POLYSACCHARIDE TISSUE FILLERS CROSSLINKED WITH RESVERATROL

Technical field

The present invention concerns polysaccharides crosslinked with activated resveratrol derivatives and processes for the preparation thereof. The crosslinked polysaccharides of the invention in the form of monophasic gels are useful in the field of cosmetic surgery and cosmetic medicine as tissue fillers and for tissue augmentation.

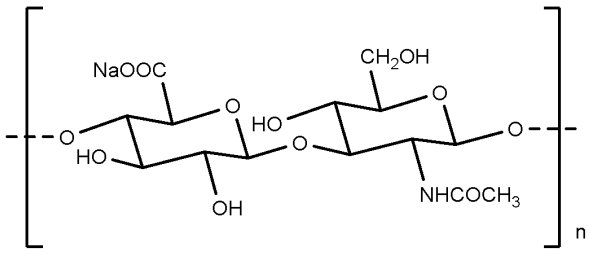
Background of the invention

Dermal fillers are used for cosmetic treatments to effectively reduce the appearance of unwanted wrinkles, to contour and create volume, and to revitalise the skin. Several dermal fillers are based on hyaluronic acid (HA), one of the fundamental components of animal tissues. HA hydrates the skin thanks to its ability to retain water and tonicity, due to its properties of aggregating the extracellular matrix. Its deficiency weakens the "scaffolding" of the skin, with a consequent reduction in tone, hydration and strength.

Increasing interest in safer products in the field of cosmetic surgery and cosmetic medicine applications has expedited the progress and development of hyaluronic acid-based compounds, as HA exhibits remarkable properties, including renewability, biodegradability, and non-antigenic, non-carcinogenic and immunogenic properties.

Hyaluronic acid (HA) is a glycosaminoglycan consisting of repeating monomers (glucuronic acid sodium salt and N-acetylglucosamine disaccharide units) linked by 1β,4' glycosidic bonds (Formula 1).

Formula 1. Repeating monomers of hyaluronic acid.

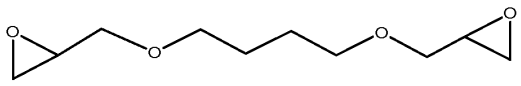


The main drawback of using hyaluronic acids of different molecular weights, in pure form, as fillers in cosmetic surgery and cosmetic medicine, is usually related to their weak mechanical properties, because in the presence of water, hyaluronic acid does not exhibit biomechanical properties, forming solutions and not gels.

In order to obtain a gel able to support the weight of a tissue and lift it, HA must be chemically transformed. The hyaluronic acid-based gels of conventional known fillers are prepared by industrial processes so as to provide suitable biomechanical properties (viscosity and elasticity), allowing their integration into the tissues.

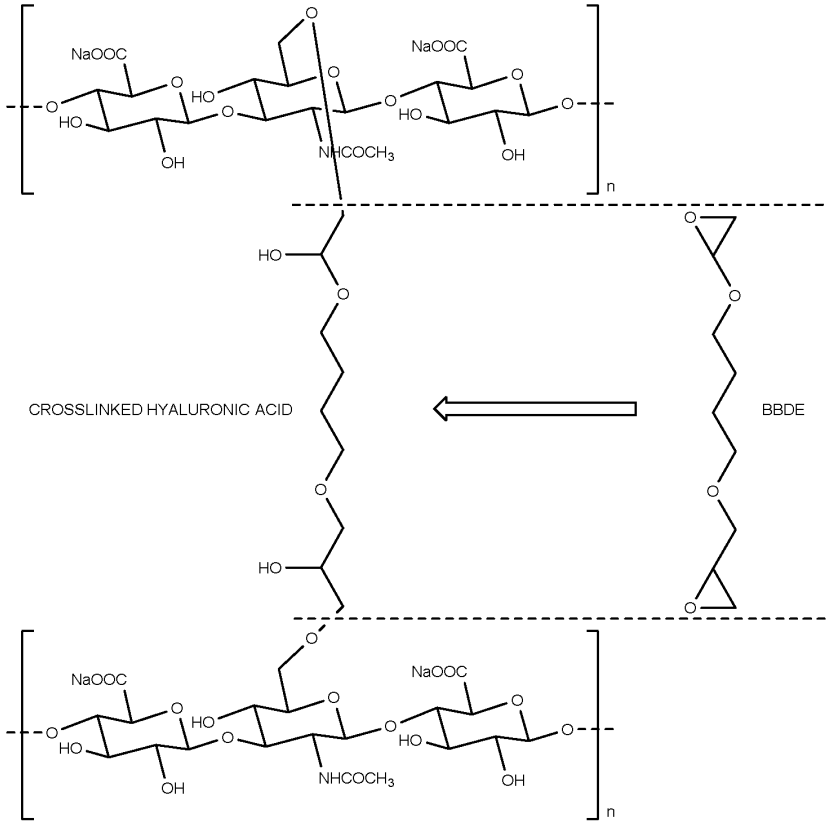
During the manufacturing process of a hyaluronic acid-based gel filler, various chemical linkers can be used, such as 1,4-butanediol diglycidyl ether (BDDE; Formula 2a) (Dermatol Surg 2013;39:1758-1766).

Formula 2a.



The number of crosslinking molecules (Scheme 1) and the type of bond they form will make the gel soft, dense or hard; the greater the strength and numerical value of the bonds, the higher the rigidity and hardness of a gel; conversely, weaker and numerically lower bonds will result in a softer gel.

Scheme 1. Crosslinking between hyaluronic acid and BDDE.

