Synthesis and characterization of Locust Bean Gum derivatives and their application in the production of nanoparticles

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Abstract (150)

The development of LBG-based nanoparticles intending an application in oral immunization is presented. Nanoparticle production occurred by mild polyelectrolyte complexation, requiring the chemical modification of LBG. Three LBG derivatives were synthesized, namely a positively charged ammonium derivative (LBGA) and negatively charged sulfate (LBGS) and carboxylate (LBGC) derivatives. These were characterized by Fourier-transform infrared spectroscopy, elemental analysis, nuclear magnetic resonance spectroscopy, gel permeation chromatography, and x-ray diffraction. As a pharmaceutical application was aimed, a toxicological analysis of the derivatives was performed by both MTT test and LDH release assay.

Several nanoparticle formulations were produced using LBGA or chitosan (CS) as positively charged polymers, and LBGC or LBGS as negatively charged counterparts, producing nanoparticles with adequate properties regarding an application in oral immunization.

Highlights 3 to 5 bullet points (max 85 characters each, including spaces)

- LBG charged derivatives (ammonium, sulfated and carboxylated) were synthesized;
- LBG-based nanoparticles, adequate for drug delivery applications, were produced;
- Only the LBG ammonium derivative demonstrated severe cytotoxicity in Caco-2 cells;
- Nanoparticles evidenced very mild effect on Caco-2 cell viability.

Keywords: Carboxylated locust bean gum; Polyelectrolyte complexation; Polymeric nanoparticles; Sulfated locust bean gum; Tetraalkylammonium locust bean gum

1. Introduction

The recent decades have brought to the market many new biomolecules that have been identified as having therapeutic potential. These molecules, which include from proteins and peptides to antigens and nucleic acids, are usually called biopharmaceuticals, meaning that they are biological in nature and manufactured using biotechnology (Rader, 2008). Although therapeutically promising, biopharmaceuticals are very unstable compounds and their administration is extremely challenging, due to inherent physicochemical and biopharmaceutical properties (Alonso, 2004; Kammona & Kiparissides, 2012). Moreover, the therapeutic action of proteins and protein-based molecules is not only limited by the potential degradation in biological environments, but also compromised by their low ability to reach the therapeutic site of action (Antosova, Mackova, Kral & Macek, 2009; Casettari & Illum, 2014; Kammona & Kiparissides, 2012). As such, a meaningful challenge for current pharmaceutical scientists has been the need to develop suitable vehicles that permit delivering macromolecules through alternative routes of administration. Polymeric nanoparticles have been demonstrating to be very promising in oral delivery of biopharmaceuticals, as many works report their effective role in the enhancement of oral drug bioavailability by facilitating cell internalization (Csaba, Garcia-Fuentes & Alonso, 2006; Kadiyala, Loo, Roy, Rice & Leong, 2010). Their reduced size provides an intimate contact with epithelia and, in several occasions, they have shown the capacity to carry the encapsulated molecules through the epithelium (Csaba, Garcia-Fuentes & Alonso, 2006; de la Fuente, Csaba, Garcia-Fuentes & Alonso, 2008). With respect to oral vaccination,

