot{\gamma}_c$, is related to shear rate at which viscosity begins to drop and s is the shear thinning index, what indicates the slope of the viscosity drop in the log-log plot. The mean values obtained for the three replicates are η_0 = 54000 ± 15000 Pa, $\dot{\gamma}_c$ = 0.0028 ± 0.0004 s^{-1} and s = 0.52 ± 0.02.

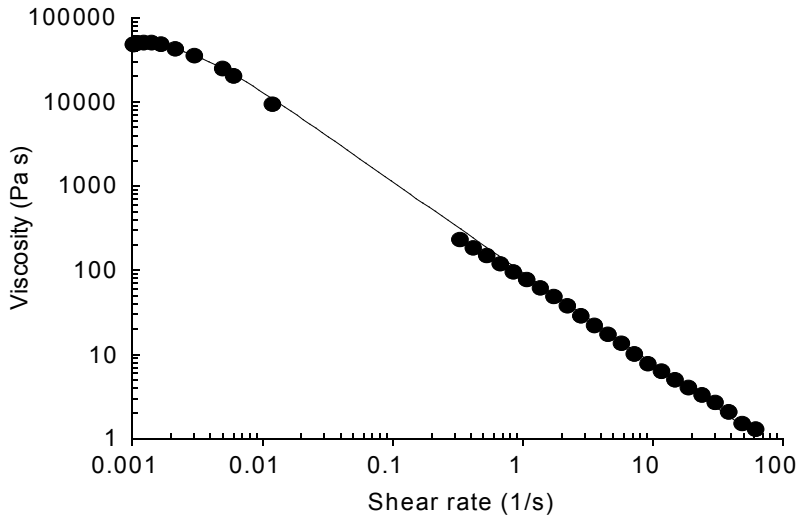

Fig. 6. Representative flow curve for the hydrogels studied. Values of viscosity obtained when applying different shear stresses and different shear rates.

The high value of zero shear viscosity is in agreement with the semisolid consistency of a hydrogel. On the other hand, due to the high shear thinning character the viscosity for rubbing velocities is only 1 Pa s, which implies it is easy extensible, as shown in next section. The hydrogel remained homogeneous within the shear rate range used, prior to centrifugation of the sample.

From oscillatory measurements, the viscoelastic moduli for different oscillation frequencies were obtained. The mean values are plotted in Figure 7. The elastic behaviour dominates the viscous one, as G' > G" [32, 33]. This is typical of gel-like consistency and indicates the existence of an internal three-dimensional network, what confirms the action of the crosslinking agent on the chitosan dispersion.

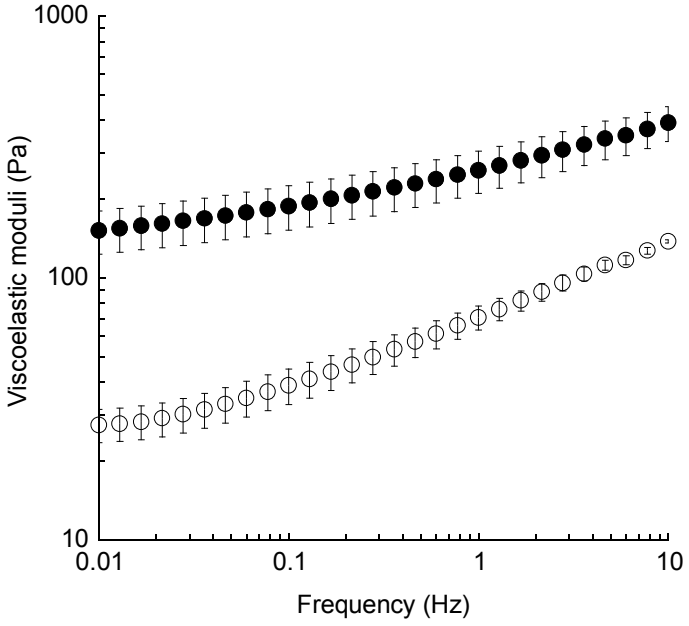

Fig. 7. Mean values and standard errors of the viscoelastic moduli as a function of the frequency of the oscillation performed (stress amplitude= 1 Pa, in LVR). Closed symbols: G', Open symbols: viscous modulus, G".