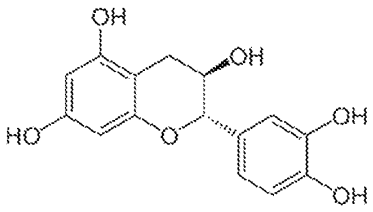


Examples of hyaluronic acid gel fillers, prepared using BDDE as crosslinking, are disclosed, for instance, in WO2017/016917, WO2005/097218; WO2012/062775, WO2013/028904, WO2013/040242, WO2016/051219, WO2009/018076; WO2017/001056, WO2017/162676, WO2016/074794, WO2013/185934, WO2017/001057, WO2018/083195, WO2017/076495.

Although the metabolism of hydrolysed BDDE is not described in the literature, it is understood to proceed through ether bond cleavage by a family of enzymes called cytochromes P450, involved in the oxidative degradation of organic molecules which catalyse the cleavage of ether bonds into alcohols. After degradation, glycerol and 1,4-butanediol are mainly obtained. The long-term effects of 1,4 butanediol are unknown; however, the literature data confirm (N Engl J Med, Vol. 344, No. 2 January 11, 2001, 87-94) that, when ingested, it is converted to γ-hydroxybutyrate, a recreational drug with depressant effects, primarily on the central nervous system.

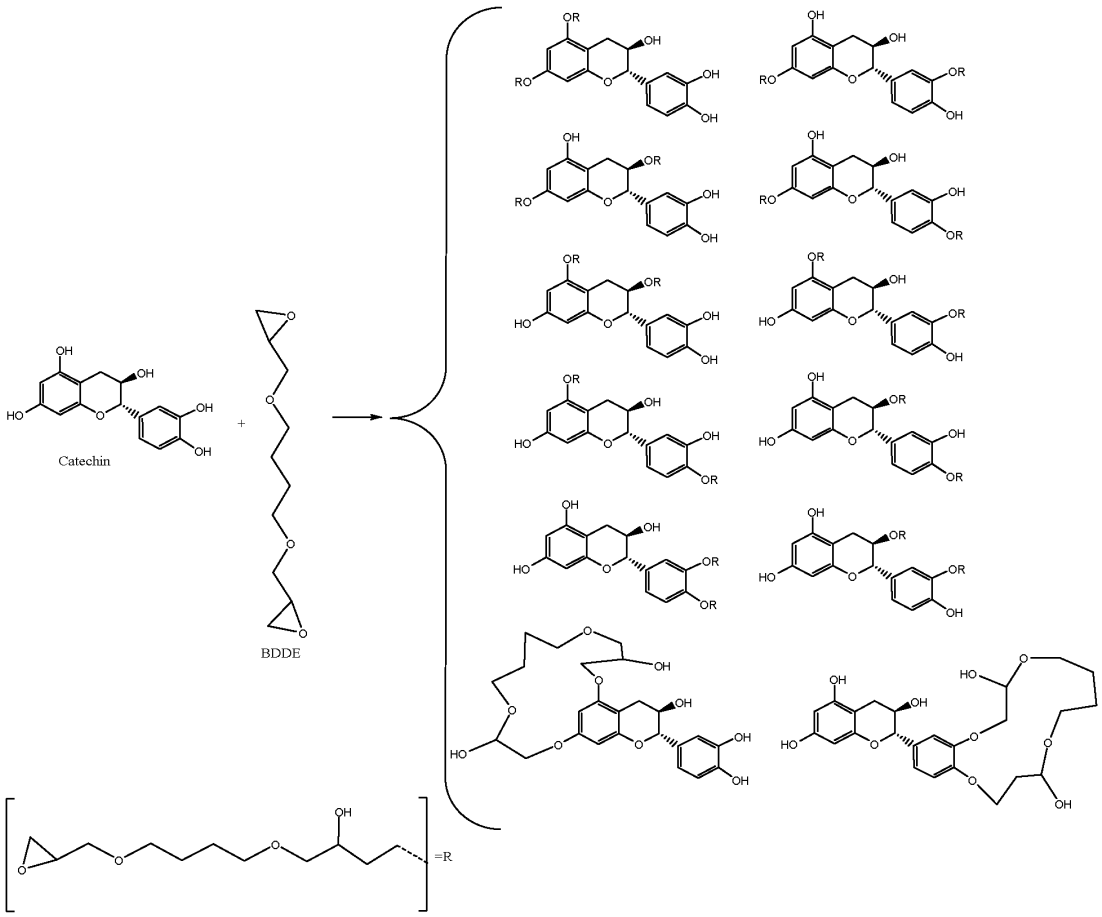
Other crosslinking agents used for the preparation of hyaluronic acid gel fillers include boronic acid derivatives, belonging to the class of alkylboronic hemiesters (WO2018/024795); diamines and polyamines and carbodiimide (WO2013/040242); citric acid (WO2018/087272); endogenous amines, like spermine and spermidine, and as coupling agent *N*-ethyl,*N*-(dimethylaminopropyl)-carbodiimide (WO2014/064632); divinyl sulphone (WO2005/066215); hyaluronic acid gels obtained by self-assembly, wherein the carboxy groups are activated to react with alcohol groups present on the same polysaccharide chain or other nearby chains (EP0341745); multicomponent condensation products obtained by reaction involving the carboxy groups and amino groups originating from partial *N*-deacetylation of HA or derivatives, together with an aldehyde and an isocyanide (WO0218450); formaldehyde, glutaraldehyde, divinyl sulphone, polyanhydrides, polyaldehydes, polyhydric alcohols, carbodiimides, carboxylic acid chlorides, sulphonic acid chlorides, epichlorohydrin, ethylene glycol, butanediol diglycidyl ether, diglycidyl ether, polyglycerol polyglycidyl ether, polyethylene glycol diglycidyl ether, polypropylene glycol diglycidyl ether and bis- or polyepoxides, preferably in the presence of butanediol diglycidyl ether or divinyl sulphone (EP1837347).

