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Preparation and characterization of a new gellan gum and sulphated hyaluronic acid hydrogel designed for epidural scar prevention

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Abstract Postsurgical adhesions are a common problem in clinical practice, causing nerve compression, pain and discomfort. A new hydrogel based on gellan gum and sulphated hyaluronic acid was synthesized, with the aim to create an effective barrier for epidural scar formation. Physico-chemical properties of the gel were analyzed, and preliminary biocompatibility data (i.e. cytotoxicity) have been collected in view of its potential clinical use. The characterization of the new material demonstrated that the hydrogel, due to its high-viscosity, could effectively act as a barrier with a long in situ residence time. In addition, the hydrogel can be easily extruded from a syringe and its structure exhibits excellent stabilizing properties. Furthermore, biological assays showed that this gel is suitable for further preclinical development.

1 Introduction

Extensive epidural scar adhesions also referred to as laminectomy membrane, often occur as a laminectomy defect following spinal surgery [1]. Fibrous astrocytes are the support tissue of the spinal cord that produce this scar tissue as a natural wound healing process. The major components of this glial tissue are collagen and sulphated proteoglycans: the first plays a structural role, while the second prevents neurite outgrowth and leaking of dura mater by electrostatic repulsion [2, 3]. Formation of thick dense scar tissue that is adherent to the surgically exposed dura mater and the adjacent nerve roots can interfere with neuromotorial processes and can result in extradural compression or dura tethering, causing recurrent radicular pain and physical impairment [4, 5].

Indeed, for some patients that undergo subsequent surgery, encasement of dura and nerve roots by dense scar tissue makes re-exposure of the same area difficult and dangerous, with a greatly increased risk of nerve root damage and dural tears.

Since scarring is a necessary process of tissue repair, one possibility of avoiding postlaminectomy complications is to reduce the density and thickness of scar tissue and limit adhesions to the dura mater and adjacent nerve roots without affecting the process of wound healing. Nevertheless, the extension of scar tissue has so far been difficult or impossible to prevent by topical or systemic medications. For decades, numerous studies in animal models have focused on the development of materials and methods capable of limiting epidural adhesions. Most of these studies were logically designed to implant a synthetic or organic material into the laminectomy site as a barrier between the exposed dura mater and surrounding muscle tissue. For example, Silastic, Dacron, methacrylate, bone graft, synthetic membranes and foams, free and pedicle fat grafts, and steroids have been utilized [1–16]. However, few of these materials are accepted due to divergent preclinical and clinical results. Only a small number of these materials, such as Gelfoam, Adcon-L, Methylprednisolone and free fat grafts, have been applied to human cases

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[13, 17–19]: furthermore, according to literature, spontaneous postoperative cerebrospinal fluid leaks and potential interference with normal bone healing has been observed for some of these materials [20, 21].

Several publications have suggested that sodium hyaluronate, a naturally occurring polymer with anti-inflammatory properties in the mammalian body that is known to decrease adhesion formation, is effective in preventing spinal adhesion formation in an animal model [22]. In particular, the use of hyaluronan has been shown to decrease epidural scar formation in a preclinical rat laminectomy model at 3 and 8 weeks [23]. In the present study, we used Sulphated Hyaluronic acid (HA-S) as a component for gel preparation; this hyaluronan derivative [24, 25] has been demonstrated to be a potent inhibitor of Tumor Necrosis Factor-alfa and -beta [26]. Thus, the sulphated HA exhibits therapeutic use as an anti-inflammatory agent in the treatment of inflammation and related pathologies. It can also be assumed that the introduction of sulphated groups in the hyaluronan backbone may improve its natural anti-adhesive properties. This is due to the presence of carboxy and sulphated groups that induce strong electrostatic repulsion in the presence of fibroblasts, thus inhibiting cell invasion of the damaged spinal tissue. This property can be thus exploited to reduce the production of an excess of scar tissue. However, sulphated hyaluronan itself cannot be used as a biomaterial for the prevention of post-surgical adhesion because its limited residence time in situ is insufficient for complete prevention of adhesions. For this reason, another component must be added which is capable of controlling the absorption of sulphated hyaluronic acid by the treated tissues, while acting as a support for the biomaterial. Therefore, the derivative was combined with gellan gum, for the preparation of a gel with improved anti-adhesive properties.

In fact, the gellan gum acts as the structural component of the device, maintaining its initial consistency for as long as it effectively exerts its role as an anti-adhesion barrier.

