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Production and physicochemical characterization of a new amine derivative of gellan gum and rheological study of derived hydrogels

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ABSTRACT

The production of an amine derivative of gellan gum, named GG-EDA, was here obtained by functionalizing the polysaccharide backbone with pendant ethylenediamine moieties. The obtained derivative was characterized by spectroscopic, colorimetric, chromatographic and rheological analyses to study the effect of the free amino groups on the physicochemical properties of the macromolecule. A titration experiment was conducted to study the acid-base dissociation constants in aqueous media for the carboxylic and amino groups in the GG-EDA and to shed light on the possibility that the derivative shows a polyampholyte structure under physiological conditions.

The rheological analysis conducted on both physical and chemical hydrogels based on GG-EDA revealed that the presence of amino groups plays a fundamental role in influencing the viscoelastic properties and stability of the produced samples.

1. Introduction

Gellan gum (GG) is a anionic hetero/exopolysaccharide with repeating tetrasaccharide units of D-glucose, D-glucuronic acid, D-glucose, and L-rhamnose, produced by *Sphingomonas elodea* during aerobic fermentation (Leone et al., 2020; Salachna, Mizielińska, & Soból, 2018). In the past decade, GG has gained much importance in the biomedical field thanks to its peculiar physicochemical features that are exploited to easily obtain physical hydrogels (Palumbo, Federico, Pitarresi, Fiorica, & Giammona, 2020). GG aqueous dispersions show thermoreversible sol–gel transition due to the conformational shift from random coil to double helix of the polysaccharide chains caused by the lowering of temperature (Prajapati, Jani, Zala, & Khutliwala, 2013). The presence of metallic cations causes the association of the helices that leads to the formation of physical hydrogels with biocompatibility, bio-adhesiveness and biodegradability properties (Aadil, Nathani, Sharma, Lenka, & Gupta, 2019; Silva-Correia et al., 2011). Divalent cations allow the formation of GG hydrogels with better mechanical properties compared with those obtained in the presence of monovalent cations. This aspect is responsible for the main limitations associated with the use of GG-based hydrogels. In fact, *in vivo*, they gradually lose structural integrity due to the exchange of divalent cations with monovalent ones contained in the physiological medium (Bacelar, Silva-Correia, Oliveira, & Reis, 2016; Coutinho et al., 2010; Xu, Li, Jiang, & Bratlie, 2018). Clearly, the rate at which this weakening occurs could be affected by hydration of the specific tissue. For this reason, exploiting the carboxylic group of glucuronic acid residues, various chemical modifications have been proposed for GG with the common goal of modifying the physicalchemical properties of the polysaccharide and obtaining GG-based hydrogels with better stability in physiological conditions

(Ferris, Gilmore, Wallace, & Panhuis, 2013). For example, methacrylated GG has been proposed for the production of chemical hydrogels for cartilage and bone tissue engineering (Vieira et al., 2019; Vilela et al., 2018) and for biomedical applications, alone or in combination with other photoreactive polymers or nanoparticles (Pacelli et al., 2015;

Abbreviations: 4-NPBC, bis(4-nitrophenyl)carbonate; bisPEG-NH_2, poly(ethylene glycol) diamine; Ch/GG-EDA, GG-EDA chemical hydrogel; DMSO, dimethyl sulfoxide; DPBS, Dulbecco's phosphate buffered saline; EDA, ethylenediamine; EDC, N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride; GG-EDA, Gellan gum-(2-aminoethyl)-carbamate; GG-TBA, Gellan gum tetrabutylammonium salt; GG, Gellan gum; MTS, CellTiter 96® AQueous One Solution Cell Proliferation Assay; NHS, *N*-hydroxysuccinimide; Ph/GG-EDA, GG-EDA physical hydrogel; TBA − OH, tetrabuthylammonium hydroxide; TNBS, 2,4,6-Trinitrobenzenesulfonic acid solution

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Sahraro, Barikani, & Daemi, 2018).

Our research group recently proposed the functionalization of the primary hydroxyl groups of GG (in glucosidic residues) with hydrophobic alkylamines demonstrating that the hydrophobic interactions that occur in an aqueous environment between these pendant moieties improve the mechanical properties of the hydrogels obtained and ensure the maintenance of temperature sensitive and ionotropic characteristic of the polysaccharide (Agnello et al., 2017; Agnello, Palumbo, Pitarresi, Fiorica, & Giammona, 2018).

Another interesting approach to obtain stable GG-based hydrogels is the formation of inter-polyelectrolyte complexes with polycations such as chitosan (CS). In this case, the electrostatic interactions that occur in the physiological pH between the carboxylate groups of GG and the protonated amino groups of CS contribute to stabilizing the double helix of GG and allow to obtain physical hydrogels with good mechanical properties and stability (de Oliveira et al., 2019) or to produce layered materials (Mat Amin et al., 2012). The advantage of this approach is the possibility of avoiding the chemical modification of the starting polymers even if the procedure for the production of complexes must be carried out at a specific pH and is not immediate. de Oliveira et al., showed that by varying the GG/CS weight ratio it is possible to modulate the surface properties of the hydrogel and hydrophilicity and influence its biological characteristics.

In particular, they found that by increasing the CS content compared to GG, it is possible to obtain hydrogels with higher cell adhesion properties since the CS reduces the density of negative charges due to GG carboxylate groups that inhibits the interaction of cells with the sample surface (de Oliveira et al., 2020). In light of these results, it is clear that the inter-polyelectrolyte interactions can play a crucial role in tailoring the features of GG containing biomaterials. With this in mind, we decided to synthesize a GG derivative with the intrinsic ability to produce intra-polyelectrolyte complexes simply when dispersed in water and giving rise to the formation of hydrogels with improved physicochemical characteristics.

For this reason, we carried out the insertion of pendant free amino groups in the backbone of the GG, to obtain a polyampholyte macromolecule. Polyampholyte polymers have been deeply studied in the past years and used to produce hydrogels for biomedical applications because of their peculiar characteristics (Bernards & He, 2014; Haag & Bernards, 2017). Functionalization with free amino groups is often performed to increase the chemical reactivity of macromolecules and in particular of polysaccharides (Fiorica et al., 2013, 2017; Hu et al., 2019; Monier, Shafik, & Abdel-Latif, 2018; Palumbo et al., 2013). Despite the growing use of GG as a biomaterial in recent years, the functionalization of this polysaccharide with free amino groups has so far not been carried out. Here a low acyl GG having a Mw 25 kDa has been used as a starting macromolecule which forms less viscous aqueous dispersions than the starting polysaccharide and therefore easier to handle and study.

The derivative, indicated as GG-EDA, was synthesized by functionalizing the GG primary hydroxyl groups with ethylenediamine (EDA) moieties. Titration experiments demonstrated that in physiological conditions the derivative is in the form of polyampholyte and thermorheological analysis revealed that GG-EDA aqueous dispersions maintain the typical thermosensitive behavior of native GG.

The physical and chemical hydrogels based on GG-EDA were obtained respectively by ionotropic gelation and chemical crosslinking by carbodiimide chemistry.

Physical hydrogels have been characterized by rheological studies to demonstrate that the presence of free amino groups can allow to obtain samples with more pronounced elastic behavior than non-functionalized GG-based hydrogels.

The physicochemical characteristics of the GG-EDA-based hydrogels (both physical and chemical) were compared by studying their rheological properties, swelling and hydrolytic degradation profile to understand whether the establishment of covalent bonds between and within the macromolecules could affect the elastic properties and stability of the hydrogel.

Finally, as a proof of concept, the cytocompatibility and cell adhesion properties of GG-EDA-based physical and chemical hydrogels were tested using 3T3 murine fibroblasts.

2. Hypothesis statement

The insertion of free amino groups in the GG leads to the obtainment of a polyampholyte derivative which gives rise to the production of hydrogels with improved strength and stability.

