Carbohydrate Polymers 185 (2018) 73–84

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Synthesis and evaluation of thermo-rheological behaviour and ionotropic crosslinking of new gellan gum-alkyl derivatives

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ARTICLE INFO

Keywords:
Gellan gum
Alkylamines
Rheology
Hydrogels

ABSTRACT

This paper reports the synthesis and the physicochemical characterization of two series of gellan gum (GG) derivatives functionalized with alkyl chains with different number of carbon, from 8 to 18. In particular, low molecular weight gellan gum samples with 52.6 or 96.7 kDa, respectively, were functionalized with octylamine (C_8), dodecylamine (C_{12}) and octadecylamine (C_{18}) by using bis(4-nitrophenyl) carbonate (4-NPBC) as a coupling agent.

Thermo-rheological and ionotropic crosslinking properties of these gellan gum-alkyl derivatives were evaluated and related to the degree of derivatization in alkyl chains. Results suggested as length and degree of derivatization differently influenced coil-to-helix gelation mechanism of GG derivatives, ionotropic crosslinking, and strength of crosslinked hydrogels obtained in $CaCl_2$ 0.102 M and NaCl 0.15 M.

Statement of hypothesis: The insertion of alkyl chains on the gellan gum backbone interferes with coil-to-helix transition mechanism and allows the production of hydrophobically assembled hydrogels.

1. Introduction

Gellan gum (GG) is a microbial anionic polysaccharide produced by *Sphingomonas elodea*. It is composed of tetrasaccharide repeating units with one residue of α-L-rhamnose (Rhap), one of β-D-glucuronic acid (GlcpA) and two β-D-glucoses (Glcp), as proposed by Jansson, Lindberg, & Sandford (1983).

GG is mostly used in the food industry because it is able to form transparent hydrogels that are more resistant to heat and acidic medium than other polysaccharide hydrogels. GG has been approved as a food additive in many countries worldwide (Morris, Nishinari, & Rinaudo, 2012). Recently, it has been investigated for biomedical applications thanks to its biocompatibility and low cytotoxicity (Oliveira et al., 2010; Silva-Correia et al., 2011). Gellan gum has been also tested as a drug delivery carrier, cell carrier, biomaterial for guided bone-regeneration and wound dressing (Chang et al., 2010; Lee, Chen, & Tsao 2010).

Main disadvantages of GG, still limiting its suitability for production of biomaterials, are related to its poor long-term stability in physiological conditions, due to the exchange of divalent cations with monovalent ones (LeRoux, Guilak, & Setton, 1999), and to its poor dissolution in water at low temperatures. In fact, high temperatures (≈90 °C) are required to dissolve GG in its coiled form; then, after lowering the temperature, GG undergoes a coil-to-helix transition thus producing double helix crosslinked hydrogels. The GG gelation is further promoted by the ionotropic crosslinking forming strong and transparent hydrogels in the presence of metal ions, especially divalent cations as Ca^{2+} (Crescenzi & Dentini, 1987; Nishinari, Miyoshi, Takaya, & Williams, 1996; Milas, Shi, & Rinaudo M, 1990; Annaka, Takahashi, Nakahira, Tokita, & Matsuura, 2001; Ogawa et al., 2001). In particular, ionic bonds are formed between carboxyl groups of the polysaccharide and Ca^{2+} (Matsukawa, Tang, & Watanabe, 1999), and this interaction is responsible for a hydrogel strengthening with formation of crystalline crosslinking points between GG helices (Morris, Gothard, Hember, Manning, & Robinson, 1996).

However, despite the attractive ionotropic behavior, the thermo-sensitive gelation process restricts the versatility of GG in terms of processing and loading of actives. In fact, the loading of thermolabile

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https://doi.org/10.1016/j.carbpol.2018.01.021
Received 11 October 2017; Received in revised form 5 January 2018; Accepted 5 January 2018
Available online 06 January 2018
0144-8617/

drugs (e.g. therapeutic proteins) as well as cell entrapment are strongly hindered, making GG less attractive than other ionotropic polysaccharides (for example alginate), that instead have found several applications for drug and cell encapsulation procedures (Tan & Takeuchi, 2007).

Several researches have been focused on obtaining GG derivatives designed to solve solubility drawbacks, without losing ionotropic gel forming properties (Bacelar, Silva-Correia, Oliveira, & Reis, 2016; Miyamoto, Tsuji, Nakamura, Tokita, & Komai, 1996; Du, Hamilton, Reilly, & Ravi, 2012).

In a previous paper (Agnello et al., 2017), the properties of a derivative obtained through the insertion of octadecyl chains on GG backbone were assayed; this chemical modification improved solubility of GG in water even at low temperatures maintaining ionotropic gel forming properties.

In last few years, many amphiphilic polymers consisting of hydrophilic polysaccharides and hydrophobic moieties have been developed and their hydrophobic association in aqueous solution has been studied (Pelletier, Hubert, Lapicque, Payan, & Dellacherie, 2000; Palumbo et al., 2014; Palumbo et al., 2017). However, hydrophobic derivatization of a high molecular weight gellan gum did not lead to a hydrophobic association, probably due to the rigidity and the high molecular weight of the polymer (D'Arrigo et al., 2012; Agnello et al., 2017).

Starting from these results, aim of this work was to investigate the properties of new GG derivatives obtained by linking alkylamines with different length to the backbone of low molecular weight GG samples. In particular, the main aim of this study was to detect how the insertion of hydrophobic tails influenced GG behavior in aqueous environment, discriminating between the interfering effect on coil-to-helix conversion typical of GG, and the associative effect mediated by the hydrophobic portions. Low molecular weight gellan gum samples (52.6 or 96.7 kDa) were first produced by alkaline hydrolysis (Nitta, Takahashi, & Nishinari, 2010), then octyl (C_8), dodecyl (C_{12}) or octadecyl (C_{18}) portions were inserted thus obtaining derivatives with increased hydrophobic character. With the aim to detect the influence of the length of pendant chains and degree of derivatization on coil-to-helix transition and on ionotropic crosslinking (as a function of saline composition), a rheological characterization was performed. Contributions of cation interaction and hydrophobic association on gelation of these GG derivatives were discriminated.