KR 2018 0010361 discloses crosslinked hyaluronic acids obtained by reacting hyaluronic acids with 1,4-butanediol diglycidyl ether (BDDE) and catechin (Formula 3).



Formula 3

The stoichiometry of the reaction disclosed in KR 2018 0010361 is 1 mole of catechin hydrate to 2 moles of BDDE, suggesting that two chains functionalised with epoxy groups are introduced on the phenolic compound (catechin), and that the subsequent reaction with HA occurs *in situ* without isolating the BDDE-activated catechin. This approach does not secure the reproducibility of the process, since of the four different equivalent phenol groups present on the catechin, only two react statistically with BDDE, yielding a complex mixture of BDDE-activated catechin. Moreover, the BDDE-activated catechin may react not only with the hydroxy groups of HA but also intramolecularly with the free phenol group of BDDE-activated catechin, reducing its ability to react with HA. This complex reaction mixture consists of at least twelve compounds (Scheme 2), which are difficult to analyse and standardise.



Scheme 2. Reaction mixture of catechin with BDDE (1:2 stoichiometry)

KR 2016 0031081 discloses hyaluronic acids functionalised with polyphenols wherein the polyphenol moiety does not act as a crosslinker.

Shin et al., Materials Today Bio, 2022, page 100386, discloses a hyaluronic acid crosslinking method that uses the "click chemistry" technique to obtain a hydrogel of crosslinked hyaluronic acid containing resveratrol not covalently bonded to the functional groups of hyaluronic acid.

In view of the cited prior art, there is a need for improved crosslinked hyaluronic acid derivatives obtainable by a more efficient synthesis approach allowing their preparation, analysis and isolation in pure form and not as a complex mixture, minimising or eliminating the intramolecular reaction that impairs the crosslinking reaction.

Description of the invention

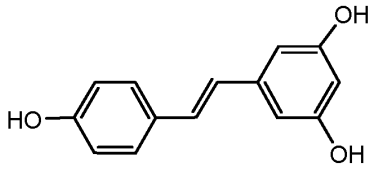
The present invention discloses gels of hyaluronic acid crosslinked with activated resveratrol, obtained by a process allowing efficient incorporation of the crosslinking agent.

The crosslinked hyaluronic acids according to the invention possess anti-inflammatory and antioxidant properties, sufficient gel strength to resist deformation and migration when implanted, exhibiting greater stability to thermal sterilisation treatments and enzymatic hydrolysis than native HAs.

A further object of the present invention is a process with modular efficiency of the crosslinking reaction.

The crosslinked hyaluronic acid gels according to the invention were prepared with various types of hyaluronic acid having a molecular weight ranging between 8 and 3.0 MDa.

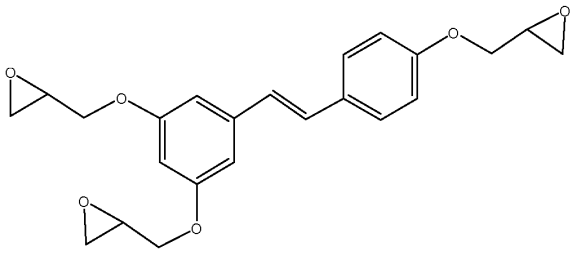
Resveratrol is a natural polyphenol existing in two isomeric forms, *cis*- and *trans*-resveratrol. The *trans* form, also named (*E*)-resveratrol, is prevalent (compound 1).



Compound 1:

Resveratrol possesses a wide range of biological properties, namely antioxidant, cardioprotective, neuroprotective, anti-inflammatory and antitumoral activities (Food Sci. Technol. 2015, 63, 1254-1260; J. Sep. Sci. 2009, 32, 2979-2984; Oxid. Med. Cell. Longev. 2015, 2015, 803971).

Tri-*O*-triglycidylresveratrol (compound 2) is known () and is commercially available (AA Blocks LLC, United States; A2B Chem, United States; Specific Polymers, France). Several syntheses of compound 2 have been disclosed (ACS Sustain. Chem. Eng. 2020, 8(37), 14137-14149; Ind. Crop. Prod. 2022, 187(Part B), 115500; J. Clean Prod. 2021, 323,129194; Chem. Eng. J. 2020, 383, 123124; Eur. Polym. J. 2021, 147, 110282; WO2014152714; US20160032043; JP2020084088; JP2020084086; FR3030514; CN115304745; CN114736354; CN114214017; CN113603884; CN113292703; CN109467674).



Compound 2

Few examples wherein resveratrol is used to form polymers with hyaluronic acid are reported. An injectable hydrogel consisting of a non-crosslinked polymer formed from oxidised hyaluronic acid (oxi-HA) and resveratrol was disclosed in J Biomed Mater Res Part A 2013:101A:3457-3466.

RU2745124 discloses a method for mechanochemical synthesis of a bioactive dry powder produced by crosslinking a hyaluronic acid salt with resveratrol. The ratio between HA and resveratrol ranges from 99.5:0.5 to 98.5:1.5 by weight. When the product is dissolved in water (2% wt. solution), a transparent gel is formed.