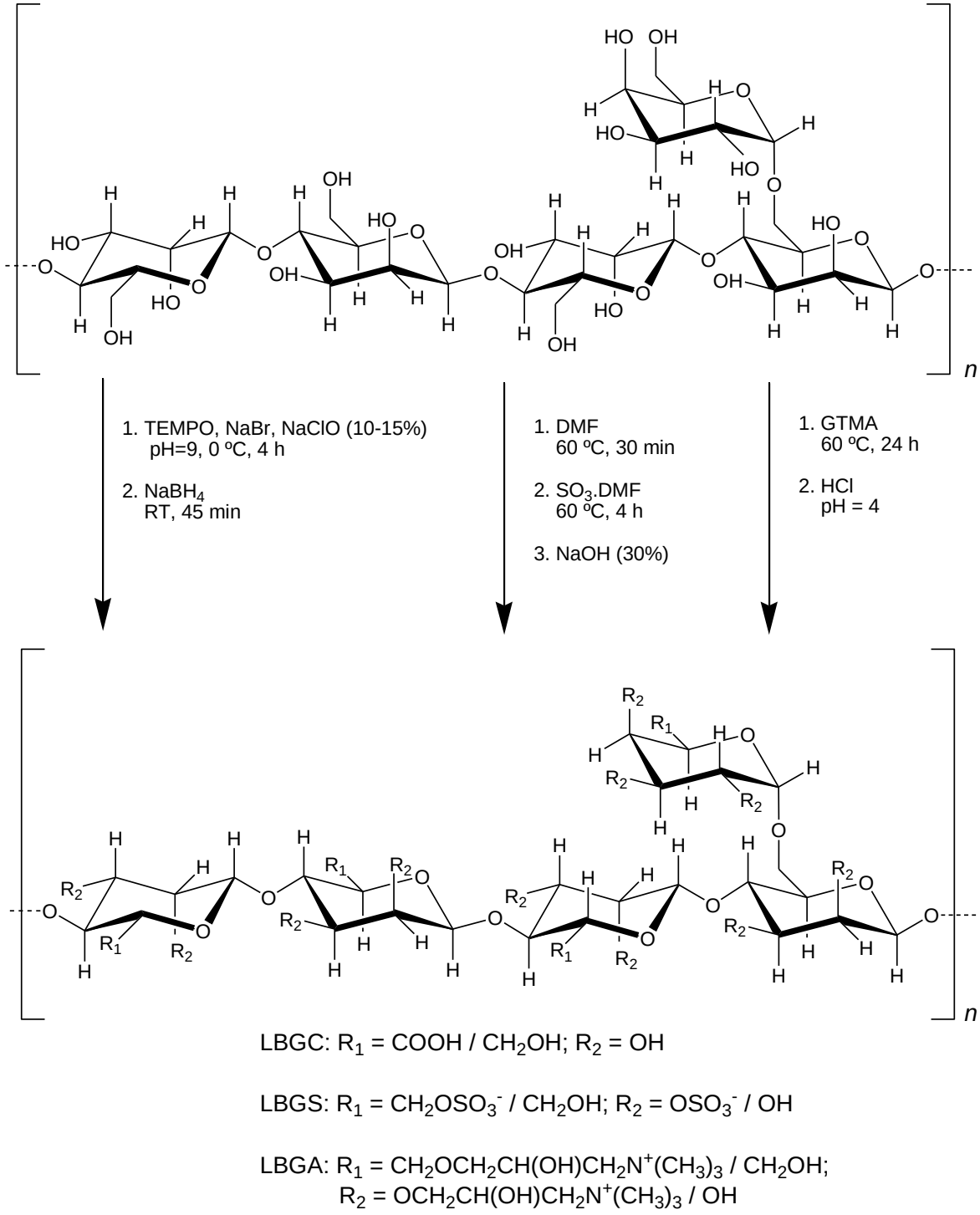
the design of suitable antigen delivery systems should focus on optimizing antigen association efficiency, ensuring the maintenance of its stability during association, tailoring release kinetics and eliciting high levels of long-lasting antibody and cellular immune responses. Nanoparticles may provide extra benefits in oral immunization strategies, because Peyer's Patches (PPs) have shown to be a predominant site for uptake of particulates (Lavelle & O'Hagan, 2006). Given their role in intestinal mediated immunization, M cells are the primary targets to consider. A careful selection of nanoparticle matrix materials may further help on the potentiation of an immune response. In this context, mucoadhesive polymers can contribute to the prolongation on the intestinal residence time (Arca, Gunbeyaz & Senel, 2009), potentiating the uptake by M cells.

Locust bean gum (LBG), also known as carob bean gum, is obtained from the endosperm of carob tree (*Ceratonia siliqua*) seeds, where it acts as reserve material. It is reported as biocompatible, biosorbable, biodegradable, non-teratogenic and non-mutagenic, presenting a mucoadhesive behaviour, and its degradation products are excreted readily (Dionísio & Grenha, 2012; Malik, Arora & Singh, 2011a; Pollard et al., 2007; Sudhakar, Kuotsu & Bandyopadhyay, 2006; Surana, Munday, Cox & Khan, 1998). Classified by the FDA as a GRAS (Generally Recognized as Safe) material, it is approved in most areas of the world for use in the food industry as thickener, stabilizer, emulsifier, and gelling agent (E410). It is also used in the pharmaceutical industry as excipient in drug formulations, and in biomedical applications (Barak & Mudgil, 2014; Dionísio & Grenha, 2012; Kawamura, 2008; Prajapati, Jani, Moradiya, Randeria & Nagar, 2013). LBG is mainly comprised of high molecular weight (approximately 50 000 – 3 000 000 Da) neutral galactomannan, consisting in a linear chain of (1-4)-linked β-D-mannopyranosyl units with (1-6)-linked α-D-galactopyranosyl residues as side

chains. The mannose and galactose contents have been reported to be 73-86% and 27-14%, respectively, which corresponds to a mannose:galactose (M/G) ratio of approximately 4:1 (Kawamura, 2008).

Recently, there has been a growing interest in the chemical functionalization of polysaccharides, particularly those non-animal derived, mainly by making use of the free hydroxyl groups distributed along their backbone, in order to create derivatives with properties tailored for the desired applications (Mizrahy & Peer, 2012).

In this paper, the chemical modification of LBG, aimed at obtain charged derivatives intended for the development of nanoparticulate carriers by polyelectrolyte complexation, is described. Two anionic (sulfate - LBGS and carboxylate - LBGC) and one cationic (trimethylammonium - LBGA) derivatives were prepared (**Figure 1**). The former were combined with the positively charged polysaccharide chitosan (CS) and the latter with LBGS in order to produce polymeric nanoparticles.



LBGC: R_1 = COOH / CH_2OH; R_2 = OH

LBGS: R_1 = $CH_2OSO_3^-$ / CH_2OH; R_2 = OSO_3^- / OH

LBGA: R_1 = $CH_2OCH_2CH(OH)CH_2N^+(CH_3)_3$ / CH_2OH;
R_2 = $OCH_2CH(OH)CH_2N^+(CH_3)_3$ / OH

Figure 1 – Scheme of the chemical modifications introduced in LBG.