Deacetylated gellan gum is natural polysaccharide capable to form physical hydrogels in the presence of mono- and divalent cations. When hot saline solutions of deacetylated gellan are cooled, a transition from random coil chains to ordered double helix is observed (Coil-Helix Transition): the double helixes then rearrange to obtain ordered junction zones (Sol–Gel Transition) by means of electrostatic interaction with cations [27]. This property makes this polysaccharide suitable as a structuring and gelling agent in foods and for several other uses, such as in the field of controlled release of bioactive materials, mainly for ophthalmic preparation [28, 29].

The target of the present study is to obtain a novel hydrogel capable of "mimicking" natural scar tissue, in order to effectively reduce epidural scar adhesions. In this regard, we prepared an hydrogel composed of gellan gum and sulphated hyaluronan, which act as "structural" and "anti-adhesive" component, respectively. In the present study, this novel potential anti-adhesive device was characterized and its preliminary biological properties were tested.

2 Experimental section

2.1 Materials

Hyaluronic acid sodium salt (HA), with a $M_\eta = 145$ kDa, and sulphated hyaluronan, with a $M_\eta = 200$ kDa and a degree of sulphatation of 3 (HA-S_3), are produced by Fidia Advanced Biopolymers. Deacetylated gellan gum (G) Kelcogel CG-LA was purchased from Kelko: according to the manufacturer, the polymer has a viscosity of $\sim$32 cP ($c_p = 2\%$ w/v in H_2O, T = 85°C), and the content of inorganic mono- and bivalent ions shows that more than 90% of the repeating units were sodium salt derivatives. L929 mouse fibroblast (ATCC CCL 1, NCTC clone 929, derived from strain L, or equivalent form) was from ATCC (American Type Culture Collection). All other chemicals were cell culture or reagent grade from Sigma and Fluka. All chemicals were used without further purification, unless otherwise specified.

2.2 Hydrogel preparation

Several tests were performed to obtain the best formulation. The hydrogel consisting of 2% w/v of gellan gum and 1% w/v of sulphated hyaluronan in physiological saline [G 2% w/v + HA-S_3 1% w/v] was chosen as the best performing composition. The resulting hydrogel is a self-supporting solid, but still extrudable from a syringe [30].

In a typical preparation, 60 mg of G and 30 mg of HA-S_3 were dissolved in 3 ml of physiological saline (NaCl 0.9% w/v H_2O), and stirred at room temperature. The temperature of the suspension was then increased to 80°C for 1 h, and the colorless clear solution obtained was left at room temperature overnight. The material was subsequently sterilized by autoclaving at 121°C for 30 min. Depending upon the type of the tests that followed, the formulation was prepared in a beaker or in a glass-syringe. For comparison, hydrogels of gellan [G 2% w/v] and of gellan and hyaluronan [G 2% + HA 1% w/v] were prepared with the same procedure.

2.3 Methods

2.3.1 Fourier transform infrared spectrophotometry

FTIR spectra have been recorded using a Shimadzu 8300 FT-IR spectrometer (Shimadzu, Yokio, Japan), equipped

with an ATR (attenuated total reflection) Golden Gate device, and controlled by the software Shimadzu IRsolution 1.10. The reported spectra are the arithmetic mean of three measurements of different parts of the same sample, each one obtained with acquisition of 10 scans. The measurements were performed on lyophilized samples.

2.3.2 Conductometric titration

Conductometric titrations were performed on samples of sulphated hyaluronan alone, or mixed with gellan gum, in order to determine the sulphated/carboxyl groups ratio SO_3^-/COO^-. The samples were dissolved in distilled water, and dialyzed against distilled water to remove free sulphated groups. The purified solutions were then ion exchanged with a Dowex resin to obtain the acidic form of the polymers, and finally titrated with 0.02 M NaOH, at 25°C [31].

2.3.3 Water uptake

The ability of a hydrogel to absorb water, or other aqueous solutions, was evaluated according to the following Swelling parameter:

$$S_{H_2O} = w_{H_2O}/w_{dry}$$

where w_{H_2O} represents the equilibrium weight of the hydrogel in water, and w_{dry} the weight of the same lyophilized dry hydrogel.

The value w_{H_2O} was obtained after 10 days of dialysis in distilled water at room temperature, after which the hydrogel was weighed; the sample was then freeze-dried, and weighed again, to obtain the value w_{dry}.

For an aqueous solution x (i.e. physiological saline, NaCl 0.9% w/v), the Swelling parameter is:

$$S_x = w_x/w_{dry}$$

where w_x represents the equilibrium weight of the hydrogel in the solution x.

w_x is obtained after 10 days of dialysis against distilled water, and 24 h against the aqueous solution at room temperature, after which the hydrogel is weighed. S_x is then calculated with the following equation:

$$S_x = w_x/w_{dry} = (w_x/w_{H_2O})/(w_{H_2O}/w_{dry}) = (w_x/w_{H_2O})/S_{H_2O}$$

Swelling measurements were performed three times, and the mean values were reported.