The intratumoral therapy using an injectable hydrogel of resveratrol-loaded click-crosslinked hyaluronic acid (Res-Cx-HA) for the treatment of triple-negative breast cancer (TNBC) was disclosed in Materials Today Bio 16 (2022) 100386, commented on above. Said formulation demonstrated effective injectability into the tumour, rapid formation of a Res-Cx-HA depot within the tumour, and the sustained presence of the depot for extended periods after injection.

In the referenced articles and patents, the tri-O-triglycidylate derivative of resveratrol was not used as crosslinking agent for HA, only for the preparation of thermosetting crosslinked epoxy resins with high thermal stability and intrinsic flame-retardant properties, for use in the electronics or aerospace industries.

Compared with BDDE, the compound most commonly used in the preparation of commercially available crosslinked hyaluronic acid polymers, the tri-O-triglycidylate derivative of resveratrol has the advantage of being able to form three bonds with the hyaluronic acid chains instead of the two bonds of BDDE, therefore providing greater crosslinking capacity. Moreover, compared with other polyphenols of natural origin used as crosslinking agents for the preparation of hyaluronic acid derivatives as dermal fillers (WO2023165165), the absence of saccharide portions in the tri-*O*-triglycidylate derivative of resveratrol greatly simplifies the preparation, since the product is soluble in organic solvents and therefore easily recoverable from the aqueous reaction medium; possible side reactions with the hydroxy groups present in the saccharide portions are also avoided, preventing the possible formation of self-condensation products.

It has now been found that tri-O-triglycidylresveratrol is an efficient crosslinker of hyaluronic acid, allowing modulation of the percentage of moles of resveratrol incorporated per unit of repeated hyaluronic acid unit (glucuronic acid sodium salt and *N-acetylglucosamine* disaccharide unit) in the range of 0.3-3.4%. Within said range, the resulting rheological parameter known as phase angle (also known as tan(δ), defined as follows: tan(δ) =G”/G’; G’

being the storage modulus and G" the loss modulus) ranges between 0.11 and 0.26; said data clearly indicate that the crosslinked hyaluronic acid derivatives obtained are in the physical form of a gel.

Detailed description of the invention

The crosslinked hyaluronic acids according to the present invention are obtained from hyaluronic acid having an average molecular weight falling within the range (Mn) of 1.5-1.8 MDa, 1.5-3.0 MDa or 250-450 kDa. The process for preparation of the crosslinked hyaluronic acid derivatives comprises the reaction of the hyaluronic acids as sodium salt in a basic aqueous solution with tri-O-triglycidylresveratrol in a water-miscible polar organic solvent selected from 1,4 dioxane, dimethyl sulphoxide, *N,N-dimethylformamide* and dimethoxy ethane, preferably dimethyl sulphoxide, at a temperature ranging from 30 to 70°C, preferably 50°C, for a reaction time ranging between 0.5 and 4 hours, preferably between 1.5 and 4 hours, and preferably between 1 and 2 hours. The basic acid solutions comprise dilute solutions of sodium hydroxide, sodium carbonate or potassium hydroxide, preferably sodium hydroxide solutions, in a concentration range between 0.10 and 0.5 molar, preferably 0.25 molar, and the final concentration of the hyaluronic acid sodium salts in the reaction mixture ranges between 2 and 10% by weight, preferably 5%. The percentage molar ratio between the activated resveratrol derivative and the repeating units of hyaluronic acid ranges from 2 to 20%, yielding a modification degree (MoD), expressed as percentage ratio between moles of resveratrol and moles of repeating hyaluronic acid units, ranging between 0.30 and 3.4. The crosslinked hyaluronic acids obtained according to the invention have a degree of crosslinking giving a ratio of viscous modulus (G") to elastic modulus (G') of less than 1.0, and a purity, determined by the carbazole method, higher than 80%. The hyaluronic acid derivatives according to the invention, obtained as powder by precipitation from water/ethanol or water/acetone mixtures and drying, can be formulated as an injectable gel in water at a concentration ranging between 1 mg/mL and 50 mg/mL, optionally in the presence of an anaesthetic, preferably lidocaine, at a concentration ranging from 0.1 to 0.4% weight/volume. In the final formulation, the crosslinked hyaluronic acid derivatives can be prepared by mixing crosslinked hyaluronic acid derivatives with molecular weights ranging between 0.25 and 3.0 MDa. Said formulations may be suitably sterilised by heat or gamma radiation treatment.

Brief description of Figures

Figure 1: Working curve of resveratrol chromophore

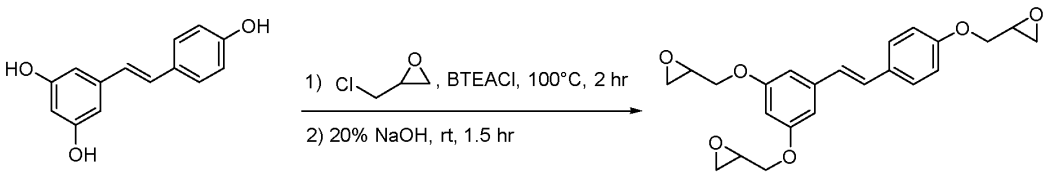
Figure 2: Working curve of hyaluronic acid