2. Materials and methods

2.1. Materials

Locust bean gum (LBG) was a kind gift from Industrial Farense (Faro, Portugal). Due to the presence of around 3-7% protein in commercial LBG (Kawamura, 2008), a

purification step, based on previously published protocols (Bouzouita et al., 2007; Wang, Wang & Sun, 2002), was performed prior to use. This purified LBG was the material used for subsequent work, unless stated otherwise. Chitosan (CS, low molecular weight, deacetylation degree = 75 – 85%), glacial acetic acid, chlorosulfuric acid ($HClSO_3$), dimethylformamide (DMF), *N*-glycidyl-*N*,*N*,*N*-trimethylammonium chloride (GTMAC), sodium hydroxide (NaOH), potassium hydroxide (KOH), 2,2,6,6-tetramethypiperidine-1-oxyl (TEMPO), sodium bromide (NaBr), sodium hypochlorite solution (NaClO), sodium borohydride ($NaBH_4$), sodium nitrate ($NaNO_3$), sodium dihydrogen phosphate (NaH_2PO_4), sodium azide (NaN_3), dialysis tubing (pore size 2000 Da), phosphotungstate dibasic hydrate, glycerol, phosphate buffered saline (PBS) pH 7.4 tablets, Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin (10000 units/mL, 10000 µg/mL), non-essential amino acids, L-glutamine 200 mM, trypsin-EDTA solution (2.5 g/L trypsin, 0.5 g/L EDTA), trypan blue solution (0.4%), thiazolyl blue tetrazolium bromide (MTT), lactate dehydrogenase (LDH) kit, sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), hydrochloric acid (HCl 37%), sodium chloride (NaCl) and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Sigma-Aldrich (Germany). Ethanol was supplied by VWR. Potassium bromide (KBr) was obtained from Riedel-del-Haën (Germany). Fetal bovine serum (FBS) was obtained from Gibco (USA). Ultrapure water (Mili-Q Plus, Milipore Iberica, Madrid, Spain) was used throughout. All other chemicals were reagent grade.

2.2. Cell line

The Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, USA) and used between passages 77-93. Cell cultures were grown in 75 cm^2 flasks in humidified 5% CO_2/95% atmospheric air incubator at 37 ºC. Cell culture

medium was DMEM supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine solution, 1% (v/v) non-essential amino acids solution and 1% (v/v) penicillin/streptomycin. Medium was changed every 2-3 days and cells were subcultured weekly.

2.3. Synthesis of Locust Bean Gum derivatives

2.3.1. Purification of Locust Bean Gum

LBG purification was performed as described elsewhere (Braz, Grenha, Ferreira, Rosa da Costa, Gamazo & Sarmento, 2017).

2.3.2. Sulfation of Locust Bean Gum

The sulfation agent, SO_3·DMF, was prepared as described before (Braz, Grenha, Ferreira, Rosa da Costa, Gamazo & Sarmento, 2017).

Method 1

LBG (500 mg) was dispersed in DMF (35 mL) and stirred at 60 ºC for 30 min, in order to provide the dispersion of LBG in the solvent. Then, the SO_3·DMF complex was added (9.3 mL) and the mixture reacted for 4 h under magnetic stirring. Subsequently, the mixture was cooled down to room temperature in an ice bath, neutralized with 30% NaOH solution until precipitation, and concentrated under reduced pressure at 60 ºC to evaporate the solvent. The residue was dissolved in distilled water (30 mL) and dialyzed against distilled water (5 L). The water was changed every 24 h and, after 3 days, the solution was concentrated under reduced pressure at 40 ºC. Then, ethanol was added to the concentrated solution, in order to precipitate the solute, and the dispersion was concentrated under reduced pressure at 40 ºC. The previous step was repeated twice,

and the last evaporation was performed until full evaporation of the solvent. The obtained powder was dried in a vacuum oven at 40 ºC for 3 days, affording 407 mg of brownish powder that was grinded and stored until further use.

Method 2

LBG sulfation by this method was performed as described before (Braz, Grenha, Ferreira, Rosa da Costa, Gamazo & Sarmento, 2017).

2.3.3. Carboxylation of Locust Bean Gum

LBG (500 mg) was dissolved in 200 mL of distilled water under stirring at 80 ºC for 30 min. After cooling down, the volume was adjusted to 200 mL and the solution was cooled in an ice bath. Then, TEMPO (10 mg) and NaBr (50 mg) were added to the solution under stirring. A 15% sodium hypochlorite solution (3.0 mL) with pH adjusted to 9.3 with 2 M HCl solution, was mixed with the polymer solution. The pH was maintained at 9.3 by addition of a 0.05 M aqueous NaOH solution for 4 h. To stop the reaction, sodium borohydride (75 mg) was added and the solution was stirred for 45 min. Then the pH of the mixture was adjusted to 8 by addition of HCl before precipitation by 2 volumes of ethanol in presence of NaCl (up to 10 g/L). The polymer was isolated by filtration under reduced pressure, washed several times with ethanol, filtered and dried in a vacuum oven at 30 ºC during 3 days. A white powder (529 mg) was obtained, grinded and stored until further use.

2.3.4. Quaternary ammonium salt of Locust Bean Gum

An aqueous solution (10 mL) of KOH (0.550 g), was prepared in a round bottom flask, under stirring, at 60 ºC. Then, purified LBG (506 mg) and 3.72 mL of GTMAC were

added. After 5 h, an equal amount of GTMAC was added to the mixture, which was allowed to react until the completion of 24 h. It was then diluted with 20 mL of miliQ water, allowed to cool down to room temperature, and neutralized with HCl (2M). The resulting solution was dialyzed for 3 days, the water being replaced every 24 h. Then, the LBGA solution was concentrated under reduced pressure at 40 ºC and ethanol was added to the concentrated solution, in order to precipitate the solute. The dispersion was concentrated under reduced pressure at 40 ºC and ethanol was added again and evaporated under the same conditions until full evaporation of the solvent. The obtained powder was dried in a vacuum oven at 40 ºC for 3 days, affording 423 mg of white powder that was grinded and stored until further use.