3. Materials and methods

3.1. Chemicals

Low acyl Gellan Gum (Gelzan™ CM), ethylendiamine (EDA), tetrabuthylammonium hydroxide (TBA-OH), bis(4-nitrophenyl)carbonate (4-NPBC), acetone, anhydrous dimethylsulfoxide (DMSO), Dowex® 50WX8 hydrogen form, sodium hydroxide (NaOH), tetramethylammonium chloride (TMACl), *N*-hydroxysuccinimide (NHS), *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC), Dulbecco's phosphate buffered saline (DPBS), poly(ethylene glycol) diamine (bisPEG-NH_2), 2,4,6-Trinitrobenzenesulfonic acid solution (TNBS), hydrochloric acid 37 %, deuterium oxide, anhydrous calcium chloride ($CaCl_2$), were purchased from Sigma-Aldrich (Italy).

CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was purchased by Promega (Italy). Live/Dead assay kit for mammalian cells was purchased from Thermo-Fischer (Italy).

3.2. Apparatus

The rheological tests were carried out using a DHR-2 TA Instrument oscillatory rheometer equipped with a flat geometry of 8 mm in diameter with radial groove and a self-heating Peltier plate.

Size exclusion chromatography was conducted with an Agilent 1260 Infinity multi-detector GPC/SEC system.

^{1}H-NMR and ^{13}C-NMR spectra were obtained with a Bruker Avance II 300 MHz spectrometer.

UV measurements were performed using an Eppendorf AF2200 spectrophotometer.

Potentiometric titration was carried out using a Hanna HI4221 pH meter.

Cell cultures were performed using an Eppendorf New Brunswik S41i incubator.

Fluorescence images were obtained with AxioVert200 (Zeiss) microscope.

3.3. Synthesis of gellan gum-(2-aminoethyl)-carbamate (GG-EDA)

The hydrolysis procedure for the production of low molecular weight GG and its tetrabutylammonium salt (GG-TBA) was performed as reported elsewhere (Agnello et al., 2018; Nitta, Takahashi, & Nishinari, 2010). Briefly, 5 g of GG (Gelzan™ CM) were dispersed by means of a blade stirrer into one liter of NaOH solution 0.1 M (pH 13) and the dispersion was kept under stirring at 50 °C for 24 h. HCl 5 N was added to neutralize the pH, the dispersion was then dialyzed against Milli-Q water (cut-off 25 kDa) and finally freeze-dried. The product, named GG (which is the sodium salt of degraded Gellan gum), was transformed into its acid form by using Dowex® 50W-X8 hydrogen-exchange resin and the dispersion was neutralized with TBA-OH to obtain the GG-TBA (Lee, Tsai, Wen, & Huang, 2012; Pawar & Edgar, 2011).

GG-EDA synthesis was carried out as follows: 500 mg of GG-TBA were dissolved in 45 mL of anhydrous DMSO (1% w/v), and the dispersion was stirred in a water bath at 40 °C for 30 min. Subsequently, 86 mg of 4-NPBC, previously dissolved in 5 mL of anhydrous DMSO,

were added dropwise and the reaction was carried out at 40 °C for 4 h. The ratio between moles of 4-NPBC and moles of repetitive units of GG-TBA correspond to 0.5. After 4 h, 189 µl of EDA were added dropwise and under vigorous stirring to the dispersion and the reaction was carried out for further 3 h at 40 °C. The molar ratio between EDA and 4-NPBC was set at 10. The reaction was stopped by adding 500 µL of saturated sodium chloride solution and stirring for 30 min.

The product was isolated by acetone precipitation then washed several times with a mixture acetone/water (8:2 v/v), and finally with acetone. The product was recovered by vacuum-drying at room temperature with a yield of 65 % based on the GG-TBA initial weight.

The derivatization degree in EDA (DD_{EDA}%) of GG-EDA was calculated both by ^{1}H-NMR analysis and with TNBS colorimetric assay.

For NMR analysis, GG or GG-EDA were dissolved at 90 °C (oven for 2 min) in D_2O at a concentration of 2% w/v.

The colorimetric assay was conducted following the supplier specification. Briefly, GG-EDA was dispersed in Milli-Q water at 0.25 % w/v. 100 µl of the analyte solution was mixed with 1 mL of TNBS reagent solution (prepared by diluting 50 times the standard solution supplied by the supplier with borate buffer pH 9.3) and the resulting solution was incubated for 2 h at 37 °C before measuring its absorbance at 500 nm. The analysis was conducted in triplicate.

A calibration curve was obtained by analyzing the absorbance at 500 nm of bisPEG-NH_2 aqueous solutions at known concentrations, incubated with the TNBS following the procedure just described for the GG-EDA. By relating the absorbance at 500 nm obtained for the GG-EDA dispersion with the calibration curve obtained from the standard solutions, it was possible to calculate the degree of functionalization in ethylenediamine groups of the obtained derivative.

The test was also conducted on the starting GG as a negative control.

3.4. Size exclusion chromatography analysis

The absolute molecular weight (Mw) and polydispersity index (PDI) of GG and GG-EDA were measured with a SEC instrument equipped with both refractive index and light scattering detector.

Tetramethylammonium chloride in water at a concentration of 0.025 M was used as a mobile phase at a flow rate of 0.8 mL/min while Polysep P-4000 (Phenomenex) was used as a stationary phase. The elution was performed at 35 °C. Pullulan (molecular weight 50 kDa) was used as a standard. All the investigated samples were dissolved at 50 °C in the mobile phase.

3.5. Potentiometric titration of GG and GG-EDA

GG or GG-EDA (20 mg) was dispersed in CO_2 free 0.01 M NaCl (40 mL), used as an ionic strength stabilizer. Then, 0.1 M HCl (5 mL) was added to the resulting solution, which was cooled to 25 ± 0.1 °C and titrated with 0.1 M sodium hydroxide free of CO_2 in a nitrogen atmosphere. The solution was thermostated at 25 ± 0.1 °C and continuously purged with ultrapure nitrogen during the titrations. The pH-meter was calibrated against a multiple pH standard buffer (pH 4.01, pH 7.04, pH 10.09, pH 9.01), exhibiting a calibration fitting with R^2 = 0.9997 and an ideality grade of 95.2 %. The pKa values were extrapolated using Microsoft Excel spreadsheets and applying the de Levie method of acid-base chemical equilibria for polyprotic acids (Ferruti et al., 2014; Mauro, Fiorica, Varvarà, Di Prima, & Giammona, 2016; Scialabba et al., 2019). The activity corrections were calculated by the Davies equation.

3.6. Thermo-rheological analysis of GG-EDA and GG aqueous dispersions

Thermo-rheological analysis was conducted on GG-EDA and GG 5% w/v dispersions in the temperature range between 5 and 50 °C. To allow the homogeneous dispersion of the polymers, the vials were placed in oven at 80 °C for 5 min and kept in a water bath at 50 °C until the analysis was performed. A parallel plate geometry with radial groove and with an 8 mm diameter upper plate was used for the experiments. Temperature dependence of complex viscosity (η*), storage modulus (G′) and loss modulus (G″) values were analyzed by cooling the samples from 50 to 5 °C at a rate of 0.5 °C/min by applying a constant strain of 20.0 % and a frequency of 0.1 Hz. The linear viscoelastic region was preliminarily assessed at predetermined temperatures by strain sweep experiments applying a constant frequency of 0.1 Hz in the range between 0.5–40 % of deformation (see Supplementary Information file, Fig. S1).