2. Experimental

2.1. Materials and apparatus

Gellan Gum (Gelrite®), octadecylamine (C_{18}-NH_2), dodecylamine (C_{12}-NH_2), tetrabuthylammonium hydroxide (TBA-OH), bis(4-nitrophenyl) carbonate (4-NPBC), diethyl ether, ethanol (EtOH), acetone, dimethylsulfoxide anhydrous (DMSO), Dowex® 50WX8 hydrogen form, sodium hydroxide (NaOH), tetramethylammonium chloride (TMACl) were purchased from Sigma-Aldrich (Italy). Octylamine (C_8—NH_2) was purchased from Fluka (Italy).

Size exclusion chromatography (SEC) was performed using a system equipped with a pump system, a 2410 refractive index detector, all purchased from Waters (TA Instruments – Waters S.p.A., Italy).

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded using a Bruker AC-300 instrument operating at 300.12 MHz.

Rheological analysis was performed with a DHR-2 TA Instruments Trios (TA Instruments –Waters S.p.A., Italy).

Fluorescence spectroscopy was performed by using a Shimadzu RF-5301PC spectrofluorophotometer.

2.2. Production of low molecular weight gellan gum samples and their tetrabutylammonium salts

Hydrolysis of gellan gum (starting molecular weight 1000 kDa) was performed in basic conditions (Nitta et al., 2010). In particular two batches of 5 g of starting gellan gum (GG) (Gelrite®), were suspended into one liter of NaOH solution 0.1 M (pH 13) and 1.0 M (pH 14), respectively. Each batch was then homogenized for 10 min with a blade stirrer at 200 rpm, then maintained at 50 °C (sample at pH 13) or 37 °C (sample at pH 14) for 24 h. After neutralization with HCl 5N, each solution was dialyzed against distilled water (cut-off 25 kDa) and finally freeze-dried. This procedure allows the production of two derivatives having a Mw lower than starting GG. The sample obtained through hydrolysis at pH 13 was indicated as GG_m (where *m* means *medium* Mw); the sample obtained through hydrolysis at pH 14 was instead indicated as GG_s, (where *s* means *small* Mw).

Both GG_s and GG_m samples were transformed in their acid form by using Dowex® 50W-X8 hydrogen-exchange resin. The Dowex® resin was activated with a large excess of HCl 1 M and washed extensively with water. Finally, both GG_s and GG_m samples were transformed in their tetrabutylammonium salts as elsewhere reported (Pawar & Edgar, 2011; Lee, Tsai, Wen, & Huang, 2012). GG_s-TBA and GG_m-TBA solutions were freeze-dried and resulting samples were used for chemical modification.

2.3. Synthesis of GG-alkyl derivatives

GG-alkyl derivatives were prepared similarly to the procedure already described to functionalize hyaluronic acid (Palumbo et al., 2015; Palumbo et al., 2016; Agnello et al., 2016).

In this procedure, octylamine (C_8-NH_2), dodecylamine (C_{12}-NH_2) and octadecylamine (C_{18}-NH_2) were employed to perform the derivatization of both GG_s and GG_m samples. Each derivative was prepared by dissolving 200 mg of GG_s-TBA or GG_m-TBA in 17.6 ml of DMSO to obtain a final concentration of 1% w/v at 40 °C. Then, 2.4 ml of DMSO containing 6.86 or 13.72 mg of 4-NPBC were added to obtain a molar ratio, X, between 4-NPBC and GG repetitive units, equal to 0.1 and 0.2, respectively. After 4 h of activation, an appropriate amount of alkylamine was added to obtain a molar ratio, Y, between alkylamine and 4-NPBC, equal to 1.2; the temperature was raised to 60 °C and the reaction maintained for 24 h. The work out of the reaction was accomplished by adding 0.2 ml of NaCl saturated solution and stirring for 30 min. The derivatives functionalized with C_8-NH_2 and C_{12}-NH_2 were recovered after precipitation into an excess of ethanol, then washed several times with ethanol/water (8:2 v/v), absolute ethanol and finally with acetone. Instead, the derivatives functionalized with C_{18}-NH_2 were recovered after precipitation into an excess of diethyl ether/ethyl acetate (1:1 v/v), then they were washed several times with hot diethyl ether/ethyl acetate, then with ethanol/water (8:2 v/v), absolute ethanol and finally with acetone. In particular, three different reactions were performed for each derivative and degrees of derivatization, calculated via ^{1}H NMR, were expressed as mean values ± standard deviation. After characterization, all samples of each derivative were mixed and solubilized in water and finally freeze-dried, then used for the characterization.

2.4. Size exclusion chromatography analysis

Values of weight-average molecular weight (Mw) and polydispersity index (PDI) of GG_s, GG_m and their derivatives with alkylamines, were determined by size exclusion chromatography (SEC). The polymers were dissolved in tetramethylammonium chloride (TMACl, 0.025 M in water) at 0.1% w/v (Gunning & Morris, 1990; Atkin, Abeysekera, Kronestedt, & Robards, 2000). The elution was performed on a Polysep P-4000 column from Phenomenex at 35 °C, using TMACl (0.025 M) as an eluent (D'Arrigo et al., 2012), at a flow rate of 0.8 ml/min. A calibration curve based on pullulan (range of Mw 5900–404,000) in TMACl (0.025 M) was used.