2.3.4 Rheological measurements

Rheological measurements were performed with a Thermo Haake Rheostress RS300, connected to a Thermo Haake DC50 water bath, and controlled by Rheowin 2.96 software. The geometry used for the tests was a cross-hatch plate device (Haake PP35/S Titanium, diameter = 35 mm). All rheological determinations were carried out within the zone of linear viscoelasticity, which was confirmed by the strain sweep tests carried out after the frequency sweep. For each sample, three different rheological measurements were performed, and the mean value was reported.

2.3.5 Extrusion measurements

The extrudability of the material was tested with an INSTRON 4520 instrument, measuring at room temperature the force necessary to extrude the hydrogel from a glass syringe, through a hole of 1.5 mm internal diameter. The linear speed of the piston was set to 60 mm/min, so that the hydrogel was completely extruded in about 30 s. A typical test is reported in Fig. 1. The values of extrusion were calculated as the mean value of the forces in the range between 7 and 30 s, after the end of the transient phase. For each sample, four tests were performed, and the mean value was reported. For a comparison, extrusion measurements were also performed with hydrogels of G alone, without the addition of HA-S_3.

2.3.6 In vitro cytotoxicity

The potential for cytotoxicity of the hydrogel [G 2% w/v + HA-S_3 1% w/v] was evaluated via the analysis of the presence of cell lysis or cytotoxicity when placed

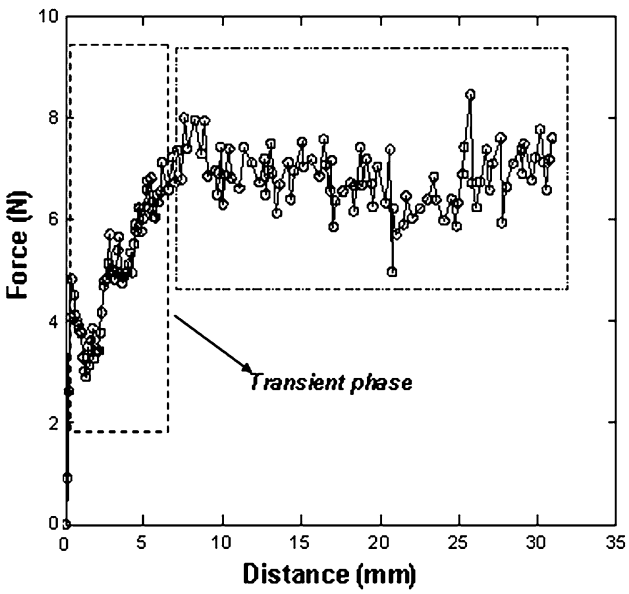

Fig. 1 Typical extrusion curve of a hydrogel from a syringe. After an initial transient phase (about 6–7 s), the force of extrusion is calculated as the arithmetic mean of the values between the seventh second and the end of the test

onto confluent monolayers of L929 mouse fibroblasts. In vitro cytotoxicity tests were performed according to the NF EN ISO 10993 standard for medical device evaluation [32]. Cell culture medium was a Minimun Essential Medium (MEM) supplemented with 10% of Fetal Bovine Serum (FBS), 1% of L-Glutamine and antibiotics.

– *Extract preparation* The biological evaluation of [G 2% w/v + HA-S_3 1% w/v] was performed on an extract of the hydrogel, since it is not soluble in culture medium. The extract was obtained according to the following procedure: the hydrogel was poured in supplemented MEM (200 mg/ml), and the resulting suspension was stirred at 37°C for 24 h. The suspension was then cooled at room temperature, shaken and centrifuged at 200×*g* for 5 min. The supernatant solution was tested immediately after extraction.

Cell cultures have been incubated at (37 ± 1) °C with (5 ± 1) % v/v CO_2 for 24 h in presence of pure and diluted extract (concentrations of 50 and 10% v/v in supplemented MEM), placed onto cell monolayers. In parallel, negative controls (High Density Polyethylene-incubated medium) and positive controls (0.64% Phenol in HDPE-incubated medium) were prepared, and tested both concentrated and diluted. The cytotoxicity has been evaluated with the Neutral Red Uptake (NRU) test. Neutral Red (NR) is a weak cationic supravital dye that readily penetrates cell membranes by non-ionic diffusion and predominately accumulates intracellularly in lysosomes. Toxic substances cause alterations of the cell surface or of the lysosomal membrane, leading to a decreased uptake and binding of NR, making it possible to distinguish between viable, damaged, or dead cells via spectrophotometric measurements. A solution of NR in supplemented MEM was poured on the incubated cell monolayers and, after addition of a lysing solution, the optical density was read at 550 nm.