3.6. Extensibility

Obtaining optimum rheological properties facilitates the extension of the formulation on the epithelial surface creating a film which allows the uniform release of the drug, ensuring the efficacy of the preparation. The extensibility of the hydrogels M60-100 and M60-275 have been compared to the uncrosslinked polymer at the same concentration and with a commercial formulation of carbomer gel (Salvacam®) (Fig. 8).

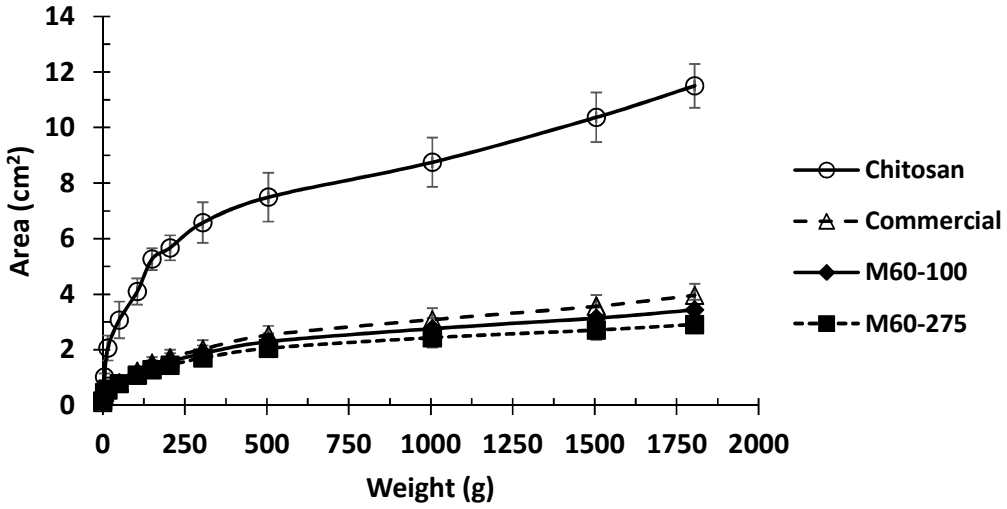

Fig. 8. Values of the extension area of commercial gel, chitosan and hydrogels M60-100 and M60-275 as a function of the applied weight

The fact that the extensibility of the hydrogel formulations is lower than that of chitosan dispersion confirms again the action of the crosslinking agent on the chitosan. As mentioned above, chitosan shows a fluid-like viscoelastic behaviour, and accordingly as expected, it showed high values in the extensibility assay. The hydrogel formulations evaluated have an extensibility similar to that of the commercial gel and are considered suitable for topical application. This property reinforce the good characteristics of the formulation prepared, when re-hydrated, the semisolid consistency of the formulation allows its spreading on skin surface without leaking.

3.6. Adhesion to the skin

The rehydrated hydrogel is a semi-solid formulation with a uniform viscous consistency. After application to the skin, in the form of a thin layer of product, the polymer network dries, so that at 20-30 minutes a transparent film is formed which adheres to the skin, adapting to its shape and with a certain elasticity that allows normal movements. The adhesion to the skin of this polymer film was analyzed by the Loop Tack test, obtaining an adhesion force of 3.17 ± 0.57 Newton.

There is no standard method for performing this measurement. The disadvantage of adhesives is that their removal causes pain, which is greater the greater the adhesive force is. The formulation proposed in this work has adequate adhesiveness to the skin and does not detach when is lightly moistened. However, it can be easily removed from the skin by rehydrating it for a few minutes with a cotton or gauze soaked in water and subsequently with a slight friction without causing pain.

3.7. Dermal irritation

The study of dermal irritation has been performed in rabbits due to the skin of these animals is more sensitive than the human skin; further it is assumed that the compounds which cause corrosion or irritation are similar in humans and in rabbits. The reason for prolonging the exposure until 4 hours is to check if the sensitivity of the skin to the compound increases, so that if it causes irritation is easier to detect. This test may be over-predictive, ie it is a conservative test for irritation/corrosion in humans.