3.7. Ionotropic crosslinking of GG-EDA and GG and rheological analysis of hydrogels

GG-EDA or GG hydrogels were produced by ionotropic crosslinking injecting the hot dispersions at 5% w/v, obtained as described earlier, in a $CaCl_2$ 0.1 M solution at 37 °C. In particular, 150 µL of the hot (80 °C) dispersions were injected in 1 mL of $CaCl_2$ solution and incubated at 37 °C in a humidified shaking incubator for 24 h. After this time, the saline solution was changed with the same volume of DPBS pH 7.4 and the samples were again incubated at 37 °C. The medium was changed every 72 h and, at predetermined time points (1, 7, 14 and 21 days), the viscoleastic properties of the hydrogels were investigated through rheological tests conducted both in strain sweep and frequency sweep at 37 °C.

Strain sweep experiments were performed a constant oscillation frequency of 0.1 Hz and a strain percentage ranging from 0.1 to 5 % (see Supplementary Information file, Fig. S2) while frequency sweep experiments were performed at a constant strain equal to 1% and variable frequencies ranging from 0.01 to 10 Hz. Each experiment was performed in triplicate.

3.8. Auto-crosslinking of GG-EDA and rheological analysis of hydrogels

Chemical auto-crosslinked GG-EDA-based hydrogels were prepared as follow: for each sample, 20 mg of GG-EDA were dissolved in 200 µL of Milli-Q water in oven at 80 °C. To the hot dispersion obtained, 125 µl of EDC aqueous solution (10 mg/ml) and 75 µl of NHS aqueous solution (10 mg/ml) were added under vortex mixing. The molar ratio between the coupling agents and free amino groups in GG-EDA was set at 0.5 while the final concentration of the polysaccharide derivative in the gelling solution was set at 5 % w/v. The dispersion was kept in the oven at 80 °C for further 5 min and then left to cool to room temperature.

Obtained chemical hydrogels (named Ch/GG-EDA) were then cured with 1 mL $CaCl_2$ 0.1 M solution at 37 °C for 24 h. After this time, the saline solution was changed with the same volume of DPBS pH 7.4 and each sample was kept into a humidified incubator at 37 °C under gentle shaking. The medium was changed every 72 h and, at predetermined points of time, strain sweep (see Supplementary Information file, Fig. S3) and frequency sweep experiments were carried out at 37 °C, as previously described, to evaluate the mechanical properties of the sample.

Physical GG-EDA hydrogel (named Ph/GG-EDA) was prepared as a control by cooling a hot dispersion of the derivative (400 µL; 5% w/v) at room temperature and curing it with 1 mL $CaCl_2$ 0.1 M solution for 24 h. The obtained hydrogel was treated and analyzed with the same conditions just described above. Each experiment was performed in triplicate.

3.9. Swelling and hydrolytic degradation of chemical and physical GG-EDA hydrogels

For the studies of swelling and hydrolytic degradation, the chemical and physical GG-EDA hydrogels, prepared following the procedures described in the previous paragraphs, after treatment with the $CaCl_2$ 0.1 M solution, were thoroughly washed with Milli-Q water to eliminate

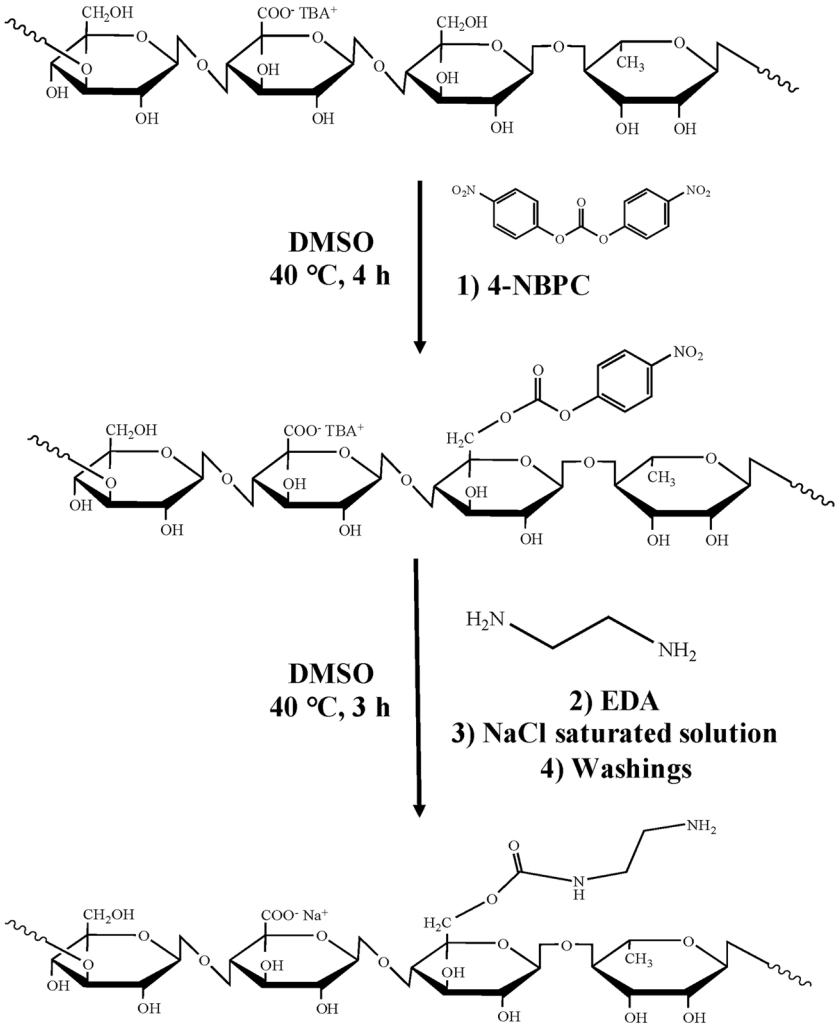

Fig. 1. Schematic representation of chemical steps used to produce GG-EDA.

the excess salt. The samples were then frozen, freeze-dried and weighed carefully.

The swelling studies were conducted by incubating the samples at 37 °C in 5 mL of DPBS pH 7.4 for 24 and 48 h. At the time of analysis, the swollen samples were weighed after removing the excess of buffer with blotting paper. The swelling percentage (Sw%) was calculated as

$$Sw\% = W_{sw} - W_d / W_d \times 100$$

Where, W_{sw} is the weight of the hydrogel after swelling and W_d is the weight of the dry sample. Each experiment was performed in triplicate and the results were expressed as mean value ± standard deviation.

Hydrolytic degradation study was performed by incubating freeze-dried samples in DPBS pH 7.4 at 37 °C. At scheduled time intervals, the samples were washed with Milli-Q water, freeze-dried and weighed carefully. The degradation of the samples was expressed as weight recovered percentage (W_r%), calculated as

$$W_r\% = W_f/W_i \times 100$$

Where, W_f is the weight of degraded sample at each time point and W_i is its initial weight.

Each experiment was performed in triplicate and results were expressed as mean value ± standard deviation.

3.10. Cytocompatibility in vitro and cell adhesion studies

Ch/GG-EDA or Ph/GG-EDA hydrogels with GG-EDA concentration equal to 5% w/v were prepared as already described. In particular, for the *in vitro* biological characterization, cylindrical hydrogels (diameter 0.8 cm, height 0.5 cm), obtained from 400 µL of gelling solution, were moved to 48 well culture plate and sterilized by UV irradiation at 254 nm for at least 1 h (30 min per side) using a 125 W UV-lamp. Then, they were conditioned by treatment with DMEM cell culture medium supplemented with 10 % v/v of FBS, 1 % v/v of penicillin–streptomycin solution, 1 % v/v of glutamine solution and 0.1 % v/v amphotericin B solution. At this point, 100 µL of cell suspension containing 2 × 104 3T3 murine fibroblasts was placed on each hydrogel and the culture plates were incubated at 37 °C in a humidified incubator with a 5% CO_2 atmosphere.

After 24 and 48 h of culture, the viability of cells in contact with chemical or physical GG-EDA hydrogels was assessed with MTS according to the manufacturer's specifications.