2.3.7 Hemolysis test

The potential hemolytic behaviour of [G 2% w/v + HA-S_3 1% w/v] was evaluated. Hemolysis tests were performed according to the NF EN ISO 10993 standard for medical device evaluation [32].

– *Extract preparation* The hemolysis tests were performed on an extract of the hydrogel, since it is not soluble in D-PBS (Dulbecco's phosphate buffer saline). The extract was obtained with the following procedure: the hydrogel was poured in D-PBS (200 mg/ml), and the resulting suspension was stirred at 37°C for 72 h. The suspension was then cooled at room temperature, shaken and centrifuged at 200×*g* for 5 min. The supernatant solution was used immediately after extraction.

Human blood (collected on anticoagulant EDTA, kept at 2–8°C and used within 48 h after collection) was obtained from three different donors, pooled and diluted in D-PBS and added to three separate tubes containing the hydrogel extracts. In parallel, negative and positive controls were similarly prepared with D-PBS and sterile water for injection respectively. The tubes were maintained in a static position for 3 h at 37°C. Following incubation, the suspensions were centrifuged. A dosage of hemoglobin was performed by measuring the absorbance of the supernatant at 540 nm. The hemolytic index of the material was calculated taking the ratio between free hemoglobin and total hemoglobin. A material having a hemolytic index up to 2% is considered non hemolytic.

3 Results

3.1 Evaluation of thermal stability for sulphated hyaluronan

The preparation of the hydrogel requires treatment at a high temperature in order to dissolve gellan gum and obtain a homogenous material after cooling of the hot solution. To study the effect of the adopted experimental conditions on the stability of the system, the thermal stability of sulphated hyaluronan was studied. In fact, the high temperature treatment could lead to a degradation of the polymer, specifically, a desulphation could occur, leading to a diminished anti-adhesive behaviour of the polymer. In addition, the presence of free sulphated groups in the hydrogel could also affect the biocompatibility of the formulation.

In order to prove the thermal stability of HA-S_3, the polymer was dissolved at three different temperatures (25, 50 and 80°C) for three hours. The solutions were then dialyzed against distilled water, and freeze-dried. The purified samples were finally analyzed by means of elemental analysis, FT-IR spectroscopy and conductometric titration.

Elemental analysis was performed on the samples dissolved at room temperature and at 80°C. The molar ratio of C/N, C/S and S/N of the samples were calculated from the results of the analysis, and compared with the theoretical values of HA-S_3. As shown in Table 1, thermal treatment does not alter these values, which are very close to the theoretical value.

The three lyophilized samples were analyzed by means of FT-IR spectroscopy and the spectra are reported in Fig. 2. The peaks related to the asymmetric stretching of S–O bond ($v = 1224\ cm^{-1}$) and C–N bond

Table 1 Molar ratio[a] of the samples of HA-S_3 dissolved at 25 and 80°C, and compared with the theoretical values

Molar ratio	T = 25°C	T = 80°C	Theoretical value
C/N	13.94 ± 0.01	13.91 ± 0.01	14
C/S	4.70 ± 0.01	4.51 ± 0.01	4.66
S/N	2.97 ± 0.01	3.08 ± 0.01	3

[a] Obtained with the results of elemental analysis

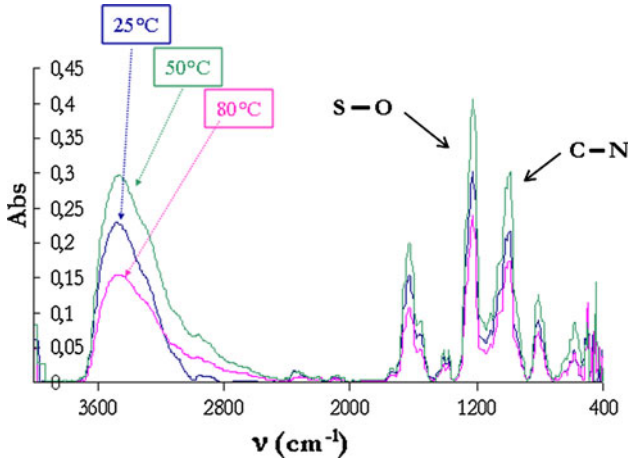

Fig. 2 FT-IR spectra of the lyophilized samples of HA-S_3 dissolved at 25°C (*blue line*), 50°C (*green line*) and 80°C (*pink line*). The peaks related to asymmetric stretching of S–O ($\nu = 1224$ cm^{-1}) and C–N ($\nu = 1020$ cm^{-1}) bonds are shown. Each reported spectrum is the arithmetic mean of 3 spectra, performed from three portions of the same sample (Color figure online)

($\nu = 1020$ cm^{-1}) were observed. A variation in the concentration of sulphated groups should lead to a variation in the ratio between the intensities of the two peaks. As reported in Table 2, the thermal treatment did not affect the ratio of the intensity of the peaks.