Figure 3: Graphs of data shown in Table 5

Figure 4: Extrusion force

Experimental section

Preparation of tri-O-triglycidylresveratrol

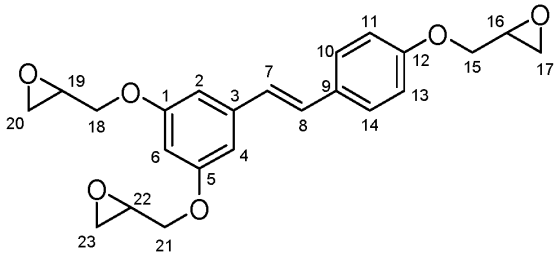


resveratrol (**1**)

tri-O-triglycidilresveratrol (**2**)
81% yield

The reaction was carried out in an inert atmosphere (argon) to avoid oxidation, on 0.5, 2 and 3 g of resveratrol **1**, obtaining **2** in 75, 81 and 81% yields respectively. A mixture of resveratrol **1** (3g, 13.14 mmol), epichlorohydrin (18 mL, 21.29 g, 231.5 mmol, 6 mol/eq for each hydroxyl group) and benzyltriethylammonium chloride (BTEACl, 300 mg, 1.32 mmol) was heated to 100°C under stirring. After 15 minutes, a yellowish solution formed, and heating was continued for 2 hours. The solution was cooled to room temperature, followed by addition of BTEACl (300 mg, 1.32 mmol) and a 20% aqueous NaOH solution (12.9 mL, 3.16 g NaOH, 78.9 mmol, 2 mol/eq for each hydroxyl group). The resulting suspension was stirred vigorously at room temperature for 90 minutes. Distilled water (15 mL) was then added, and the resulting mixture was extracted with ethyl acetate (2 x 30 mL). The combined organic phases underwent brine washing (2 x 30 mL), subsequent drying over Na_2SO_4, and evaporation to dryness at 75°C under low pressure. Said process yielded a yellowish oil, which was further dried at 12 mmHg for 3 hours. The resulting crude product (5.15 g) was dissolved in CH_2Cl_2 (20 mL) and loaded onto a silica gel column (30 g; 230-400 mesh, column diameter 3.5 cm, silica fill height 8 cm) packed with CH_2Cl_2. Elution with CH_2Cl_2 (700 mL) yielded pure tri-O-triglycidylresveratrol **2** (4.21 g, 81% molar yield) as a white solid. The compound obtained exhibited a single spot in TLC. The elution was carried out using CH_2Cl_2/methanol in ratios of 100/1 or 100/2 *v/v*. Detection was performed by applying a solution consisting of *p*-anisaldehyde (9.3 mL), acetic acid (AcOH, 3.8 mL), ethanol (EtOH, 340 mL) and 96% sulphuric acid (12.5 mL), followed by heating at 100°C for 1 minute. The resulting pure tri-O-triglycidylresveratrol **2** exhibits m.p. = 88.5-89.6°C (crystallised from EtOH, 200 mg in 10 mL); literature: m.p. = 53-68°C (ACS Sustain. Chem. Eng. 2020, 8(37), 14137-14149), yellow oil (J. Clean Prod. 2021, 323,129194),

pale yellow solid (CN 113292703), m.p. 75°C (CN 114736354), yellow oil (CN 113603884), pale yellow solid (CN 115304745) and pale yellow viscous solid (JP 2020-84088). The ^{1}H- and ^{13}C-NMR spectra acquired were in agreement with those described in the literature (Chem. Eng. J. 2020, 383, 123124; Ind. Crop. Prod. 2022, 187 (Part B), 115500). The numbering adopted for the description of the spectra obtained is reported below.



Trans-tri-*O*-triglycidilresveratrol (**2**)

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.428 (2H, d, J = 8.7 Hz, H-10 and H-14), 7.013 (1H, d, J = 16.2 Hz, H-8), 6.904 (2H, d, J = 8.7 Hz, H-11 and H-13), 6.875 (1H, d, J = 16.2 Hz, H-7),6.675 (2H, d, J = 2.2 Hz, H-2 and H-4), 6.422 (1H, t, J = 2.2 Hz, H-6), 4.228 (3H, dd, J = 11.0 and 3.1 Hz, H15a, H-18a and H-21a), 3.974 (1H, dd, J = 8.7 Hz, H-11 and H-13).974 (1H, dd, J = 11.0 and 5.6 Hz, H-15b), 3.968 (2H, dd, J = 11.0 and 5.7 Hz, H-18b and H-21b), 3.351 (3H, m, H-16, H-19 and H-22), 2.906 (3H, dd, J = 4.8 and 4.8 Hz, H-17a, H-20a and H-23a), 2.756 (3H, dd, J = 2.6 and 4.8 Hz, H-17b, H-20b and H-23b); ^{13}C NMR (125 MHz, $CDCl_3$): δ 159.80 (C-1 and C-5), 158.31 (C-12), 139.79 (C3), 130.33 (C-9), 128.87 (C-8), 127.82 (C-10 and C-14), 126.53 (C7), 114.86 (C-11 and C-13), 105.60 (C-2 and C-4), 100.94 (C-6), 68.89, 68.88, 68.81 (C-15, C-18 and C-21), 50.06 (C-16, C-19 and C-22), 44.67, 44.65 (C-17, C-20, and C-23).

Crosslinking reactions with tri-O-triglycidylresveratrol

The crosslinking reactions were carried out using various percentages (2%, 5% and 10%) of resveratrol triglycidylate (*tri-O-triglycidylresveratrol*) at 50°C for both 1 hour and 2 hours, using the method shown below.

In a 100 mL Erlenmeyer flask equipped with a magnetic stirrer, hyaluronic acid sodium salt with a molecular mass of 1.5-1.8 MDa (500 mg, 1.25 mmol repeating units) was added to an 0.25 M aqueous solution of sodium hydroxide (10 mL). The resulting suspension was stirred at room temperature for 50 min (after 15 minute stirring, the suspension becomes a transparent viscous solution).