In particular, at scheduled times, the culture medium was changed with 250 µL of MTS reagent (CellTiter 96® AQueous One Solution Cell Proliferation Assay) in culture medium (volume ratio 1:5). The plate was incubated at 37 °C for 40 min then the absorbance at 492 nm of medium was read with UV–vis spectrophotometer.

Cell viability was expressed as a percentage of viability compared to control cells cultured in tissue culture plastic wells. The Live/Dead assay was also performed on each sample to study both cell viability and morphology.

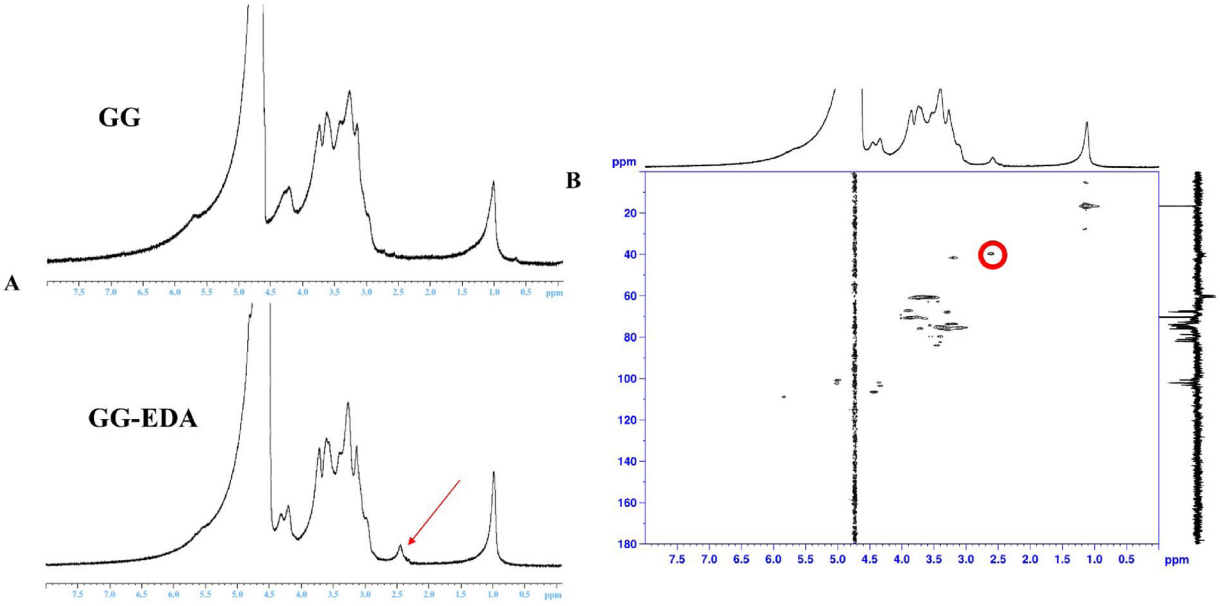

Fig. 2. ^{1}H-NMR spectra of GG and GG-EDA (A); HMQC spectrum of GG-EDA (B).

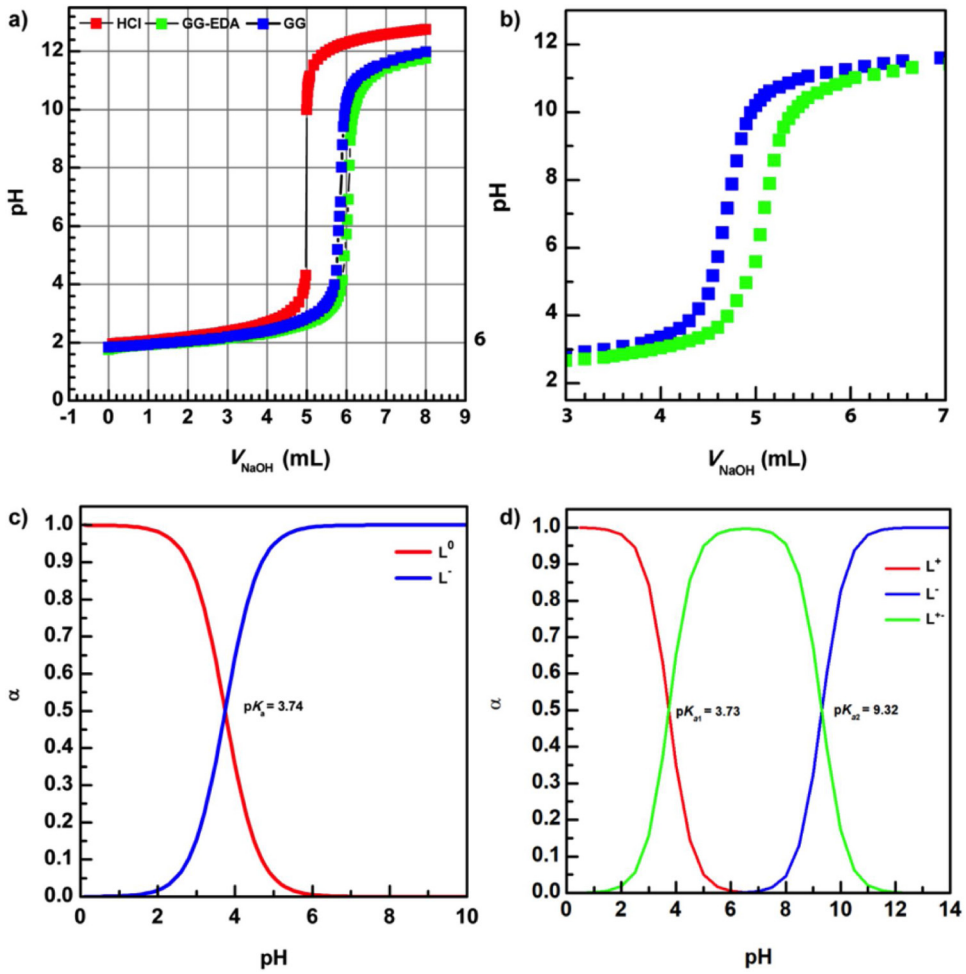

Fig. 3. Forward potentiometric titration curves of 0.1 M HCl (5 mL), GG and GG-EDA (a): de Levie fitting curves are reported in black solid lines. Detail of potentiometric titration curves for GG and GG-EDA in the pH range 3–7(b). Speciation curves calculated for GG (c) and GG-EDA (d).

