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Short communication

Synthesis and characterization of N-(2-aminoethyl)-2-acetamidyl gellan gum with potential biomedical applications

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ABSTRACT

N-(2-aminoethyl)-2-acetamidyl gellan gum (GCM-EDA) was prepared by carboxymethylation (*via* nucleophilic substitution of primary hydroxyl groups of the β-D-glucose unit of gellan gum, in the presence of alkali and chloroacetic acid) and reaction with *tert*-butyl N-(2-aminoethyl) carbamate (N-Boc-EDA) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) as an activator, followed by deprotection with trifluoroacetic acid. The structural confirmation and characterization of N-(2-aminoethyl)-2-acetamidyl gellan gum was performed by spectroscopic, rheological and thermogravimetric analysis, and *in vitro* tests showed a lack of cytotoxicity which is indicative of the potential of this material to be used in biomedical applications.

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1. Introduction

Gellan gum (G) is an FDA-approved anionic polysaccharide obtained by aerobic fermentation of *Sphingomonas elodea* that is of interest in both food and cosmetic industries as well as in biomedicine (Prajapati, Jani, Zala, & Khutliawala, 2013). Its potential in this field has only recently started being explored fully and applications include so far tissue engineering (Tang, Sun, Fan, & Zhang, 2012), drug delivery systems (Jana, Das, Nayak, Sen, & Basu, 2013), and dental applications (Chang, Huang, Yang, Kuo, & Lee, 2012). Here we report on the preparation and characterization of a novel G derivative that appears to have promising perspectives for biomedical applications.

2. Experimental

2.1. Materials

Gellan (G) (KelcoGel®, low acyl, $M_w = 1 \times 10^6$ g/mol) was purchased from CP Kelco company, chloroacetic acid (CA) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) from Sigma–Aldrich, tert-Butyl N-(2-aminoethyl)carbamate (N-Boc-EDA) from Aldrich, trifluoroacetic acid (TFA) (Merck) and dichloromethane (DCM) were purchased from Fluka. The isotonic phosphate buffer solution (PBS, pH = 7.21) was prepared in house (1.36×10^{-1} M NaCl, 2.6×10^{-3} M KCl, 1.46×10^{-3} M KH_2PO_4 and 8×10^{-3} M Na_2HPO_4).

2.2. Synthesis of GCM and GCM-EDA

Carboxymethyl-gellan gum derivatives (GCM) were obtained according to literature (Miyamoto, Tsuji, Nakamura, Tokita, & Komai, 1996): 0.5 g of G were dissolved in 10 mL NaOH at various concentrations (10, 20, 30 and 40% (w/v)) and stirred for 5 h at room temperature. 5 mL of aqueous 2 M CA solution were then added dropwise and the reaction mixture was kept under stirring overnight at 4 °C. The product was dialyzed against water (until constant conductivity) and then freeze-dried to afford pure GCM. The obtained yields decreased from 81.00% to 48.72% with increasing of carboxymethylation reaction alkalinity. For further chemical modification only GCM with minimal degradation (as determined from rheology) was used.

GCM-EDA-N-Boc was synthesized by adding 0.48×10^{-3} mol EDAC to a solution of 50 mg of GCM in 25 mL water and stirring the reaction mixture for 2 h at 4 °C. A solution of N-Boc-EDA (3.48×10^{-3} mol in 5 mL of water, pH adjusted at 5.00 with HCl