Conductometric titrations were performed on the aqueous solutions of the three samples: the values of sulphated/carboxyl groups ratio (SO_3^-/COO^-) are reported in Table 2, and show that the thermal treatment does not affect these values, confirming results of elemental analysis and of FT-IR spectroscopy.

In conclusion, the data collected clearly indicate the stability of HA-S_3 undergoing the adopted experimental thermal treatment.

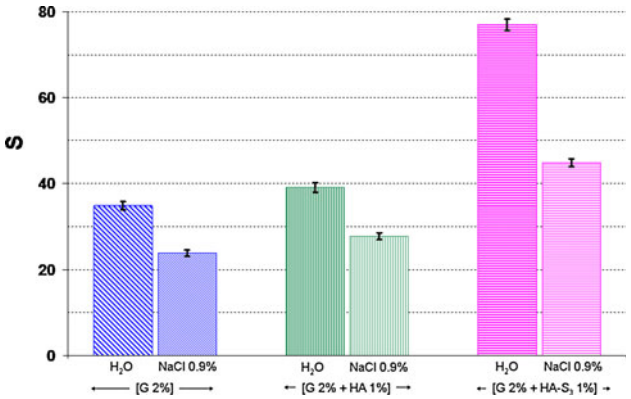

Fig. 3 Water uptake behaviour of different samples in water (*bold lines*) and in physiological saline (*thin lines*). [G 2% w/v]: *diagonal blue line*; [G 2% + HA 1% w/v]: *vertical green line*; [G 2% + HA-S_3 1% w/v]: *horizontal pink line*. Data are presented as mean ± SD (Color figure online)

3.2 Water uptake

Water uptake of a hydrogel represents its hydrophilic nature, and it is an important characteristic for materials used in biomedical applications.

Water uptake was observed, in water and in physiological saline, for different samples: [G 2% w/v], [G 2% + HA 1% w/v] and [G 2% + HA-S_3 1% w/v], all prepared in NaCl 0.9% w/v. For each sample, three different swelling measurements were performed: results are reported in Fig. 3.

The introduction of HA, and HA-S_3, to a greater extent, in the gellan gum scaffolds, causes an increase in the water uptake capacity of the hydrogel. This increases the hydrophilic character of the sample, due to the presence of the negative charges of mucopolysaccharides. Water

Table 2 FT-IR and conductometric measurements of samples of HA-S_3 dissolved at 25, 50 and 80°C

T (°C)	FT-IR measurements[a]			Conductometric measurements[b]
	$A_{S=O}$ (1224 cm^{-1})	$A_{C=N}$ (1020 cm^{-1})	$A_{S=O}/A_{C=N}$	SO_3^-/COO^-
25	0.302	0.208	1.452	3.07 ± 0.10
50	0.409	0.281	1.456	3.09 ± 0.10
80	0.240	0.166	1.445	2.93 ± 0.10

[a] Values of absorbance, and ratio, of the peaks related to the asymmetric stretching of S–O bond ($\nu = 1224$ cm^{-1}) and C–N bond ($\nu = 1020$ cm^{-1}), from the lyophilized samples of HA-S_3 dissolved at 25, 50 and 80°C

[b] SO_3^-/COO^- ratio for the samples of HA-S_3 dissolved at 25, 50 and 80°C

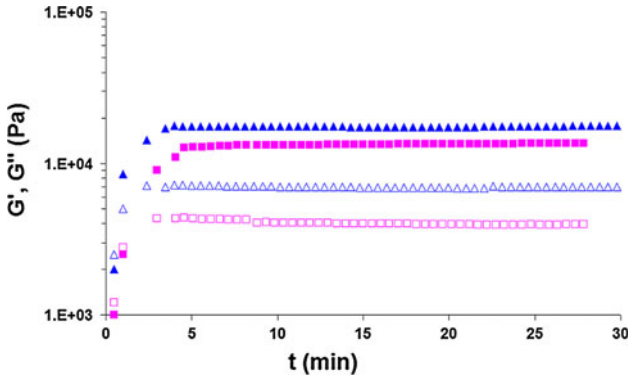

Fig. 4 Gelation kinetics of samples G 2% w/v (*blue triangle*) and G 2% + HA-S_3 1% w/v (*pink square*). Measurements were performed at a constant frequency (f = 0.1 Hz) and temperature (T = 25°C). G′: filled symbols, G″: empty symbols (Color figure online)

uptake in water (S_{H_2O}) is greater than that in NaCl 0.9% w/v (S_{NaCl}). In physiological saline, the hydrogels shrink, due to the presence of Na^+ ions, which act as counterions to the negative charges of the polymeric chains, thus reducing their hydrophilic behaviour.