2.4. Chemical characterization of Locust Bean Gum derivatives

2.4.1. Fourier transform infrared (FTIR) spectroscopy

For recording FTIR spectra of purified LBG and their derivatives, samples were grounded with KBr in a mortar and compressed into discs. For each spectrum, a 32-scan interferogram was collected in transmittance mode with a 4 cm^{-1} resolution in the 4,000-400 cm^{-1} region.

2.4.2. Elemental analysis

Elemental analysis data were obtained in a Thermo Finnigan, FLASH EA 1112 Series (C, N, S) or in a Fisons Instruments, EA 1108 CHNS-O (O) elemental analyzer.

2.4.3. Nuclear magnetic resonance (NMR) spectroscopy

All liquid NMR spectra were acquired in a Bruker Avance III 400 spectrometer equipped with a temperature control unit and a pulse gradient unit capable of producing

magnetic field pulsed gradients in the z-direction of 56.0 G/cm, operating at 400.15 MHz for hydrogen, 100.61 MHz for carbon, using a multinuclear reverse 5 mm probe (TXI). The samples where dissolved in D_2O. Solid state NMR spectra were acquired in a Bruker Avance III 300 spectrometer equipped with a BBO probehead, operating at 300.15 MHz for hydrogen, 75.00 MHz for carbon. The sample was spun at the magic angle at a frequency of 5 kHz in a 4 mm-diameter rotor at room temperature.

^{1}H NMR spectra were recorded with 8.22 KHz spectral window digitized with 64 K points. The **^{13}C** spectra were recorded between 0 and 238 ppm using 24,000 Hz spectral window digitized into 64 K points.

Two-dimensional ^{1}H-^{1}H correlation spectroscopy (**COSY**) spectra were acquired using 32 transients and 16 dummy scans, with a spectral width of 5000 in a total of 2K data points in *F*2 and 128 data points in *F*1, the relaxation delay was set to 1.5 s. Heteronuclear Single Quantum Coherence-Total Correlation Spectroscopy (**^{1}H/^{13}C HSQC-TOCSY)** spectra were acquired using the following parameters: 2K data points in F2 with a spectral width of 5000 Hz, 512 data points in F1 with a spectral width of 17 KHz, a relaxation delay of 2 s, MLEV-17 sequence with a mixing time of 40 ms, 16 transients and 16 dummy scans. The phase-edited heteronuclear single quantum correlation (**^{1}H/^{13}C HSQC-DEPT**) spectra were acquired in 2K data points in F2 with a spectral width of 5000 Hz, 512 data points in F1 with a spectral width of 17 KHz, a relaxation delay of 2 s, 2 to 8 transients and 16 dummy scans. The Heteronuclear Multiple Bond Correlation (**^{1}H/^{13}C HMBC**) spectra were acquired using the following parameters: 1K data points in F2 with a spectral width of 5000 Hz, 256 data points in F1 with a spectral width of 22 KHz, a relaxation delay of 2 s, 24 transients and 16 dummy scans.

The ^{13}C MAS NMR experiments were acquired with proton cross polarization (CPMAS) with a contact time of 1.2 ms, and a recycle delay of 2.0 s.

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2.4.4. GPC/SEC^3 analysis

Triple detection Gel Permeation Chromatography (GPC/SEC^3) analysis was performed in a modular system constituted by a degasser, HPLC pump (K-1001) and RI detector (K-2300) from Knauer, and a viscometer and RALLS from Viscotek (Trisec Dual
270 Detector Model 270), using two PL aquagel-OH mixed 8 μm, 300 x 7.5 mm columns. For purified LBG, LBGC and LBGS the eluent was 0.2 M $NaNO_3$, 0.01M NaH_2PO_4, 0.1% w/v NaN_3, pH=7, at 1mL/min; the samples were dissolved in the eluent at 1 mg/mL. For LBGA the eluent was 0.5 M $NaNO_3$, 0.01M KH_2PO_4, 0.1% w/v NaN_3, pH=2, at the same rate; the sample was dissolved at 1mg/mL in 10^{-2} M HCl.

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2.4.5. X-ray diffraction (XRD)

Powder X-ray diffractograms were recorded on a Panalytical X'Pert Pro diffractometer, operating at 45 kV and 35 mA. The patterns of the pristine and modified samples were recorded in the range 5-45 degrees (2θ) with a step size of 0.0167 º and a time per step
280 of 2 000 seconds, using CuKα radiation filtered by Ni and an X'Celerator detector. Prior to the analysis, samples were reduced to a fine powder by grinding in a mortar.

2.5. Production, characterization and safety evaluation of Locust Bean Gum-based nanoparticles

285 All nanoparticles were prepared by polyelectrolyte complexation, which consists in the electrostatic interaction between the positive and negative charges of the different polymers (Bhattarai, Gunn & Zhang, 2010).

2.5.1. Production of CS/LBGS and CS/LBGC nanoparticles

Several mass ratios of CS/LBGC and CS/LBGS (see **Table 1**) were used to prepare the nanoparticles by polyelectrolyte complexation. The stock solution of CS, dissolved in 1% (w/w) acetic acid, was prepared to reach a final concentration of 1.0 mg/mL, while those of LBGC and LBGS, dissolved in ultrapure water, had a final concentration of 2.0 mg/mL. Nanoparticles were prepared as described before (Braz, Grenha, Ferreira, Rosa da Costa, Gamazo & Sarmento, 2017).