The study was carried out in two rabbits since, according to OECD TG 404, the reduction of 3 to 2 animals tested had no impact on the classification and in the two animals tested the same results were obtained. The rehydrated hydrogel has demonstrated to be non-irritating.

3.8. Permeability

The evaluation of hydrogels as modified drug delivery systems in skin has been performed by determining the amount of drug present in skin and receptor and the permeability constant (Kp). The rehydrated M6P-275 hydrogel has been evaluated in two media of different composition. In formulation 1, the medium is composed of 10% propylene glycol; in formulation 2, the medium is composed of 10% propylene glycol, 10% glycerol and 10% isopropyl alcohol. Table 4 shows the values of permeate per cm^2, the permeability constant and the amount of drug in the skin at 24 hours for these formulations.

			Formulation 1	**Formulation 2**
Permeated drug (mg/cm^2)	**Time (h)**	**14**	$8.34 \cdot 10^{-4} \pm 9.40 \cdot 10^{-5}$	$9.98 \cdot 10^{-5} \pm 3.33 \cdot 10^{-5}$
		16	$9.10 \cdot 10^{-4} \pm 1.11 \cdot 10^{-4}$	$1.73 \cdot 10^{-4} \pm 9.60 \cdot 10^{-5}$
		18	$1.04 \cdot 10^{-3} \pm 1.11 \cdot 10^{-4}$	$2.06 \cdot 10^{-4} \pm 1.32 \cdot 10^{-4}$
		20	$1.15 \cdot 10^{-3} \pm 1.51 \cdot 10^{-4}$	$2.20 \cdot 10^{-4} \pm 1.08 \cdot 10^{-4}$
		22	$1.26 \cdot 10^{-3} \pm 1.95 \cdot 10^{-4}$	$2.82 \cdot 10^{-4} \pm 1.22 \cdot 10^{-4}$
		24	$1.37 \cdot 10^{-3} \pm 1.73 \cdot 10^{-4}$	$2.98 \cdot 10^{-4} \pm 1.21 \cdot 10^{-4}$
Kp (cm/s)			$1.11 \cdot 10^{-5} \pm 2.51 \cdot 10^{-6}$	$3.97 \cdot 10^{-6} \pm 2.23 \cdot 10^{-6}$
Drug in skin (mg/cm^2)			$1.31 \cdot 10^{-2} \pm 8.72 \cdot 10^{-3}$	$3.20 \cdot 10^{-2} \pm 9.89 \cdot 10^{-3}$

Table 4. Amount of drug permeated at each time, permeability constant and amount of drug in skin at 24 hours for formulations 1 and 2

The permeability constant of formulation 1 is significantly higher than that of formulation 2, so the amount reaching the receptor is higher. In contrast, the amount of drug retained on skin at 24 hours is significantly greater for formulation 2.

Based on the results obtained it is concluded that the hydrogel M60-275 allows the administration of drugs at the topical level, without being absorbed significantly. The

permeability of the drug in the skin can be regulated by modifying the rehydration medium of the hydrogel. This polymer system could serve as a vehicle for the topical treatment of cutaneous diseases (such as alterations of pigmentation, bacterial or viral infections, mycoses, ulcers, lesions) or as a cosmetic formulation, being able to limit transdermal absorption to minimize systemic effects.

3.9. Wound healing

Due to the haemostatic, antimicrobial and healing properties of chitosan [36] and regeneration properties of 6-PG^{-}Na^{+},[20] 'lyophilized hydrogel' has been considered as dressing for wound healing. For this, the evolution of surgically created full thickness wounds at dorsum of Wistar rats has been evaluated. The animal model used is one of the most common for healing studies [37-39]. The size of wounds created (3 cm in diameter) has been established based on previous studies [11] which show that when the area is smaller self-regeneration of the tissue dominates the process and the healing effect of the formulations tested is not appreciated. In any case, due to the self-regeneration the most important information of these results is obtained the first days after causing the injury.