3.3 Rheological measurements

Rheological characterization was performed on samples [G 2% w/v] and [G 2% + HA-S_3 1% w/v] in physiological saline. In the first test design, hot solutions were poured onto the plates of the rheometer, and the gelation kinetics were observed (Fig. 4). After complete formation of the hydrogel, a frequency sweep test was performed (Fig. 5a). The introduction of HA-S_3 reduces the G′ value of the hydrogel, interfering with the formation of more stable junction zones of gellan gum.

Frequency sweep tests were also performed on the hydrogels extruded from glass syringes (Fig. 5b). In this case, measurements show lower values of G′, due to the fact that the samples are not a compact hydrogel. Nevertheless, the high G′ values obtained confirm the "solid" nature of the extruded material, essential for clinical applications.

3.4 Stability analysis

The shelf-life of [G 2% w/v + HA-S3 1% w/v] was evaluated, in order to determine the long term stability of this material.

To obtain this result, stability analysis was performed on different samples stored at room temperature (for 3, 6, 9 and 12 months), or at 40°C (for 1, 2, 3 and 6 months). Measurements of extrusion and determination of sulphated/carboxyl groups ratio were performed on the various samples.

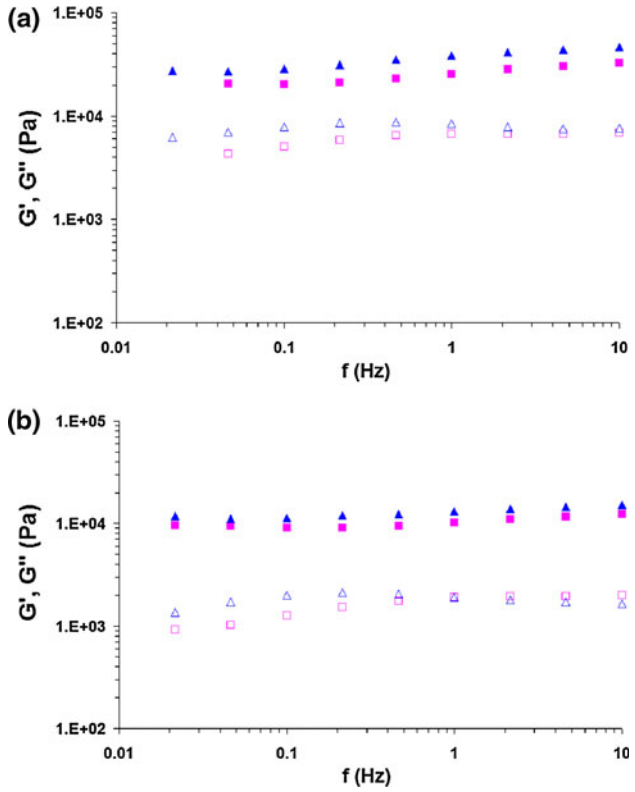

Fig. 5 **a** Frequency sweep test of hydrogels G 2% w/v (*blue triangle*) and G 2% + HA-S_3 1% w/v (*pink square*). Measurements were performed at constant deformation (strain = 1%) and temperature (T = 25°C). G′: filled symbols, G″: empty symbols. **b** Frequency sweep test of extruded hydrogels G 2% w/v (*blue triangle*) and G 2% + HA-S_3 1% w/v (*pink square*). Measurements were performed at constant deformation (strain = 1%) and temperature (T = 25°C). G′: filled symbols, G″: empty symbols (Color figure online)

Three different batches (A, B and C) with a polymeric concentration of G (2 ± 0.2) % w/v + HA-S_3 (1 ± 0.1) % w/v in physiological saline were prepared. The hot solutions were poured into glass syringes and the obtained hydrogels were then sterilized and kept inside the storage chamber.

The results of extrusion measurements are presented in Table 3. The values of extrusion force are the mean of four different measurements, performed on four different hydrogels. The different force values observed between batches A, B and C is due to their different polymeric concentrations.