2.5.2. Production of LBGA/LBGS nanoparticles

Three mass ratios of LBGA/LBGS (2/1, 1/1 and 1/2) were used to prepare the nanoparticles by polyelectrolyte complexation. The stock solutions of LBGA and LBGS were prepared by dissolving the polymers in ultrapure water, at final concentrations of 0.5 mg/mL and 1.0 mg/mL, respectively. Nanoparticles were prepared as described before (Braz, Grenha, Ferreira, Rosa da Costa, Gamazo & Sarmento, 2017).

2.5.3. Characterization of Locust Bean Gum-based nanoparticles

2.5.3.1. Size, polydispersion index, ζ potential and production yield

The size, ζ potential, polydispersion index (PdI) and production yield were determined as described before (Braz, Grenha, Ferreira, Rosa da Costa, Gamazo & Sarmento, 2017).

2.5.3.2. Morphological analysis

The morphological examination of LBGA/LBGS nanoparticles was conducted by transmission electron microscopy (TEM; JEM-1011, JEOL, Japan). The samples were

stained with 2% (w/v) phosphotungstic acid and placed on copper grids with carbon films (Ted Pella, USA) for TEM observation.

2.5.4. Safety evaluation

The *in vitro* cell viability and cytotoxicity of bulk LBG, purified LBG and the synthesized derivatives was assessed in Caco-2 cells by the MTT and the LDH release assays, respectively. LBGA/LBGS nanoparticles were evaluated using the MTT assay. The assays were performed as described before (Braz, Grenha, Ferreira, Rosa da Costa, Gamazo & Sarmento, 2017).

2.6. Statistical analyses

The t-test and the one-way analysis of variance (ANOVA) with the pair wise multiple comparison procedures (Holm-Sidak method) were performed to compare two or multiple groups, respectively. All analyses were run using the SigmaStat statistical program (Version 3.5, SyStat, USA) and differences were considered to be significant at a level of $P < 0.05$.

3. Results and discussion

3.1. Synthesis and chemical characterization of Locust Bean Gum derivatives

The syntheses of the three charged LBG derivatives were made by adapting procedures described in the literature for the modification of other polysaccharides. To perform the sulfation reaction, SO_3·DMF was chosen as sulfating agent (Yuan et al., 2005), as it presents advantages over methods involving the manipulation of either pyridine or sulfur trioxide (Alban, Schauerte & Franz, 2002; Mähner, Lechner & Nordmeier, 2001; Mihai, Mocanu & Carpov, 2001). For the synthesis of the sulfate derivative, two

approaches were performed as described in the methodology. The difference mainly resided in the processing of LBG prior to the addition of SO_3·DMF. For the introduction of trimethylammonium groups in LBG, GTMAC was used as alkylating agent, which proved to be efficient in the alkylation of other polysaccharides (Dionísio, Braz, Corvo, Lourenço, Grenha & da Costa, 2016; Qin et al., 2004; Rekha & Sharma, 2009; Simkovic, Yadav, Zalibera & Hicks, 2009).

For the transformation of LBG into the corresponding polyuronic acid, TEMPO, a stable nitroxyl radical, was chosen as oxidizing agent (Sierakowski, Milas, Desbrières & Rinaudo, 2000). This has proved to possess a high efficiency in the conversion of high molecular weight polysaccharides. A highly selective oxidation of C-6 primary hydroxyl to carboxylic groups can be achieved in an aqueous solution of the polysaccharide at pH 9-11 with NaClO and catalytic amounts of TEMPO and NaBr (Cunha, Maciel, Sierakowski, Paula & Feitosa, 2007; da Silva Perez, Montanari & Vignon, 2003; Sierakowski, Milas, Desbrières & Rinaudo, 2000).

As shown in **Figure 2**, LBG sulfate functionalization (LBGS) was confirmed by FTIR, through the appearance of a S=O asymmetric stretching band (Yuan et al., 2005) at 1255 cm^{-1} and that of C-O-S symmetric stretching (Alban, Schauerte & Franz, 2002) at 817 cm^{-1}. In the carboxylate derivative (LBGC), the absorption bands at 1601 cm^{-1} and 1415 cm^{-1} are attributed to asymmetric and symmetric stretching vibration of $–COO^-$, respectively (Cunha, Maciel, Sierakowski, Paula & Feitosa, 2007). Since the quaternary ammonium groups do not display characteristic IR absorption bands (Nakanishi, Goto & Ohashi, 1957), evidence for formation of the amino functionalized derivative (LBGA) comes from the broadening of the band at 1088 cm^{-1} (ether C-O symmetric stretching) and the new bands at 1479 and 914 cm^{-1} (C-H scissoring in methyl groups of the ammonium and ether C-O asymmetric stretching, respectively) (Qin et al., 2004).