Fig. 9 shows the evolution of the wounds for 21 days in the animals treated with the hydrogel and the formulations compared (without treatment, treated with chitosan or with 6-PG^{-}Na^{+}).

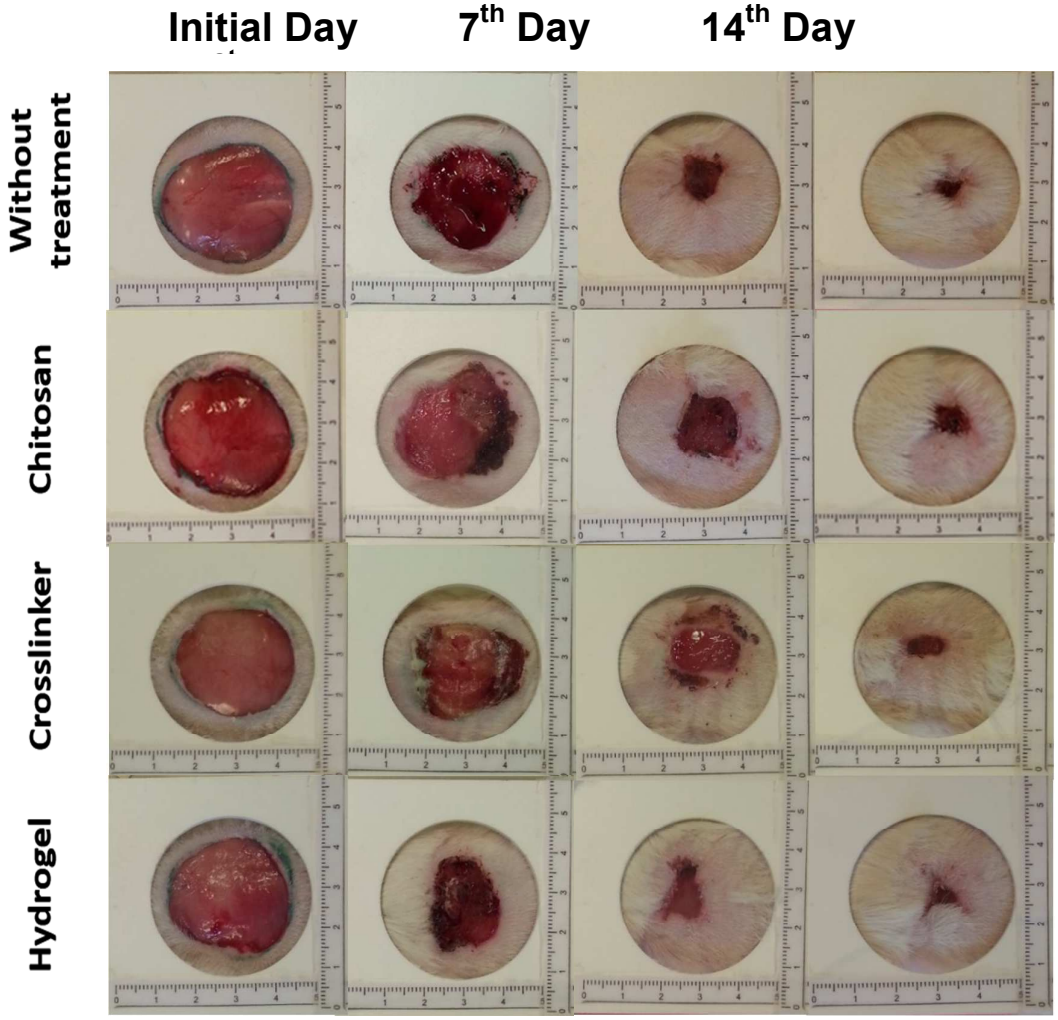

Fig. 9. Evolution of full thickness wounds in the absence of treatment, and in the ones treated with chitosan, with a solution of 6-PG^{-}Na^{+} and with the hydrogel M60-275. Images taken on the day 0 (skin excision), 7, 14 and 21

The percentage of wound closure, referring to the initial area, is collected in table 5 for each time studied.