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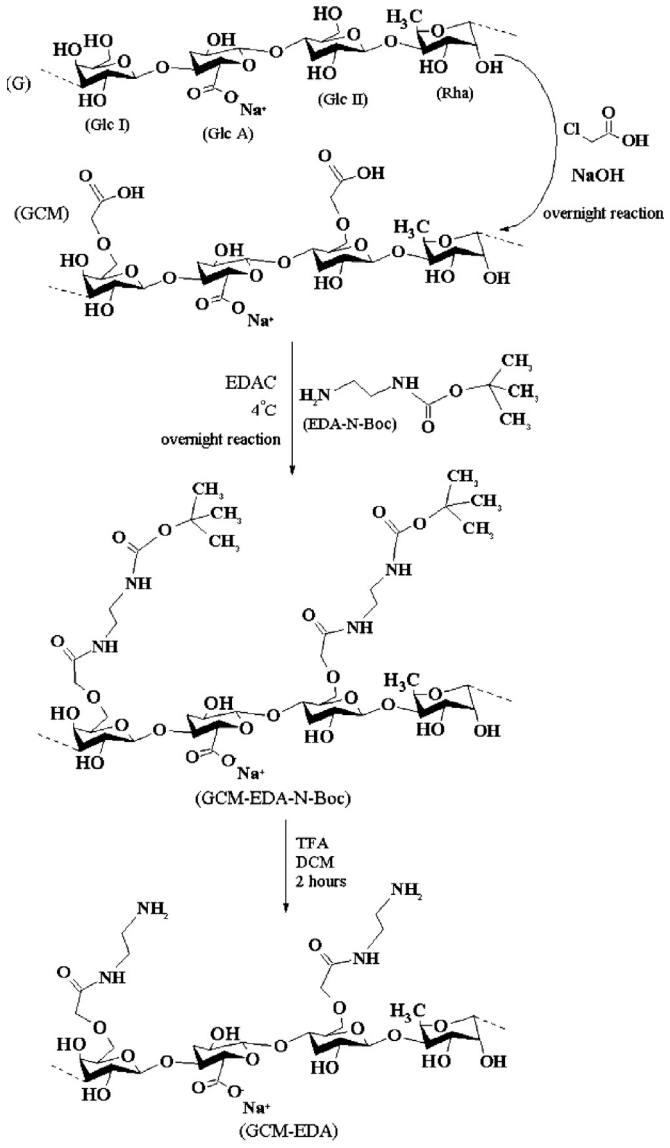

Fig. 1. Synthesis of aminated carboxymethyl-gellan gum.

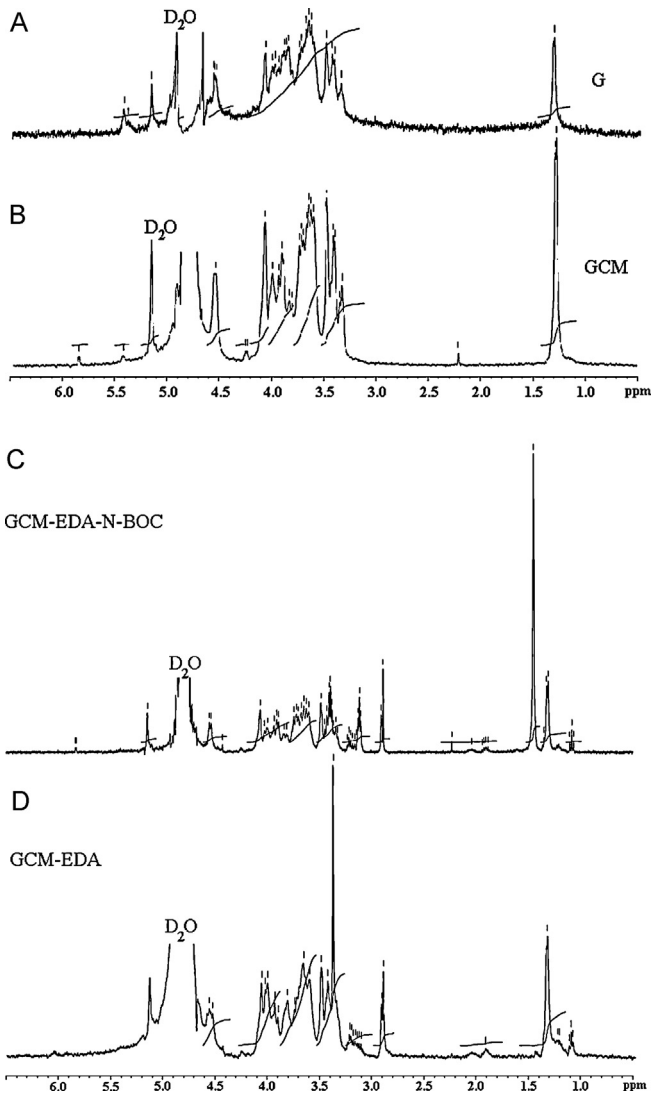

Fig. 2. (A and B) ^{1}H NMR spectra for gellan gum (G) and the carboxymethylated derivative (GCM). (C and D) ^{1}H NMR spectra for GCM-EDA-N-Boc and for GCM-EDA deprotected with TFA.