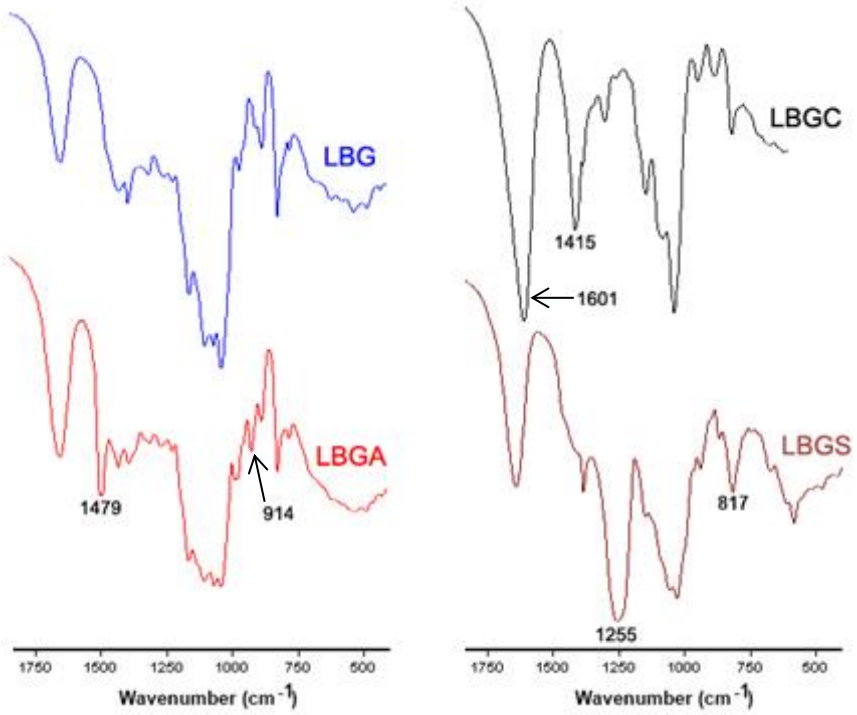


Figure 2 – FTIR spectra of purified Locust Bean Gum (LBG) and its ammonium (LBGA), carboxylate (LBGC) and sulfate (LBGS-M1) derivatives.

In the elemental analysis, the weight percentages found for the analysed elements are compiled in **Table S1**.

For LBGS, different degrees of substitution were obtained, even under the same reaction conditions. For the sample of LBGS obtained by method 1 (LBGS-M1), a C:S molar ratio of 8.78 was obtained, which corresponds to a degree of substitution (DS) of 3.5. Therefore, if sulfate groups are assumed to be in the form of sodium salts, a molecular formula between $C_{30}H_{47}S_3O_{34}Na_3$ and $C_{30}H_{46}S_4O_{37}Na_4$, to which corresponds a mean molecular weight of 1166 g/mol, is derived. On the other hand, the samples of LBGS obtained by method 2 presented a high variability on C:S molar ratio, ranging from 26.76 in batch 1 (LBGS-M2-B1) to 6.55 in batch 2 (LBGS-M2-B2), and batch 3 (LBGS-M2-B3) presenting a value of 10.24. These values corresponded to values of DS of 1.22, 4.63, and 3, and to the mean molecular weights of 932, 1282, and 1111 g/mol,

respectively. As indicated in materials and methods, and stated above, the difference between the two methods only refers to a preliminary treatment of LBG before the sulfation reaction. In the second method, a better dispersion of LBG was promoted before the contact with the sulfating agent in an attempt to improve the reaction. The need for this pre-treatment was motivated by the poor solubility of LBG in DMF. Since in the sulfation reaction the polymer is used as a dispersion in the solvent, it would be expected that a more effective dispersion would favour the reaction. Quite surprisingly, it was observed that, although the pre-treatment afforded the highest value of DS (4.63), it also gave the lowest substitution (1.22), while in its absence an intermediate value of DS was obtained. This variation in DS translates, in the FTIR spectra of the various samples, in different intensities of the band at 1255 cm^{-1} relative to other bands in the spectrum, with more substituted samples presenting a more intense band (**Figure S1**). Assuming that better dispersion of LBG leads to higher reaction efficiency and affords higher values of DS, it seems that the dispersibility of LBG in the reaction medium does not directly correlate to the method used in its dispersion. One reason for the observed variability in DS may be the fact that, contrary to what is observed in the reactions described below (oxidation and alkylation), in which LBG progressively dissolves as the reactions proceed, in this case a total solubilisation is never reached. This renders the outcome of this reaction quite unpredictable and, therefore, this issue will have to be tackled in future work. In fact, the reaction of LBG activated by pre-soaking in DMF and dispersed in the same solvent, with solid SO_3·DMF complex, bellow 15 ºC, led to a DS of approximately 4 (Maiti, Chowdhury, Chakraborty, Ray & Sa, 2014). On the other hand, sulfation of LBG dispersed in formamide with SO_3·pyridine complex, under diverse conditions of reaction time, temperature, and amount of sulfating agent, led to DS varying between approximately 2 and nearly 5 (Wang et al., 2014). Again, the

soaking of LBG with the solvent prior to the reaction led to an intermediate DS relative to the range obtained without any pre-treatment, although in the latter case a different reagent and solvent were used. Nevertheless, only one batch per reaction conditions seems to have been obtained in both these works and, therefore, the state of dispersion of LBG in each case may well be the factor governing the substitution obtained, instead of the parameters analysed. Moreover, in the latter work, no correlation or trend between molecular weights of the obtained derivatives or depolymerization of the parent polysaccharide and degree of substitution is observed. On the contrary, a very erratic dispersion of molecular weights with growing DS is obtained, pointing to a random behaviour in this reaction.