While stirring, dimethylsulphoxide (DMSO, 7 mL for the 2% and 5% reactions, or 8 mL for the 10% reaction) was added. The mixture was stirred at room temperature for 10 minutes and then, slowly at 50°C and under stirring, 10 (2%), 25 (5%) or 50 mg (10%) of resveratrol triglycidylate (*tri-O-triglycidylresveratrol*) dissolved in DMSO (3 mL) was added. A highly viscous transparent mass was obtained; in the case of the 10% reaction, the mass started as a white suspension but turned into a transparent colourless solution after 20 minute heating at 50°C. The reaction mixture was kept at 50°C by means of an oil bath, with continuous stirring for 1 or 2 hours. Subsequently, the oil bath was removed and water (5 mL) was introduced into the reaction mixture. The mixture was stirred at room temperature for 3 minutes and then cooled to room temperature, using an ice bath. The pH value of the resulting transparent viscous solution was precisely adjusted to 7 using 1M HCl (about 2.2-2.4 mL, added dropwise). A transparent gelatinous mass formed, which was stirred for a few minutes before testing the pH. Once neutralised, the mass was left to swirl for 5 minutes at room temperature, rechecking the pH and adjusting it if necessary. By dropwise addition of EtOH (40 mL) under vigorous stirring (about 20 min), a white fibrous product formed.

The suspension obtained was vigorously stirred for 5 minutes at room temperature and for an additional 5 minutes in an ice bath. The suspension was homogenised with a mixer at a speed of 5,000-6,000 rpm for about 2-3 minutes, and then centrifuged in 50 mL tubes for 10 min at 5,000 rpm. The supernatant is removed and the product is dispersed in a mixture of phosphate buffer solution (PBS; pH 7.4) (5 mL) and EtOH (15 mL) by stirring with a spatula. The mixture is then stirred with a magnetic stirrer for 30 minutes and then centrifuged at 5,000 rpm for 10 minutes. The treatment is repeated twice. The product is then washed by adding a 4:1 EtOH/H_2O mixture (10 mL), dispersing the solid with a spatula, stirring with a magnetic stirrer for 15 minutes and then centrifuging at 5,000 rpm for 10 minutes. Said washing procedure was repeated 2 or 3 times until the absorbance at 321 nm, measured directly on the supernatant filtered through an 0.22 μm nylon syringe filter, is less than 0.0500. Finally, two washes were performed with EtOH (10 mL each), again dispersing the solid, stirring for 15 minutes and then centrifuging for 10 minutes at 5,000 rpm.

The white solid was placed in a vacuum desiccator at about 10 mmHg overnight. A white powder was obtained.

Similarly, other crosslinking reactions were carried out using higher percentages (i.e. 15% and 20%) of resveratrol triglycidylate at 50°C for 1 hour and 2 hours.

The relevant experimental data are summarised in Table 1.

Table 1. Crosslinking tests with tri-O-triglycidylresveratrol.

Test number	% Res[1]	Ratio[2]	Time (h) at 50°C	Weight yield	% purity[3]	Modification degree (MoD)[4]
1	2	1:50	1	86	82	0.30
2	2	1:50	2	90	81	0.36
3	5	1:20	1	86	85	0.67
4	5	1:20	2	82	85	0.83
5	10	1:10	1	83	84	0.95
6	10	1:10	2	88	84	1.61
7	15	1:6.7	1	83	86.5	1.6
8	15	1:6.7	2	90	82.6	3.1
9	20	1:5	1	91	85.8	3.2
10	20	1:5	2	90	82.2	3.4

[1]% Res= mol resveratrol triglycidylate/mol repeating units of HA-Na x 100

[2]Ratio = mol triglycidylated resveratrol: mol repeating units of HA-Na

[3]Purity was determined by the carbazole method

[4]Modification Degree (MoD) = mol chromophore/mol repeating units of Ha-Na x 100

Hydrolysis of polymers for resveratrol chromophore determination by UV spectrometry and hyaluronic acid determination by the carbazole method

Various hydrolysis conditions for the crosslinked polymers were investigated to determine the optimal conditions enabling complete hydrolysis of the polymers. Said completeness is crucial for the spectroscopic quantification of the resveratrol chromophore within them. At the same time, care was taken to avoid any alteration of the chromophore. Complete hydrolysis is necessary because the presence of residual colloidal-sized fragments of the crosslinked polymer, undetectable upon observation, would result in photon scattering causing overestimation of the chromophore content of resveratrol.

Of the different hydrolysis conditions tested, hydrolysis carried out with 1M H_2SO_4 at 100°C for 10 min was considered the best. There is no alteration of the chromophore and the treatment of hyaluronic acid does not lead to significant interference in absorbance at 321 nm, selected for quantification of the resveratrol chromophore.

Hydrolysis of polymers, solution C preparation

The precisely weighed polymer (15 mg) is poured into a 10 mL glass test tube with a Teflon screw cap; a 1M aqueous solution of H_2SO_4 (5 mL) is then added, and the polymer is allowed to hydrate for 10 min, then heated to 100°C for exactly 10 min. The resulting reaction mixture

is cooled in an ice bath, yielding solution C. Said solution serves the dual purpose of determining the mmol of the resveratrol chromophore and quantifying the mg of hyaluronic acid in the sample measured.