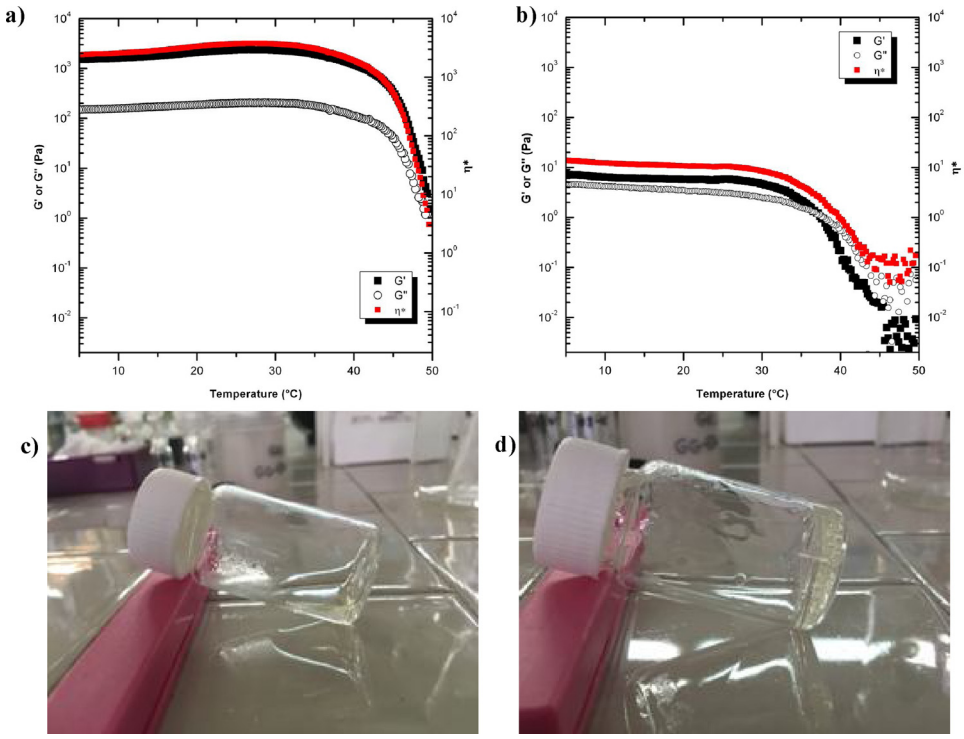

Fig. 4. Temperature dependence of complex viscosity (η*), storage modulus (G′) and loss modulus (G″) during cooling process for GG-EDA (a) and GG (b) dispersed in water at 5% w/v. Experiments were performed at 0.1 Hz and 20 % of strain. Images of GG-EDA 5% w/v dispersion at 50 °C (c) and at 25 °C (d).

3.11. Statistical analysis

Swelling, hydrolytic degradation and cytocompatibility data are presented as means ± standard deviation (SD). *t*-Test was used for the statistical analysis, p values lower than 0.05 were considered as statistically significant.

4. Results and discussion

4.1. Synthesis and physicochemical characterization of gellan gum-(2-aminoethyl)-carbamate (GG-EDA)

The chemical procedure used to insert pendant primary amino groups in the GG backbone is similar to that already described by our research group to functionalize hyaluronic acid with alkyl amines (Giammona, Palumbo, & Pitarresi, 2010; Pitarresi, Fiorica, Licciardi, Palumbo, & Giammona, 2013) and also used to insert alkyl chains in the same GG (Agnello et al., 2017, 2018,).

The initial GG was degraded under alkaline conditions to allow its complete deacetylation and to obtain a derivative with a lower molecular weight which forms less viscous dispersions than the starting polysaccharide and therefore easier to handle and study.

The functionalization procedure, shown in Fig. 1, involves the activation of primary hydroxyl groups of the glucosidic residues of GG with 4-NBPC and the subsequent reaction with EDA.

The insertion of pending EDA groups was evaluated by ^{1}H-NMR analysis, by comparing the GG-EDA and GG spectra and by 2D HMQC analysis which correlates the ^{1}H and ^{13}C signals of the obtained amine derivative. Fig. 2A shows the GG-EDA ^{1}H NMR spectrum with a new peak at δ 2.5 (indicated by the red arrow) assigned to the methylene protons near the free amino group of EDA.

The $DD_{EDA\%}$ was calculated both with the analysis of the GG-EDA ^{1}H-NMR spectrum, by comparing the integral of the peak at δ 2.5 with that at δ 1 (3H, s, of the $-CH_3$ of rhamnose), and through the TNBS colorimetric assay, resulting equal to 43 ± 3 mol% (result expressed as the mean value of those obtained by the two analytical techniques). Considering that the theoretical molar ratio for the activation of primary hydroxyl groups (moles of 4-NBPC/moles of repeating units GG-TBA) has been set at 0.5, the reaction efficiency is higher than 80 %.

HMQC 2D (Fig. 2B) analysis shows the presence of a cross peak of the methylene carbon (between 40 and 45 ppm) and protons (red circle) and gives a further confirmation of the insertion of EDA moieties into the polysaccharide backbone.

SEC analysis revealed that the molecular weight of GG and GG-EDA is 25.33 kDa (polydispersity index 1.38) and 25.55 kDa (polydispersity index 1.42), respectively. This means that in the reaction conditions used, the GG does not undergo a significant degradation process.

Since the mechanical properties and the rheological behavior depend strictly on the molecular weight of the starting macromolecules, this result allows to study in parallel GG-EDA and GG comparing any different physicochemical characteristics that can be related only to the difference in the chemical structures.

The De Levie approach was used to study the acid-base dissociation constants of GG-EDA and GG in aqueous media, since this is the most reliable and flexible method to take into account all the titration parameters by optimizing the calculations using a calculation iterative including ionic strength balance combined with mass balance of all species present in solution.

Furthermore, using this method it is possible to introduce a series of additional conditions such as the presence of excess hydrochloric acid in addition to the polymer studied, obtaining manageable equations capable of faithfully describing the experimental titration curve without any approximation.

The rationale for the interpretation of GG-EDA and GG titration using the de Levie approach is reported in detail in the Supplementary Information file. As shown in Fig. 3, the experimental and calculated curves for GG-EDA and GG are in agreement, suggesting a good fit with the calculated pKa values (3.74 and 3.73/9.32 for GG and GG-EDA, respectively).

Interestingly, a quantitative composition of the acidic and basic functions was achieved by comparing the titration curves of GG-EDA and GG at the end point indicating the presence of approximately 1.25

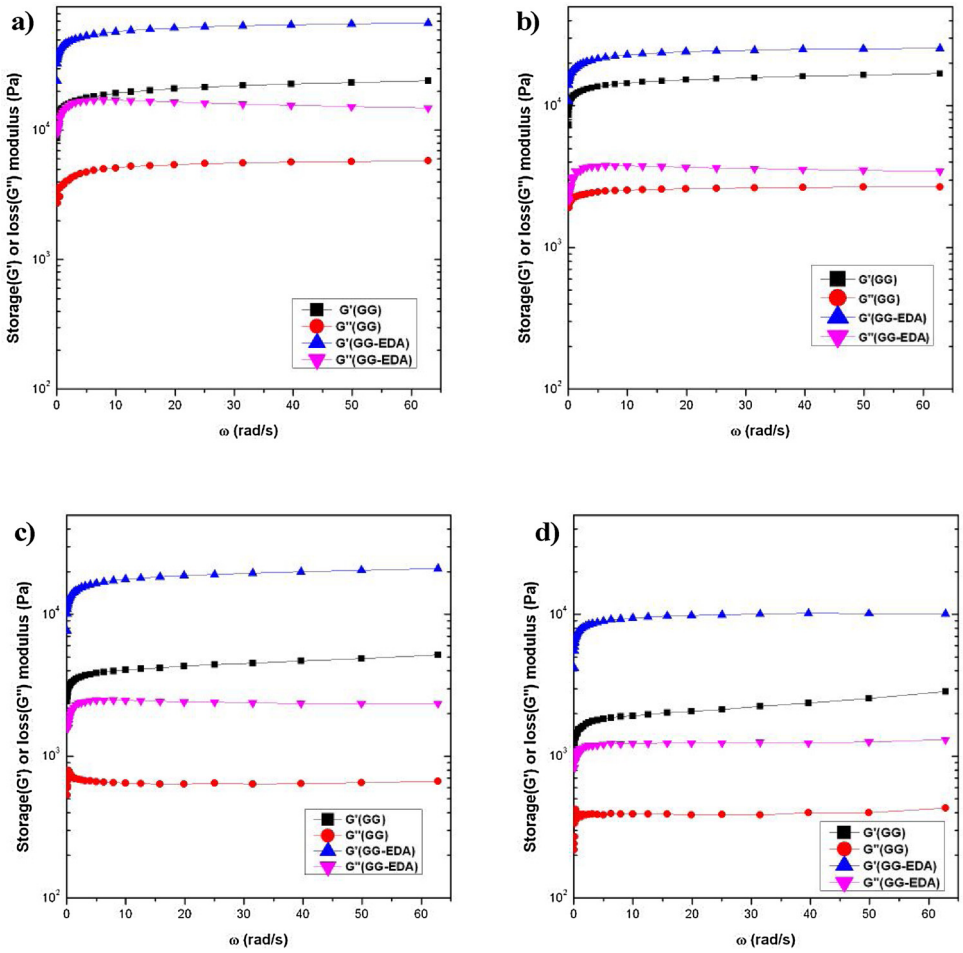

Fig. 5. Frequency sweep rheograms performed at 1% of strain for GG-EDA or GG ionotropic crosslinked hydrogels after 24 h of incubation at 37 °C in CaCl 0.1 M (a) and after 7 (b), 14 (c) and 21(d) days of incubation at 37 °C in DPBS pH 7.4.

eq g^{-1} of amino groups (43 % on a molar basis), thus confirming ^{1}H NMR and TNBS data (Fig. 3a).