For LBGC, a C:O ratio of 1.02 was found, which corresponds to a degree of oxidation (DO) of 4, meaning that all the free C-6 must have been oxidized. Assuming all carboxylate groups to be in the sodium salt form, the molecular formula would be $C_{30}H_{38}O_{29}Na_4$, and the molecular weight 955 g/mol. This value is not surprising, in view of the effectiveness of the oxidizing system, although somewhat higher than DO values observed for other galactomannans, which typically lay below 70% of the free units (Cunha, Maciel, Sierakowski, Paula & Feitosa, 2007).

In LBGA, the C:N molar ratio was found to be 13.16, corresponding to a DS of 4.24. If all the ammonium groups are in the form of chloride salt, this corresponds to a molecular formula between $C_{54}H_{106}O_{29}N_4Cl_4$ and $C_{60}H_{120}O_{30}N_5Cl_5$, and the mean molecular weight of 1454 g/mol. This corresponds to a full reaction of the free C-6 hydroxyl groups, along with reaction on some secondary hydroxyls, in line with what was observed by us in a similar modification performed in pullulan (Dionísio, Braz, Corvo, Lourenço, Grenha & da Costa).

The analysis of the ^{1}H NMR and ^{13}C spectra of LBG and the obtained derivatives (**Figure S2**) allowed us to obtain a molecular view on the success of the transformations. However, the broadened signals in the ^{1}H spectra do not allow the evaluation of the derivatization locations and, therefore, spectral assignment was performed through 2D NMR (^{1}H,^{1}H-COSY and ^{13}C,^{1}H-HSQC-DEPT) experiments. **Figure 3** shows the ^{1}H/^{13}C HSQC-DEPT NMR spectra of untreated LBG **(a)**, LBGS-M2-B1 **(b)**, LBGC **(c),** and LBGA **(d)**. In untreated LBG, the two anomeric carbons C-1 from mannose and galactose residues resonate at 102.8 and 100.3 ppm, respectively. The unsubstituted C-6 positions of main chain mannose exhibited a chemical shift at 61.4 ppm.

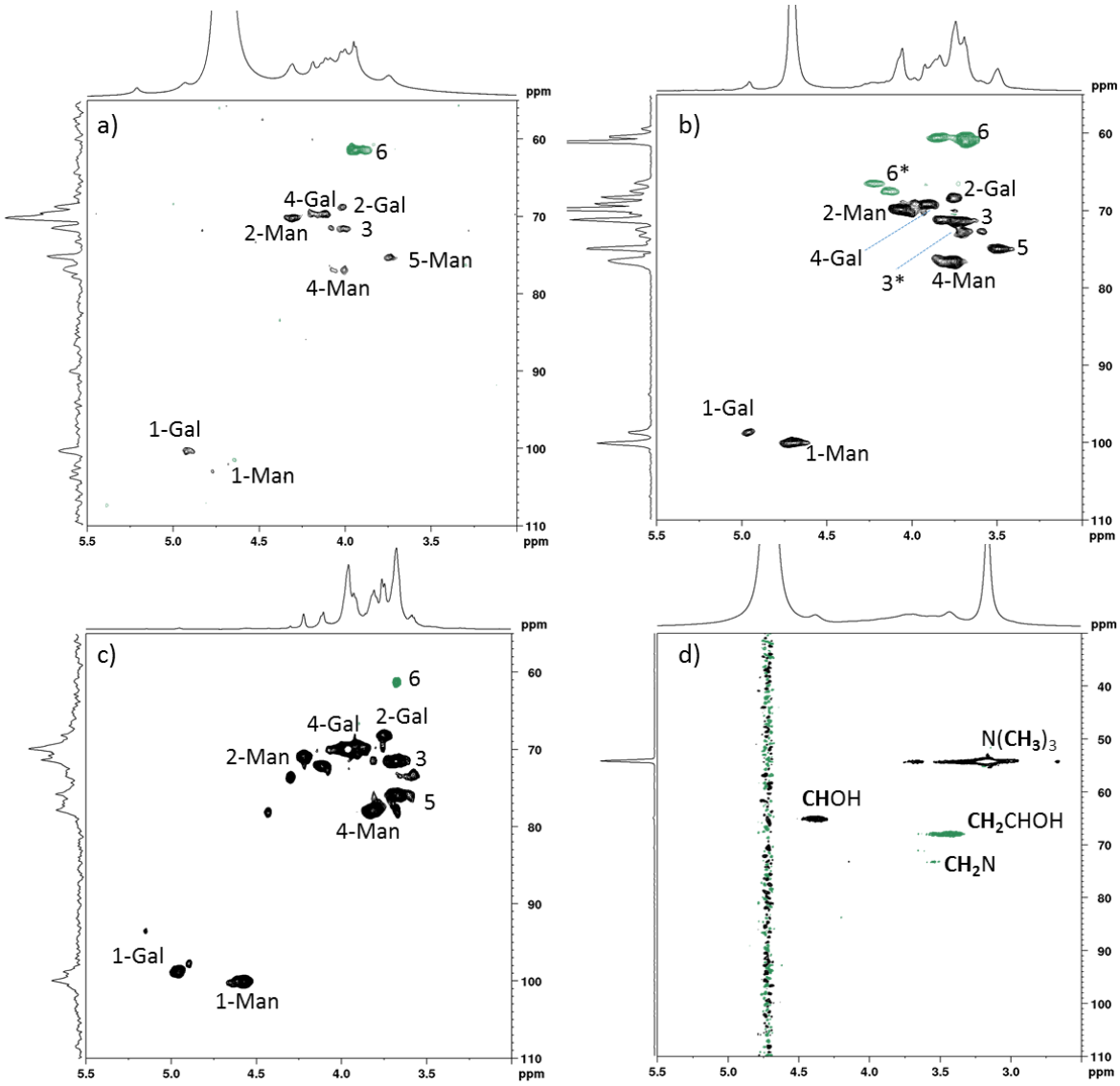


Figure 3 – $^{1}H/^{13}C$ HSQC-DEPT spectra of (a) LBG, (b)LBGS-M2-B1, (c) LBGC, and (d) LBGA; (*) Assignments attributed to the sulfate derivative.