Considering the single batches, a slight decrease in the value of force of extrusion is observed with time, (i.e., after 12 months at room temperature and after 6 months at 40°C). This behaviour is probably related to a partial degradation of the gellan gum chains. A slight decrease in the molecular weight of the polymer could in fact result in a weaker hydrogel that is extrudable with a lower force.

For comparison, extrusion measurements were performed on different hydrogels of gellan gum alone, G

Table 3 Extrusion forces of batches A, B and C (see the text), stored for 3, 6, 9 and 12 months at room temperature, and for 1, 2, 3 and 6 months at 40°C[a]

Batch A				Batch B				Batch C			
T = 25°C		T = 40°C		T = 25°C		T = 40°C		T = 25°C		T = 40°C	
Time[b]	$Force_{Ext}$ (N)	Time[b]	$Force_{Ext}$ (N)	Time[b]	$Force_{Ext}$ (N)	Time[b]	$Force_{Ext}$ (N)	Time[b]	$Force_{Ext}$ (N)	Time[b]	$Force_{Ext}$ (N)
0	6.9 ± 0.6	0	6.9 ± 0.6	0	11.0 ± 1.0	0	11.0 ± 1.0	0	15.3 ± 0.6	0	15.3 ± 0.6
3	8.1 ± 0.8	1	7.4 ± 0.5	3	10.4 ± 0.4	1	8.1 ± 0.5	3	17.0 ± 2.0	1	19.0 ± 3.0
6	6.7 ± 0.3	2	7.9 ± 0.6	6	11.0 ± 1.0	2	7.7 ± 0.5	6	16.5 ± 0.5	2	16.0 ± 1.0
9	6.6 ± 0.3	3	6.8 ± 0.7	9	9.2 ± 0.3	3	8.2 ± 0.3	9	15.0 ± 1.0	3	14.0 ± 2.0
12	6.9 ± 0.3	6	4.6 ± 0.5	12	9.2 ± 0.6	6	7.2 ± 0.3	12	14.7 ± 0.6	6	12.0 ± 2.0

[a] Each value represents the arithmetic mean of 4 measurements (±SD)

[b] Time in months

Table 4 SO_3^-/COO^- ratio of the batches A, B and C (see the text), stored for 3, 6, 9 and 12 months at room temperature, and for 1, 2, 3 and 6 months at 40°C[a]

Batch A				Batch B				Batch C			
T = 25°C		T = 40°C		T = 25°C		T = 40°C		T = 25°C		T = 40°C	
Time[b]	SO_3^-/COO^-	Time[b]	SO_3^-/COO^-	Time[b]	SO_3^-/COO^-	Time[b]	SO_3^-/COO^-	Time[b]	SO_3^-/COO^-	Time[b]	SO_3^-/COO^-
0	2.14 ± 0.10	0	2.14 ± 0.10	0	1.97 ± 0.10	0	1.97 ± 0.10	0	1.91 ± 0.10	0	1.91 ± 0.10
3	1.79 ± 0.10	1	2.60 ± 0.10	3	1.87 ± 0.10	1	1.81 ± 0.10	3	2.76 ± 0.10	1	2.39 ± 0.10
6	2.58 ± 0.10	2	1.93 ± 0.10	6	2.45 ± 0.10	2	2.13 ± 0.10	6	2.44 ± 0.10	2	2.40 ± 0.10
9	1.84 ± 0.10	3	1.88 ± 0.10	9	2.05 ± 0.10	3	2.32 ± 0.10	9	2.69 ± 0.10	3	2.44 ± 0.10
12	2.55 ± 0.10	6	2.45 ± 0.10	12	2.21 ± 0.10	6	2.35 ± 0.10	12	2.58 ± 0.10	6	2.55 ± 0.10

[a] Each value represents the arithmetic mean of 3 measurements (±SD)

[b] Time in months

(2 ± 0.2) % w/v in physiological saline. It was observed that for these materials the forces of extrusion (16.0 ± 1.0 N for the lower concentrations, 19.0 ± 1.0 N for the higher ones), were greater than those for the hydrogels containing HA-S_3. These results confirm that the sulphated hyaluronan chains interfere with the formation of more stable junction zones of gellan gum, thus leading to a more easily extrudable hydrogel.

The values for sulphated/carboxyl groups ratio obtained by conductometric titrations are reported in Table 4.

These values are the mean of three different measurements, performed on three different hydrogels. The differences between the batches A, B and C are due to their different polymeric concentrations, as already shown for the extrusion measurements. A slight increase in the SO_3^-/COO^- ratio is observed with time, within the single batch, (i.e., after 12 months at room temperature and after 6 months at 40°C). This behaviour, besides confirming the results of the extrusion tests, is probably related to a partial degradation of gellan gum chains. The low molecular weight chains of gellan gum are lost during the dialysis process, leading to a slight decrease of carboxyl concentration, and to an increase of the SO_3^-/COO^- ratio.