37%) was then added dropwise, and the mixture was left stirring overnight at 4 °C. The product was dialyzed against water and freeze-dried to afford GCM-EDA-N-Boc. For the deprotection reaction, 40 mg of GCM-EDA-N-Boc were dispersed in 10 mL DCM and 0.049×10^{-3} mol TFA were added; after stirring for 2 h followed by the removal of solvent under vacuum, the yellow residue was dialyzed against water and freeze-dried to afford GCM-EDA.

2.3. Physicochemical characterization

2.3.1. Potentiometric titration

Titrations with 0.01 M NaOH were carried out for a quantitative determination of the -COOH groups. GCM derivatives were dissolved in bidistilled water (0.02%, w/v) and passed through Amberlite IR120. Titrations were performed with Titralab TIM854 potentiometric titrator, 0.04 mL addition increments, until pH = 8.7. The degree of carboxymethylation % (DC) was calculated from the following equation:

$$\mathrm{DC\%} = \frac{[\mathrm{AGU}] \times n\mathrm{COOH}}{mp\text{-}59 \times n\mathrm{COOH}} \times 100, \quad (1)$$

where AGU (g/mol) = G monosaccharidic unit molar mass, nCOOH (mol) = moles of COOH determined from the equivalent volume of titrant (0.01 M NaOH) and 59 g/mol represents the net increase of molar mass of one AGU for each substituted carboxymethyl group.

2.3.2. NMR spectroscopy

^{1}H/^{13}C NMR spectra were recorded with a Bruker Avance DRX 400 MHz spectrometer using D_2O as solvent (at 70 °C for G and 50 °C for G derivatives). The degree of substitution % (DS) for GCM-EDA was determined from proton spectra as follows:

$$\mathrm{DS\%} = \left[\frac{\mathrm{A[2H\text{-}C10]}}{\mathrm{A[3H\text{-}C6]}} \times \frac{3}{2}\right] \times 100, \quad (2)$$

where A[2H-C10] is the integral of the signal of the methylene protons adjacent to the amino group (δ 2.89 ppm) in position C10 of Gcl I and Glc II units, and A[3H-C6] is the integral of the signal attributed to the methyl protons of the Rha unit (δ 1.32 ppm).

2.3.3. Rheology

Rheological measurements (oscillatory tests) were performed with a Haake RheoStress 300 Rotational Rheometer equipped with a Haake Thermostat DC10 (using a cone-plate with 0.053 mm

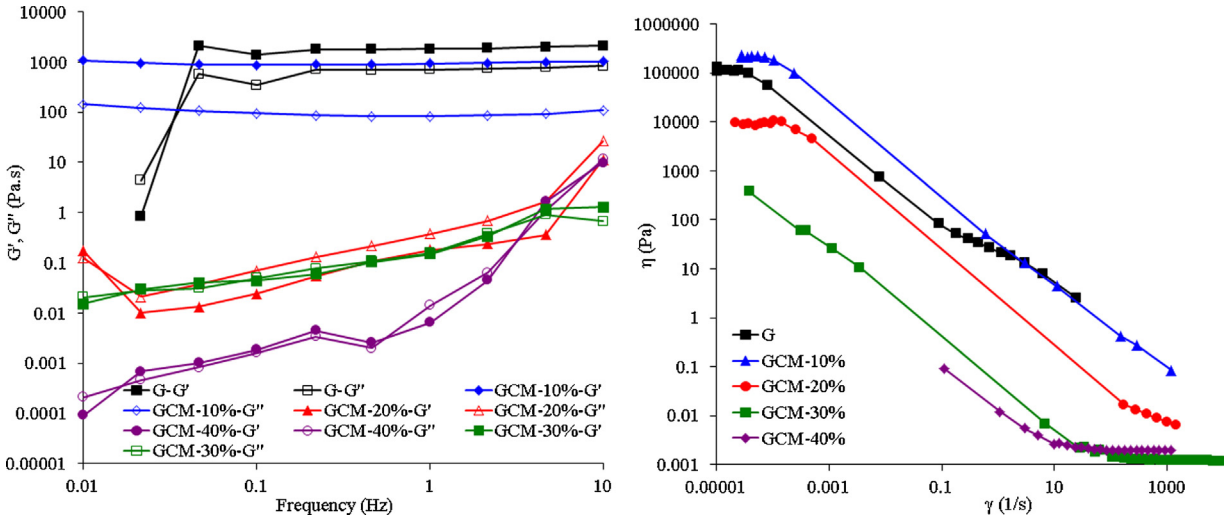

Fig. 3. Frequency sweep and shear viscosity of native G and GCM derivatives.