Day	% wound closure			
	Without treatment	**Chitosan**	**6-PG$^-$Na$^+$**	**Hydrogel**
7	26.10 ± 10.68	13.19 ± 10.76	10.68 ± 6.05	39.79 ± 11.21
14	84.93 ± 5.05	80.84 ± 6.12	74.62 ± 2.01	82.34 ± 7.24
21	96.17 ± 2.19	92.87 ± 2.34	90.26 ± 8.37	91.97 ± 3.24

Table 5. Percentage of wound healing at 7, 14 and 21 days from skin excision in untreated rats and those treated with chitosan, 6-PG$^-$Na$^+$ solution and hydrogel M60-275

Observing the percentages of wound closure, the treatment of the groups with chitosan or with 6-PG$^-$Na$^+$ did not present any advantage over the control group. In the case of the group treated with the hydrogel, at 7 days the area of the wound is lower than the one of the group without treatment, with a percentage of wound closure of 39.79% compared to 26.10% of the group without treatment. At 14 and 21 days after surgical excision of the skin, both groups show a similar percentage of closure, due to the self-regeneration of the tissue. However, microscopic examination of skin samples at the end of the assay reveals that healing is more effective in the hydrogel treated group. Fig. 10.A shows the appearance of healthy skin, in which can be distinguished the dermis and the epidermis with the germinative, granular, spinous layer and stratum corneum. In Fig.10.B is shown the untreated wound, where granulation tissue and a large number of inflammatory infiltrate cells are distinguished; neovascularization is also observed, although the dermis and epidermis are not differentiated. This same observation is applied for the appearance of the wound treated with chitosan and with 6-PG$^-$Na$^+$ (Figure 10. C and D, respectively. The appearance of the wound treated with the

hydrogel is shown in Figure 10.E. In this case below the crust (that is separating from the tissue) the epidermis can be distinguished, with the germinative, granular and spinous layers, and in the dermis neovascularization can be observed.