Resveratrol chromophore determination

Using a syringe, solution C (400 µL) is added to H_2O (1.6 mL). The absorbance is measured directly against water at 321 nm for the chromophore, and at 500 nm to confirm the lack of absorbance in the visible range. Said process ensures that there is no photon scattering caused by the existence of colloidal-sized polymer fragments, which are invisible to the naked eye. From the measured absorbance, the mmols of chromophore in the polymer are obtained by means of the pre-prepared working curve using the following equation, wherein 28431 is the slope of the working curve:

$$\text{mmols chromophore in polymer} = A321 \times 25/28431$$

Working curve of the resveratrol chromophore

The standard solution of resveratrol in DMSO (about 3 mg/mL) was prepared by dissolving resveratrol (19.2 mg) in DMSO (6 mL), yielding a solution of 3.2 mg/mL. Solutions with known concentrations were prepared by adding the volumes (µL) indicated in Table 2 to water (1.9 mL). The resulting concentrations are expressed in mmoles of chromophore per mL (mmol chromophore/mL), calculated on the basis of the ratio between the mg of resveratrol and the molecular weight of resveratrol (228.25 g/mol). The corresponding absorbances at 321 nm are measured against H_2O (Table 2). Subsequently, a working curve (Figure 1) is constructed, represented by the linear equation $y = 28431x$, with an R^2 value of 0.9998.

Table 2. Data of working curve of resveratrol chromophore

µL in 1.9 mL of H_2O	mmol of chromophore/mL	A_{321}
1	0.000007	0.2149
2	0.000015	0.4126
3	0.000022	0.6290
4	0.000030	0.8341
5	0.000037	1.0481
6	0.000044	1.2644

Determination of hyaluronic acid content in the weighed polymer by the carbazole method

The method was developed by adapting the one reported in the European Pharmacopoeia (European Pharmacopoeia 11.0, p. 3995) and in two literature articles (J. Pharm. Biomed. Anal. 1999, 21, 491-496 and Anal. Biochem. 1962, 4, 330-334).

Solution for analysis: 100 µL of solution C is taken up with a syringe and added to 2.9 mL of H_2O, yielding a solution of about 100 µg/mL of polymer.

Carbazole analysis method

Preparation of the reagent solutions

Solution A $Na_2B_4O_7.10H_2O$ (PM 381.37) in 0.025M H_2SO_4

Dissolve 0.474 g of $Na_2B_4O_7.10H_2O$ in 50 mL of H_2SO_4

Solution B β-carbazole 0.125%, stable 12 weeks, (3 months) at 4°C in the dark

Dissolve 62.5 mg of β-carbazole in 50 mL of anhydrous EtOH

Carbazole method working curve

The stock solution of glucuronic acid was prepared with 50.0 mg of glucuronic acid dried in a mechanical pump for 3 days to constant weight, made up to 50 mL with distilled water in a 50 mL volumetric flask. The solutions for the working curve were prepared by weighing with an analytical balance.

- aGLu6: 1.0102 g stock solution made up to 10.1020 g with H_2O (concentration: 100 µg/g)
- aGLu5: 1.9996 g stock solution made up to 30.7725 g with H_2O (concentration: 64.98 µg/g)
- aGLu4: 3.9933 g aGLu5 solution made up to 9.9920 g with H_2O (concentration: 25.97 µg/g)
- aGLu3: 1.9886 g aGLu5 solution made up to 9.9384 g with H_2O (concentration: 13.00 µg/g)
- aGLu2: 1.5080 g aGLu5 solution made up to 10.0670 g with H_2O (concentration: 9.73 µg/g)
- aGLu 1: 1.0163 g aGLu5 solution made up to 10.1586 g with H_2O (concentration: 6.50 µg/g)

As glucuronic acid (MW = 194.1) corresponds to one repeating unit (RU) of hyaluronic acid sodium salt (RU HA-Na, MW = 401.3) and the density of the dilute solutions is 1 g/mL,

the concentrations of the working solutions can be expressed (Table 3) in mg/mL HA-Na by the equation:

mg/mL hyaluronic acid = concentration (µg/g) of glucuronic acid solution/1000 x 401.3/194.1

400 µL of the working curve solutions is processed in duplicate by the carbazole analysis method described above, resulting in the absorbances shown in Table 3.

Table 3. Data of carbazole method working curve.

solution	HA-Na mg/mL	A_{530}
aGLu1	0.0134	0.1025
aGLu1	0.0134	0.1056
aGLu2	0.0201	0.1572
aGLu2	0.0201	0.1502
aGLu3	0.0269	0.2015
aGLu3	0.0269	0.2147
aGLu4	0.0537	0.4085
aGLu4	0.0537	0.4033
aGLu5	0.1343	1.0348
aGLu5	0.1343	1.0350
aGLu6	0.2067	1.5840
aGLu6	0.2067	1.5892

The working curve (Figure 2) represented by the line y=7.6778x, with R^2= 0.9999, was obtained.

Analysis method

Using a syringe, 400 µL of the test solution (or 400 µL of H_2O for the blank) is taken up and placed in a 10 mL glass tube with a teflon screw cap.

Using a graduated pipette, 2 mL of solution A is added while cooling in an ice bath.

- Stopper and vortex.
- Heat to 100°C for exactly 15 minutes.
- Cool in an ice bath.
- Add 80 µL of solution B with a syringe and vortex.
- Heat to 100°C for exactly 15 minutes.
- Cool in an ice bath.

- Swirl in the vortex and read off the absorbance at 530 nm against the blank (400 µL of H_2O) directly in a 1 mL plastic cuvette. The colour remains stable for at least 16 hours.