preset gap). Dynamic viscosity (η), viscoelastic parameters G′ (storage modulus) and G″ (loss modulus) were determined at 25 °C for the G and GCM derivatives dissolved in PBS (pH 7.21) at 2%, w/v.

2.3.4. Thermogravimetric analysis

Thermogravimetric analysis was performed using a Mettler Toledo TGA-SDTA851e system, in nitrogen atmosphere, with a flow of 20 mL/min, at a heating rate of 10 °C/min using a 4–6 mg sample.

2.3.5. Cytotoxicity measurements

Rat brain endothelial cells (bEnd3) were employed to measure cell viability following incubation with a suspension of the material to be tested (*i.e.* GCM-EDA); for comparison purposes, Chitosan (Cs; M_w = 700 kDa), Poloxamer 407 (P-407) and Dextran (M_w = 6000 Da) were also investigated. The materials to be tested were dispersed in PBS (1 mg/mL) and the resulting suspensions added prior to incubation of the cells.

Rat brain endothelial cells (bEnd3) were seeded in 96 well plates at 10,000 cells per wall in 200 μL Dulbecco's Modified Eagle Medium (DMEM supplemented with 10% fetal bovine serum, to maintain this particular cell-line viable). After 12 h at 37 °C treated cells were further incubated for 2 h with (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), after which the medium was removed and 100 μL DMSO was added into each well. The absorbance at 570 nm was determined after 10 min using a microplate reader (Optima- BMG Labtech 96). Viability of untreated cells was set as having value 1. Zero cells and digitonin were employed as positive controls, while cells with no treatment were employed as a negative control.

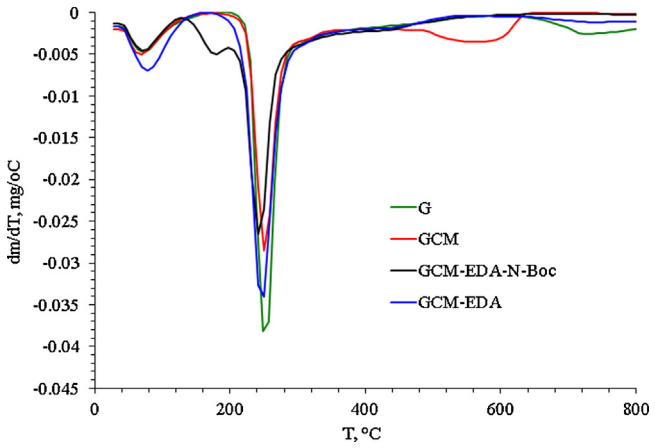

Fig. 4. DTG curves for GCM, GCM-EDA-N-Boc and GCM-EDA.

3. Results and discussion

GCM-EDA was prepared (yield 70%) *via* a facile 3-step chemical modification of G (Fig. 1).

Its structure was confirmed by NMR spectroscopy. The 1.32 ppm signal in the 1H-NMR spectra of G (Fig. 2A and B) was attributed to the methyl protons of the Rha unit, the signal at δ 4.53 ppm to the hydrogen bound to C1 of Glc II and the peak at δ 5.15 ppm to the hydrogen bound to C1 of the Rha unit; the protons of the tetrasaccharide repeating unit formed by Gcl I, Glc A, Glc II and Rha gave peaks in the region δ 3.34–4.25 ppm (Bosco, Miertus, Dentini, & Segre, 2000).