From the speciation diagrams shown in Fig. 3c and d, calculated using the pKa values obtained as described above, it can be seen that while GG is a pure polyanionic compound (L-) under physiological conditions (pH 7.4), for GG-EDA, the anionic fraction (L-) and the zwitterion form (L + -) coexist in a delicate balance that depends on the amount of repetitive amino-functionalized units. In particular, for GG-EDA containing 43 mol% of EDA functions, the L-/L + - ratio is almost 3:1, thus demonstrating the ampholytic nature of the derivative at physiological pH.

4.2. Thermo-rheological analysis of GG-EDA and GG aqueous dispersions

As previously discussed, the thermotropic characteristics of GG are linked to its ability to form, at low temperatures, ordered double helix structures which are stabilized by ionic bonds in the presence of cations. The sol-gel transition temperature of the aqueous dispersions of GG depends on the molecular weight of the starting polysaccharide and its concentration and is of fundamental importance for producing hydrogels for biomedical applications (Ogawa, Takahashi, Yajima, & Nishinari, 2006).

GG-EDA and GG 5% w/v aqueous dispersions were prepared at 80 °C since at room temperature both GG and GG-EDA form coarse dispersions. After heating to 80 °C for 5 min, both dispersions became clear.

The thermo-rheological analysis carried out on the obtained dispersions, clearly revealed that GG-EDA maintains the thermotropic behavior since during the cooling of the system, a strong increase in the values of η *, G′ and G″, which starts already at 50 °C, can be observed (see Fig. 4a).

As can be seen, the temperature at which these parameters reach a plateau for the dispersion of GG-EDA is 40 °C while for GG it is about 30 °C (Fig. 4b).

This indicates that the aggregation between macromolecules for GG-EDA occurs at higher temperatures due to the ionic interactions between primary ammonium and carboxylate groups which facilitate the coil to helix transition and stabilize these ordered structures.

The higher values of η*, G′ and G″ obtained for the dispersion of GG-EDA (two orders of magnitude compared to GG) are further confirmation of the presence of a greater number of interactions within and between the polysaccharide chains in the aqueous environment.

Macroscopically, the dispersion of GG-EDA flows freely at temperatures above 50 °C and stops when the temperature drops below 40 °C (see Fig. 4c and d). The inversion tube test was performed to demonstrate that this behavior is reversible.

4.3. Ionotropic crosslinking of GG-EDA and GG and rheological analysis of hydrogels

To study the effect of the presence of inter- and intra-polyelectrolytic interactions on the mechanical properties and on the physiological stability of GG-EDA hydrogels compared to GG, the ionotropic crosslinking of the aqueous dispersions described above was carried out by injecting them in a 0.1 M CaCl solution.

Hydrogels are formed immediately after the injection, giving rise to

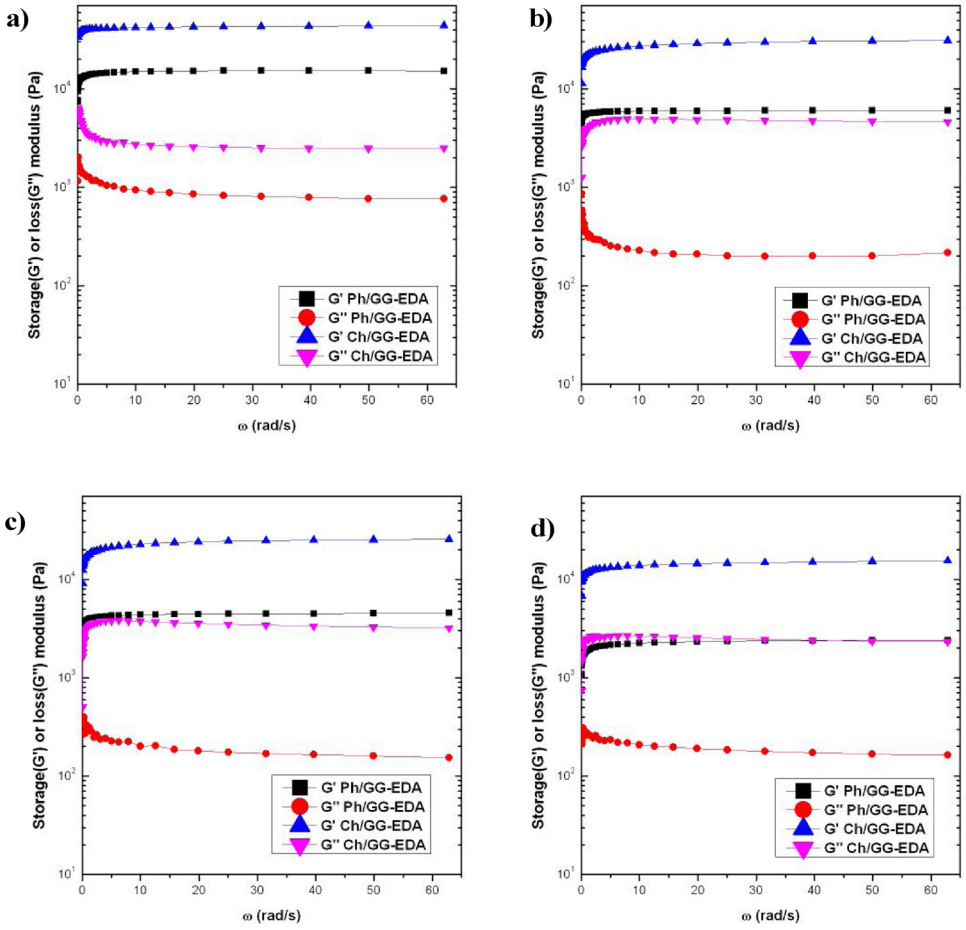

Fig. 6. Frequency sweep rheograms performed at 1% of strain for Ch/GG-EDA or Ph/GG-EDA after 24 h of incubation at 37 °C in CaCl 0.1 M (a) and after 7 (b), 14 (c) and 21 (d) days of incubation at 37 °C in DPBS pH 7.4.

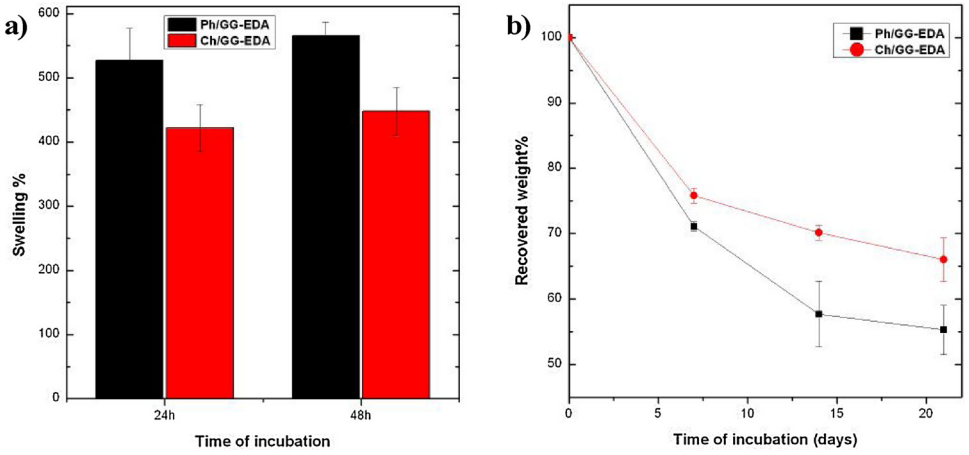

Fig. 7. Swelling percentage of Ch/GG-EDA or Ph/GG-EDA hydrogels after 24 and 48 h of incubation at 37 °C in DPBS pH 7.4 (a). Recovered weight % of Ch/GG-EDA or Ph/GG-EDA hydrogels as a function of incubation time in DPBS pH 7.4.

the formation of spherical whitish structures. The samples were kept at 37 °C in the curing solution for 24 h, then moved to DPBS at pH 7.4. This phosphate buffer does not contain divalent cations and was chosen to mimic the physiological conditions in which calcium is exchanged with monovalent cations in the GG-based hydrogel.