The amount of hyaluronic acid present in the initially weighed polymer was calculated from the absorbance measured, using the following equation, wherein 7.6778 is the slope of the linear regression.

mg hyaluronic acid = A530 x 150/7.6778

Application of analyses to polymers crosslinked with triglycidylresveratrol

For the synthesised polymers, the resveratrol chromophore content and the hyaluronic acid purity of the tested polymers (Table 4) were determined by applying the analysis methods developed and detailed above. The results made it possible to determine the Degree of Modification (MoD) of the polymers, expressed as mol of chromophore/mol of repeating units (RU) of hyaluronic acid (RU HA-Na) x 100.

The MoD only indicates the total content of crosslinking molecules present in the polymer, whether they crosslink or are simply bound, without forming crosslinks.

Table 4. 1-6 polymers with triglycidylresveratrol and HA-Na 1500-1800 kDa.

Test number	% in reaction[a]	Ratio[b]	hours at 50°C	% purity of polymer	MoD
1	2	1:50	1	82.3	**0.30**
2	2	1:50	2	80.9	**0.36**
3	5	1:20	1	85.2	**0.67**
4	5	1:20	2	84.6	**0.83**
5	10	1:10	1	83.7	**0.95**
6	10	1:10	2	84.5	**1.61**

[a]mol of resveratrol in the reaction/mol of repeating units of HA-Na x 100

[b]mol resveratrol in the reaction: mol of repeating units of HA-Na

[c]mol resveratrol bound to the polymer/mol repeating units of HA-Na x 100

Determination of rheological properties of gels obtained by hydration of polymers crosslinked with triglycidylresveratrol

The preparations for the rheological tests were made by allowing polymers 1-6 (120 mg) to hydrate in physiological saline solution (PBS, 5 mL) for 2 days at room temperature (25°C), thus obtaining the corresponding hydrogels with a concentration of approximately 20 mg/mL (considering an average polymer purity of 83%).

The hydrogels were directly used for rheological tests. The rheological parameters: G* complex modulus, G' storage modulus, G" loss modulus, tan(δ) phase angle (phase angle, G"/G') and η* complex viscosity were determined with an Anton Paar rheometer (MCR 302) with 35 mm plates having a plate spacing of 1 mm and a frequency range of 10 to 0.1 Hz. The values shown in Table 5 and plotted in Figure 3 were measured at 1 Hz at 37°C.

Table 5. Rheological data.

Test number	Reaction conditions	$G^*_{(1Hz)}$ [Pa]	$G'_{(1Hz)}$ [Pa]	$G''_{(1Hz)}$ [Pa]	tan(δ) (1Hz)	$\eta^*_{(1Hz)}$ [Pa.s]
1	2%, 50°C, 1h	368	362	62	0.17	9
2	2%, 50°C, 2h	822	810	143	0.18	21
3	5%, 50°C, 1h	368	360	75	0.21	11
4	5%, 50°C, 2h	238	231	60	0.26	9
5	10%, 50°C, 1h	1397	1388	156	0.11	23
6	10%, 50°C, 2h	453	449	59	0.13	9

Evaluation of extrusion force of a syringe needle

The syringe needle extrusion force (needle size G27 x ½") of gels obtained from tri-O-triglycidylresveratrol crosslinked polymers, at the concentration of 20 mg/ml in PBS, was evaluated. The results showed a range between 10 and 15 Newton values (Figure 4), which is comparable to those obtained with commercial fillers.

CLAIMS

1. Crosslinked hyaluronic acids obtained by reacting hyaluronic acid or salts thereof with an activated derivative of resveratrol.

2. Crosslinked hyaluronic acids according to claim 1, wherein the activated derivative of resveratrol is tri-O-glycidylated resveratrol.

3. Crosslinked hyaluronic acids according to any one of claims 1 to 2, obtained from hyaluronic acid having an average molecular weight Mn of 1.5-1.8 MDa.

4. Crosslinked hyaluronic acids according to any one of claims 1 to 2, obtained from hyaluronic acid having an average molecular weight Mn of 250-450 kDa.

5. Crosslinked hyaluronic acids according to any one of claims 1 to 2, obtained from hyaluronic acid having an average molecular weight Mn of 1.5-3.0 MDa.

6. Crosslinked hyaluronic acids according to one or more of claims 1 to 5, having a degree of crosslinking providing a ratio of viscous modulus (G'') to elastic modulus (G') of less than 1.0.

7. Crosslinked hyaluronic acid derivatives prepared according to claims 1-6 with a modification degree (MoD), expressed as percentage ratio between moles of resveratrol and moles of repeating units of hyaluronic acid, ranging from 0.30 to 3.4.

8. A process for the preparation of crosslinked hyaluronic acids according to claims 1-7, comprising the reaction of hyaluronic acids in aqueous solution with the activated derivative of resveratrol in solution at a temperature ranging from 30 to 70°C, preferably 50°C.

9. Process according to claim 8, wherein the percentage molar ratio between the activated resveratrol derivative and repeating units of hyaluronic acid ranges from 2 to 20%.

10. Intradermal or intra-articular injectable compositions in the form of sterile gels containing the crosslinked hyaluronic acids according to claims 1-7.

11. Injectable compositions according to claim 10, comprising a mixture of crosslinked hyaluronic acids of different molecular weights.

12. Injectable compositions according to any one of claims 10 to 11, comprising 1 mg/mL to 50 mg/mL of crosslinked hyaluronic acids, optionally in the presence of an anaesthetic, preferably lidocaine, at a concentration of 0.1 to 0.4% weight/volume.