The ^{1}H NMR spectrum of GCM-EDA-N-Boc (Fig. 2C) shows two peaks at δ 3.10 and δ 2.89 ppm corresponding to the two pairs of methylene protons adjacent to the amino groups; a high intensity peak assigned to the tert-butyl group appears at δ 1.45 ppm near the peak corresponding to the methyl (δ 1.32 ppm) of GCM Rha proving the successful linkage of N-Boc-EDA onto GCM. ^{1}H NMR spectrum of GCM-EDA, carried out after TFA deprotection (Fig. 2D) shows the disappearance of the tert-butyl group while shifted signals at δ 3.36 ppm and δ 2.89 ppm assigned to EDA methylene protons were maintained, thus confirming the formation of the aminated GCM derivative (GCM-EDA) with a calculated DS (according to Eq. (2)) of 46.58%.

The degree of carboxymethylation (DC) determined using potentiometric titration (according to Eq. (1)) did not show significant differences between GCM derivatives having values around 23.8%, thus we can assert that DC was not influenced by the variation of carboxymethylation reaction alkalinity.

Frequency sweep spectra (γ = 0.01) of G and GCM derivatives exhibited the classical behavior of viscoelastic gels (G′ > G″); from shear viscosity data obtained (Fig. 3) it can be concluded that all tested materials have a non-Newtonian behavior. The dynamic viscosity of GCM derivatives progressively decreased with increasing concentration of NaOH employed in the reaction. The GCM obtained with 10% (w/v) NaOH had a similar behavior with that of the starting material (G), which indicates minimal degradation.

Differential thermogravimetric curves (DTG) indicated that thermal decomposition of these gellan gum derivatives takes place in several steps (Fig. 4). All tested materials showed mass loss due to dehydration in the first step, providing information about the

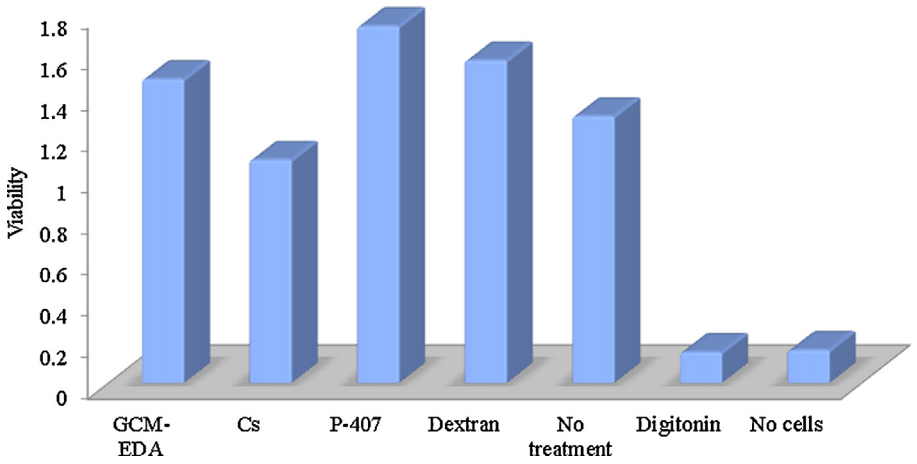

Fig. 5. Cytotoxicity – comparative results of the MTT assay (SD = 0.2%, $n = 3$).

water content. When compared to native G, GCM showed similar decomposition temperature for the main degradation step (T_{peak}), while the third step was found to be shifted to a lower temperature (approx. 500 °C). GCM-EDA-N-Boc showed a distinct intermediate decomposition process at 176 °C, prior to the main degradation step, and which can be attributed to the loss of the N-Boc moiety (Seo et al., 2009).

Preliminary cytotoxicity tests using bEnd3 cells and a MTT assay indicated that GCM-EDA is non toxic, with cell viability results comparable to FDA-approved poloxamer 407 and dextran (Fig. 5).

4. Conclusions

N-(2-aminoethyl)-2-acetamidyl gellan (GCM-EDA) was prepared as a new material using a 3-step procedure involving carboxymethylation of gellan gum and reaction with *tert*-butyl N-(2-aminoethyl) carbamate followed by deprotection with trifluoroacetic acid. Spectroscopic data confirmed the successful synthesis of aminated carboxymethyl gellan (GCM-EDA) while rheological tests evidenced a direct relationship between the alkalinity of the carboxymethylation reaction and the dynamic viscosity of GCM derivatives. MTT cytotoxicity assays performed on bEnd3 cells showed the GCM-EDA lack of toxicity for this type of cells, which is indicative of the promising use of this material in drug delivery for central nervous system and treatment of cerebral vascular tumors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.04.085.

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