To study the effect of calcium loss from the three-dimensional networks on the strength of the samples and elastic behavior, rheological analyses were conducted on both GG-EDA and GG hydrogels at scheduled times.

The analysis conducted in frequency sweep showed that the G′ values for the GG-EDA hydrogels are higher than those of the GG hydrogels at all the time points studied (Fig. 5).

Clearly, the presence of ionic interactions of the polyampholyte leads to a three-dimensional network with a greater number of cross-links which improve the elastic performance of the sample (Banerjee et al., 2009).

The comparison between the values of the G' modulus obtained at different time intervals for both samples from strain sweep experiments

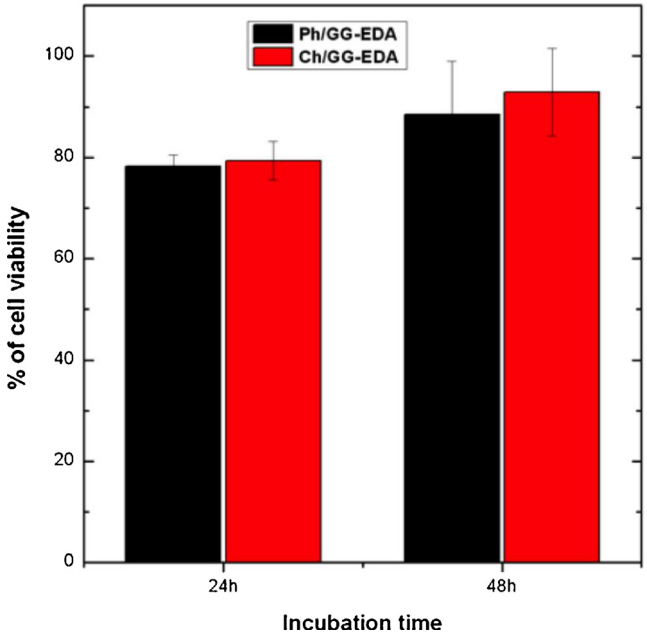

Fig. 8. Viability of 3T3 murine fibroblasts cultured on Ph/GG-EDA or Ch/GG-EDA hydrogels after 24 and 48 h of culture. Results are expressed as a percentage of viability compared to control cells cultured in tissue culture plastic wells.

(see Supplementary Information file, Fig. S2), allowed to highlight that the decrease in the elastic behavior of the hydrogels, attributable to the depletion of calcium from the three-dimensional structure, it is more pronounced for the GG hydrogel.

This result shows that the elastic behavior of GG hydrogel is mainly conferred by the saline bonds between the calcium ions and the carboxylate groups.

The depletion of calcium from the GG-EDA hydrogels causes a milder loss of the G′ modulus values due to the presence of polyampholyte ionic interactions which contribute to making the sample more stable under physiological conditions.

Overall, the rheological analyses carried out on ionotropic crosslinked GG and GG-EDA hydrogels have confirmed that the polampholyte nature of GG-EDA allows to produce, using the same simple and reproducible procedure usually used for the production of GG-based hydrogels, samples with

higher elastic behavior and higher stability in physiological conditions.

4.4. Auto-crosslinking of GG-EDA and rheological analysis of hydrogels

Chemical crosslinking has been widely used to obtain GG-based hydrogels with improved mechanical and stability characteristics.

For example, the insertion of methacrylic portions allows to crosslink the polysaccharide by UV irradiation or *via* thiol-ene chemistry (Bacelar et al., 2016; Xu et al., 2018). In addition, chemical hydrogels of GG have been obtained by treating temperature-induced hydrogels with an excess of EDC (2 times compared to the carboxyl groups of GG) in an aqueous environment with controlled pH (Dentini et al., 2001).

In addition to conferring the nature of polyampholyte, the pendant primary amino groups in GG-EDA can be used to carry out a chemical crosslinking that could take place under milder conditions than those described so far in the literature.

The procedure used here to crosslink the GG-EDA required the addition of EDC/NHS (molar ratio between activating agents and free amino groups of GG-EDA equal to 0.5, which corresponds to less than 25 % of the GG carboxylic groups) to hot dispersion (80 °C) at 5% w/v.

After the addition of the activating agents, the system underwent a sudden sol-gel transition, observable by a rapid loss of fluidity, induced by the formation of amide bonds between amino groups and carboxylic groups activated by the NHS.

Since the speed with which the chemical hydrogel is formed makes difficult to inject the dispersion into the $CaCl_2$ 0.1 M solution, the ionotropic crosslinking of the system was carried out by incubating the preformed chemical hydrogel in the saline solution for 24 h at 37 °C.

Rheological analyses were carried out on the chemical crosslinked hydrogel (Ch/GG-EDA) after the curing and the incubation times in DPBS at pH 7.4 to study the influence of the formation of chemical bonds on the trend of G' modulus and therefore on the stability of the sample. As a control, a physical hydrogel (Ph/GG-EDA) was prepared in a similar way by cooling the dispersion of GG-EDA to 5% w/v at room temperature and by treating the temperature-induced GG-EDA hydrogel with the solution of CaCl 0.1 M as described above.

The oscillation frequency test shown in Fig. 6 showed that, as expected, the values of the G′ modulus for the Ch GG-EDA hydrogel are higher than those of the Ph/GG-EDA sample at all the time points studied. This result shows that the formation of chemical bonds within the three-dimensional hydrophilic network leads to a more rigid structure with higher elastic performances.

As further confirmation, the Ch/GG-EDA hydrogel showed a mild decrease in the G′ modulus over time compared to the physical one, as is better observable by strain sweep experiments (see

Supplementary Information file, Fig. S3). This highlights the stabilizing effect of covalent bonds on the mechanical properties of the sample. It is probable that four different types of interactions in the chemical hydrogel contribute to give the sample the mechanical characteristics: amide bonds, physical interactions that occur during the thermally induced coil to helix transition, ionic interactions between the opposite charged functional groups and finally the saline bonds between the calcium and carboxylate groups.

Interestingly, in the chemical hydrogel only 50 % of the amino groups (molar ratio between EDC/NHS and amino groups equal to 0.5) is theoretically involved in the formation of amide bonds, which means that in the final sample there are still free amino groups potentially exploitable for the functionalization of the hydrogel with different active molecules such as adhesion and chemoattractant molecules.

4.5. Swelling and hydrolytic degradation of chemical and physical GG-EDA hydrogels

Freeze-dried GG-EDA hydrogels (both chemical and physical) appear macroscopically as dried sponges and show, as can be seen from the SEM images reported in the Supplementary Information file (Fig. S4), a typical porous structure, potentially able to swell in aqueous medium.