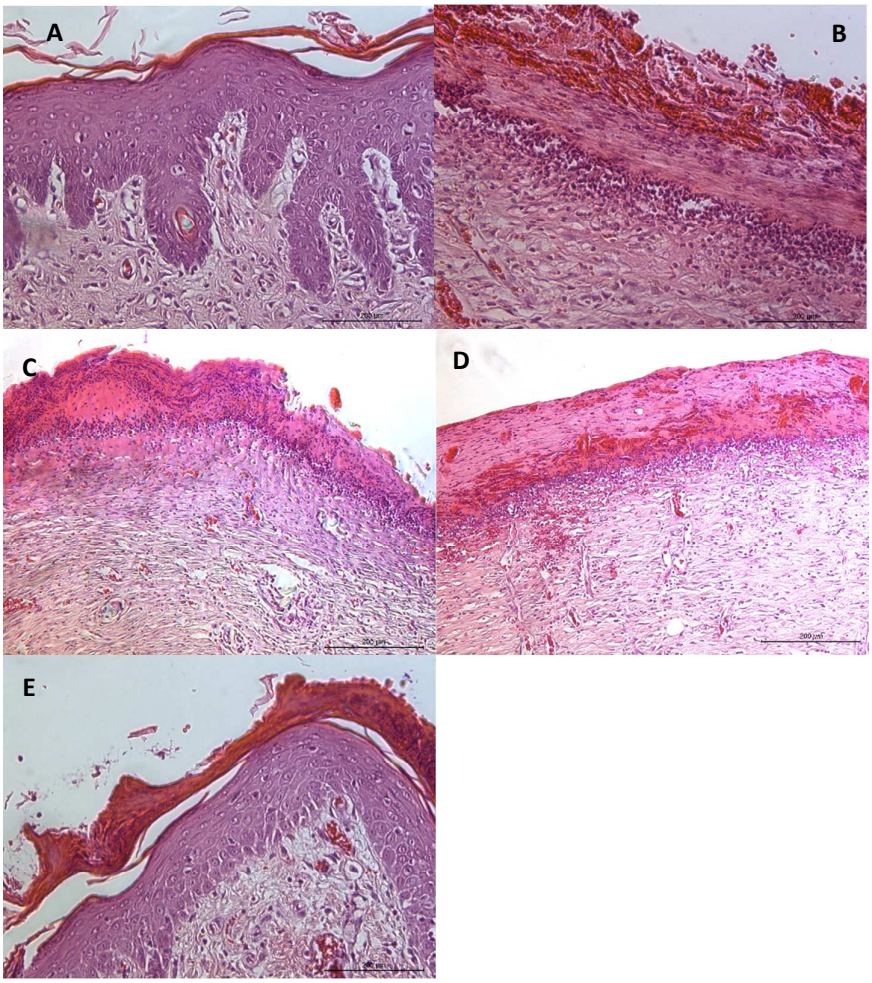

Fig. 10. Histology of skin of the different treatment groups, obtained at day 21. For comparison a full thickness healthy skin has been included (A). B: Wound not treated; C: Wound treated with chitosan dressing; D: Wound treated with 6-PG-Na; E: Wound treated with M60-275 dressing.

Overall, the hydrogel perform several actions that favour wound regeneration, as it creates a permanent moist medium that stimulates cellular activity in all stages of the healing process,

and absorbs the exudate and secretions. The moist medium created by the hydrogel allows the debridement of necrotic tissue, helps tissue regeneration in the granulation stage and favours cell division and activity [40]. Polymer network also serves as an extracellular matrix that facilitates reepithelization [11].

In conclusion, the hydrogel M60-275 applied to full thickness wounds does not cause any damage to the tissues, but favours healing process, so it can be used as dressing for the application of therapeutic substances that promote healing.

4. CONCLUSIONS

This work has shown that the molecule 6-$PG^{-}Na^{+}$, soluble in water, is a promising new agent for chitosan crosslinking and, potentially, for other cationic polymers. The crosslinking reaction between this molecule and the polymer is based on ionic interaction between the anionic groups of the phosphoacid and cationic amino groups. Characterization studies have shown the interaction between chitosan and 6-$PG^{-}Na^{+}$, producing a reduction of thermal stability due to crosslinking and the formation of a polymer framework. It has been found that at pH ≤ 4.5 hydrogel disintegrates within a few hours, probably due to loss of the negative charges of the crosslinker molecule. However, at pH values near neutrality the hydrogel maintains its integrity. The lower the crosslinking percentage, the higher the swelling is. These results agree with those obtained in release studies because the lower the pH and the lower the crosslinking percentage, the faster the drug release is. At pH 7.5 the hydrogel M6P-275 has released 90% of the drug within 7 hours. The release profiles follow a first order kinetics.

It has been also shown that the proposed system is non-toxic in Caco-2 cells and its application does not produce skin irritation. In addition, it has been found that the extensibility properties of the rehydrated formulation are suitable for the cutaneous administration. Its application

over the skin creates a polymeric film like a patch with good adhesiveness properties. This polymer film allows the application of active substances topically, limiting its systemic action.

The lyophilization of the formulation would allow product preservation until its application, keeping stable the hydrogel and the active substance contained therein. This lyophilised formulation can be applied as wound dressing, without previous rehydration, over full thickness wounds. With this mode of use it has been shown, at the microscopic level, that the hydrogel improves healing and regeneration of injured skin. Thus it can be used as wound dressing for wound healing, either alone or, preferably, as a drug application system for substances that promote healing. Moreover, the system can be useful to administrate different drugs on the skin or mucous membranes in order to obtain controlled release profiles.

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The product was patented in Spain in 2016 by authors of this paper. Patent application 201631463.

Author contribution

M. M. M. performed experimental studies, G.R.B performed the synthesis of the hydrogel, A.C. supervised chemical study, M.B provided advice on experimental design and data analysis, I.G.A design *in vitro* and *in vivo* study, data analysis and conclusions of the study she is the corresponding author of the work, M.J.H. performed rheological tests, V.M. designed and performed hydrogel testing *in vitro* and M.G.A. design and supervised the study. MT. M. M.,

I.G.A, M.J.H., V.M and M.G.A. wrote the manuscript. All authors reviewed and commented on the manuscript.

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