3.5 Biological tests

3.5.1 In vitro cytotoxicity

In order to evaluate the potential cytotoxicity of [G 2% w/v + HA-S_3 1% w/v], samples of gel extracts were tested via an in vitro mammalian cell culture test. Under the tested conditions, the hydrogel extract of met the requirements of the test. In this respect, a decrease in cell density of over 25% was not detected in any of the tested dilutions. Negative and positive controls behaved as expected, and the results are reported in Table 5. Each value represents the arithmetic mean of three measurements (±SD).

3.5.2 Hemolysis test

This study was carried out to evaluate the potential hemolysis behaviour of [G 2% w/v + HA-S_3 1% w/v]

Table 5 Results of cytotoxicity tests, obtained by measurements of Optical Density (OD) of Neutral Red, at a wavelength of 550 nm

	[G 2% w/v + HA-S_3 1% w/v] extract (%)	Positive control[a] (%)	Negative control[b] (%)
Pure material	<25	95.1	0
Conc = 50% v/v	<25	96.6	0
Conc = 10% v/v	<25	44.3	0

[a] 0.64% Phenol in High Density Polyethylene-incubated medium

[b] High Density Polyethylene-incubated medium

Table 6 Results of hemolysis tests, obtained by spectrophotometric reading at 540 nm of the released hemoglobin

[G 2% w/v + HA-S_3 1% w/v] extract (%)	Positive control[a] (%)	Negative control[b] (%)
0.94	99.29	0.17

[a] Distilled water for injection

[b] D-PBS

through contact with human red blood cells. The mean hemolytic index for the hydrogel extract was 0.94%, indicating that it is not haemolytic. Negative and positive controls behaved as expected, and the results are reported in Table 6. Each value represents the arithmetic mean of three measurements (±SD).

4 Discussion

The rheological measurements show that the behaviour of gellan gum hydrogels [G 2% w/v] and [G 2% w/v + HA-S_3 1% w/v] is typical of strong hydrogels.

The introduction of HA-S_3 reduces both the G′ value of the hydrogel, and its force of extrusion, interfering with the formation of more stable junction zones of gellan gum, thus leading to a decrease of the hydrogel brittleness.

Moreover, the introduction of HA-S_3 increases the hydrophilic character of the sample, due to the presence of the negative charges of mucopolysaccharides.

Frequency sweep tests performed on the hydrogels extruded from glass syringes confirm the "solid" nature of the extruded material, essential for clinical applications: the lower values of G′ are due to the fact that the samples are not a compact hydrogel.

The extrusion measurements and the conductometric titration, performed to evaluate the shelf life of the material, show that for all the different batches a slight decrease in the value of force of extrusion and a slight increase in the SO_3^-/COO^- ratio are observed with time: this behaviour is probably related to a partial degradation of the gellan gum chains.

The evaluation of the thermal stability of HA-S_3, studied by means of elemental analysis, FT-IR spectroscopy and conductometric titration, shows that the sulphated polysaccharide is not affected by the adopted experimental thermal treatment.

Finally, the biological tests performed on extracts of [G 2% w/v + HA-S_3 1% w/v] showed biocompatible properties: in the in vitro cytotoxicity test a decrease in cell density of over 25% is not detected in any of the tested dilutions, and the hemolysis tests indicates that the sample is not haemolytic.

5 Conclusions

In order to face the problem of post-surgical epidural adhesion, a new hydrogel composed of G 2% w/v + HA-S_3 1% w/v in physiological saline was prepared. The presence of the sulphated polymer adds hydrophilic properties to the gellan gum structure and, by interfering with the junction zones of the gellan gum, allows to obtain an hydrogel with improved mechanical and rheological properties, more easily extrudable from a syringe for surgical use.

The material maintains a very high elastic modulus value, fundamental to ensure a long residence time in situ. Accordingly, it shows good stability even when stored at room temperature up to 12 months. The variations in the force of extrusion and (SO_3^-/COO^-) ratio is minimal, and does not affect the rheological behaviour of the hydrogel, or the entanglement of the sulphated hyaluronan within the gellan gum structure, and consequently its anti-adhesive properties.

Biological testing demonstrated encouraging biocompatible properties, therefore extensive biocompatibility and biofunctionality studies have been designed in order to assess the efficacy of the hydrogel in preventing epidural fibrosis formation and to confirm material tolerability profile in in vivo models.

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