The swelling studies performed on Ch/GG-EDA and Ph/GG-EDA freeze dried hydrogels, shown in Fig. 7a, have revealed that although both samples can absorb large quantities of aqueous medium, the chemical hydrogel shows a lower swelling percentage, significant ($p < 0.05$) after 48 h of incubation, probably due to the more compact structure obtained following the formation of covalent bonds. In agreement with rheological studies, hydrolysis experiments (Fig. 7b) have shown that the degradation of Ch/GG-EDA hydrogels is slower than Ph/GG-EDA thus confirming the greater stability of the first sample under physiological conditions. In particular the recovered weight % for the chemical hydrogel was significantly ($p < 0.05$) higher than the physical one after 14 and 21 days of incubation.

4.6. Cytocompatibility in vitro and cell adhesion studies

The characteristics reported so far for GG-EDA-based hydrogels make them optimal candidates as materials for biomedical applications. However, together with the mechanical and degradability properties,

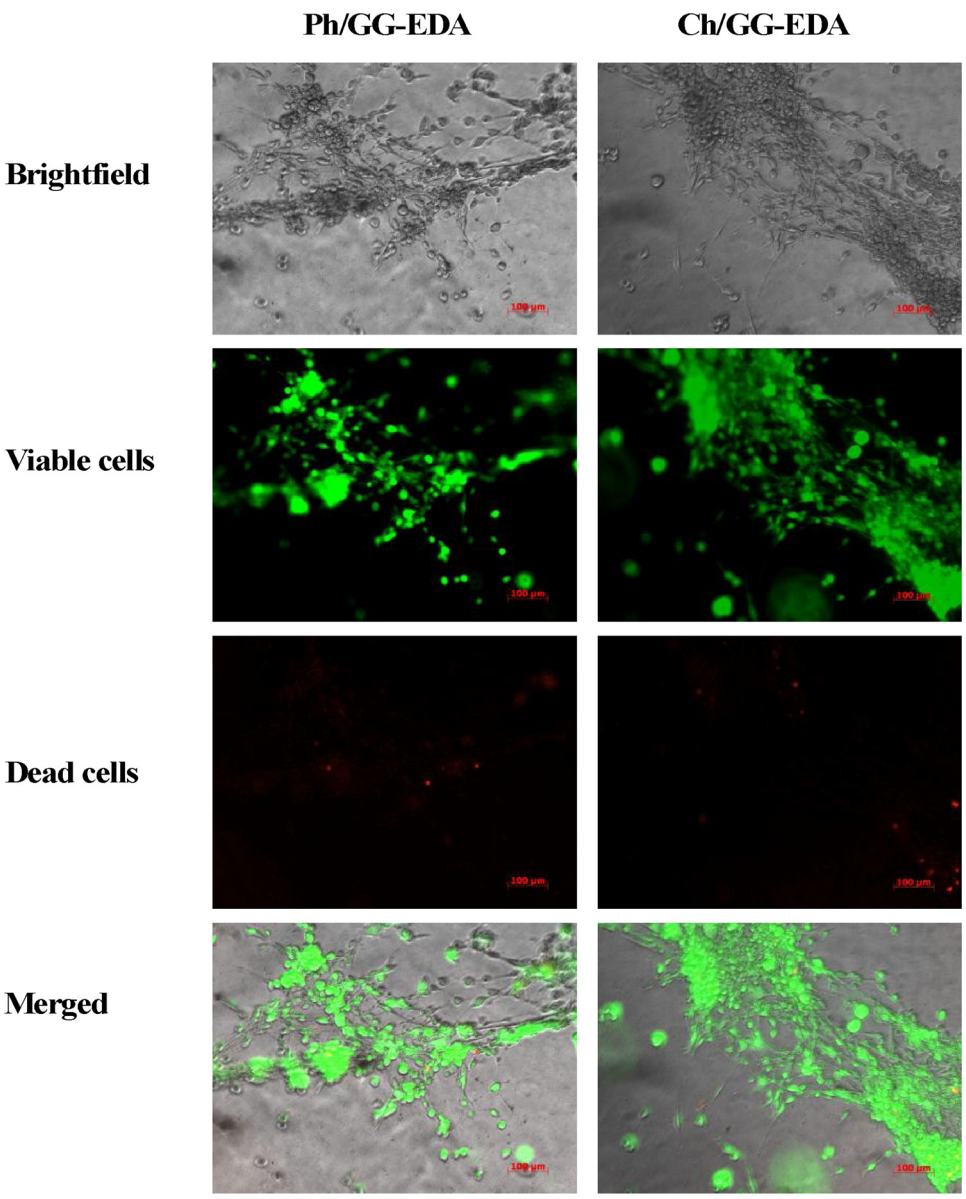

Fig. 9. Brightfield, fluorescent and merged images of Live/Dead assay conducted for 3T3 murine fibroblasts cultured for 48 h on Ph/GG-EDA or Ch/GG-EDA hydrogels.

cytocompatibility is another crucial aspect that must be considered in the design of a biomaterial.

To study the cytocompatibility of GG-EDA-based hydrogels, murine 3T3 fibroblasts were seeded on the surface of Ch/GG-EDA or Ph/GG-EDA prepared as described above and sterilized by UV irradiation. Fig. 8 shows that the viability (determined through the MTS test) of cells cultured on the hydrogel at both time intervals studied. No significant differences were observed between crosslinked physical and chemical samples revealing that both hydrogels do not interfere with cell viability.

The Live/Dead assay qualitatively confirmed the MTS results since, as can be seen from Fig. 9, very few dead cells can be observed on the hydrogels.

Furthermore, this assay allowed to observe that most of the cells adhered on both hydrogels show a typical elongated morphology which provides evidence that the samples are adhesive for cells. SEM images of cells cultured on chemical and physical GG-EDA hydrogels also confirmed the typical spindle-like shape of fibroblasts (see Supplementary Information file, Fig. S5).

5. Conclusions

The insertion of pendant primary amino groups in the GG backbone was carried out by means of a reproducible chemical procedure. The synthesized derivative, GG-EDA, has shown physicochemical properties that can be exploited for the production of hydrogels with better stability and elastic behavior than hydrogels obtained from non-functionalized polysaccharide.

In particular, through titration experiments it has been possible to demonstrate that GG-EDA shows, in the physiological pH, a nature of polyampholyte characterized by the simultaneous presence of primary ammonium and carboxylate groups that can establish ionic interactions with each other. The thermo-rheological analysis revealed that GG-EDA shows the typical thermotropic behavior of GG giving rise to the production of a temperature-induced hydrogel with a higher G' modulus than GG alone due to its polyampholyte nature. The physical interactions in the GG-EDA between opposite charged functional groups also stabilize the ionotropic crosslinked hydrogel which has led to higher G' values over time in physiological conditions than the GG hydrogel.

As proof of the concept, to demonstrate that it is possible to exploit the chemical versatility of the primary amino groups inserted, the chemical crosslinked GG-EDA hydrogel was produced by the EDC/NHS chemical procedure. Rheological analysis has shown that the establishment of intra and inter chemical bonds allows the formation of a hydrogel with further stability over time in physiological conditions in terms of G' modulus and degradation profile.

Finally, the preliminary *in vitro* biological characterization has shown that GG-EDA-based hydrogels are cytocompatible and adhesive for cells.

Overall, the results obtained suggest that GG-EDA can be considered a useful starting biomaterial for obtaining hydrogels for biomedical applications.

CRediT authorship contribution statement

Calogero Fiorica: Conceptualization, Methodology, Investigation, Data curation, Writing - review & editing, Visualization. **Giovanna Pitarresi:** Conceptualization, Writing - review & editing, Supervision. **Fabio Salvatore Palumbo:** Conceptualization, Methodology, Writing - review & editing, Supervision. **Nicolò Mauro:** Methodology, Investigation, Data curation, Writing - review & editing. **Salvatore Federico:** Investigation, Data curation, Visualization. **Gaetano Giammona:** Conceptualization, Supervision, Writing - review & editing, Funding acquisition.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.carbpol.2020.116033.

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