The attachment of sulfate groups to the hydroxyls results usually in downfield shifts of the carbons bearing the sulfates and the protons linked to them (Duus, Gotfredsen & Bock, 2000). LBG primary hydroxyl groups in C-6 position are clearly the most reactive towards the sulfation reaction, as would be expected. In LBGS **(Figure 3-b)**, the C-6 resonances exhibit a downfield shift to 66.4 ppm, indicative of C-6 sulfation. It is also noticeable a sulfate introduction in position C-3. The lower steric hindrance in the branched galactose residues in comparison to the main chain mannose leads to the assumption that sulfate introduction would have taken place preferentially in the

hydroxilated positions of the former, and as such 3-Gal would have been preferentially substituted (Muschin et al., 2016). The carboxylation of LBG imposes a different effect on the $^{1}H/^{13}C$ HSQC-DEPT spectra, since the resonances of the positions that indeed react are expected to disapeer from the original location. From **Figure 3-c** it is possible to observe that C-6 resonance is almost absent, which means that once again this was the reaction location. Unreacted positions C-2,C-3 and C-4 appear unchanged, while additional resonances appear downshifted from the original envelope of resonances, most likely due to oxidized C-6 positions. The carboxylation is also confirmed by the presence of the carbonyl in the ^{13}C NMR spectra at 175.3 ppm **(Figure S2-f)**. The attachment of *N*-(2-hydroxypropyl)-*N,N,N*-trimethylammonium to LBG produces a derivative with a high swelling capability. Here, the introduction of the ammonium group is confirmed by the well resolved N-methyl resonances at 3.146 ppm. However, these resonances dominate the whole spectrum **(Figure 3-d)** and all ^{1}H signals are very broadned. As such, this sample was analysed in the solid state **(Figure S2-e)**, where the resonances of the ammonium group at 54.62 ppm, the anomeric carbons (102.17 and 100.71 ppm) and the remaining polysaccharide chain between 90 and 60 ppm could be detected.

The average molecular weights, polydispersity index (PdI), and radius of gyration (*R*g) of LBG and its derivatives are presented in **Table S2**. For the parent polysaccharide (LBG), these are in general agreement with the literature (Dakia, Blecker, Robert, Whatelet & Paquot, 2008; Kawamura, 2008). Upon chemical modification, an increase in both molecular weight and R_g was observed in LBGA, and a big decrease in these parameters was patent in LBGC and in the analysed sample of LBGS-M1. The increase identified in LBGA is attributable to the presence of the introduced pendant chains, which led to an increase in the molar mass of the repeating unit and force the polymer,

once in solution, and similarly to what happens in the crystalline state (XRD results), to adopt a conformation that is suitable to accommodate such bulky groups. The results observed in the LBGC and LBGS-M1 derivatives suggest the occurrence of depolymerization during the chemical modification, a common observation when the conditions of either the oxidation (Cunha, Maciel, Sierakowski, Paula & Feitosa, 2007) or the sulfation reaction (Alban, Schauerte & Franz, 2002) are applied. The latter was already stated in a similar modification performed in pullulan (Dionísio, Braz, Corvo, Lourenço, Grenha & da Costa). Moreover, at least in the analysed sample, and as verified in the referred sulfation of pullulan, no additional dehydration reactions, with intra- and/or intermolecular crosslinking leading to a fraction of high molecular weight chains, observed in sulfation reactions carried out at higher temperatures (Mihai, Mocanu & Carpov, 2001), occurred in this case.

Figure 4 shows the XRD patterns of the pristine and modified LBG samples. The pattern of LBG, with a broad peak centered at *ca.* 20º 2θ with shoulders at *ca.* 7.5 and 15º 2θ, reflects the predominantly amorphous nature of the material. These shoulders vanish in the pattern of LBG modified with sulfate (LBGS-M2-B2), probably due to some changes in the organization of the polymer chains imposed by the sulfate groups. In what concerns the ammonium derivative, the pattern clearly shows an increase of intensity for higher d-spacings, which is compatible with an increase of the distance between the polymer chains, due to the long chain bearing the ammonium group (Dionísio, Braz, Corvo, Lourenço, Grenha & da Costa). When compared with the other modifications, the introduction of carboxylate groups gives rise to the highest degree of disruption of the long-range order of the LBG polymer chains. The intensity of the peak that appears at 20º 2θ in the pattern of the original polymer (LBG) is substantially reduced and new broad peaks are now present at ca. 12 and 25º 2θ. This is not

surprising, as the conversion of galactose and mannose units into the corresponding uronic acids would enormously affect the conformation of the polysaccharide chains and, consequently, the way they pack in the solid phase.

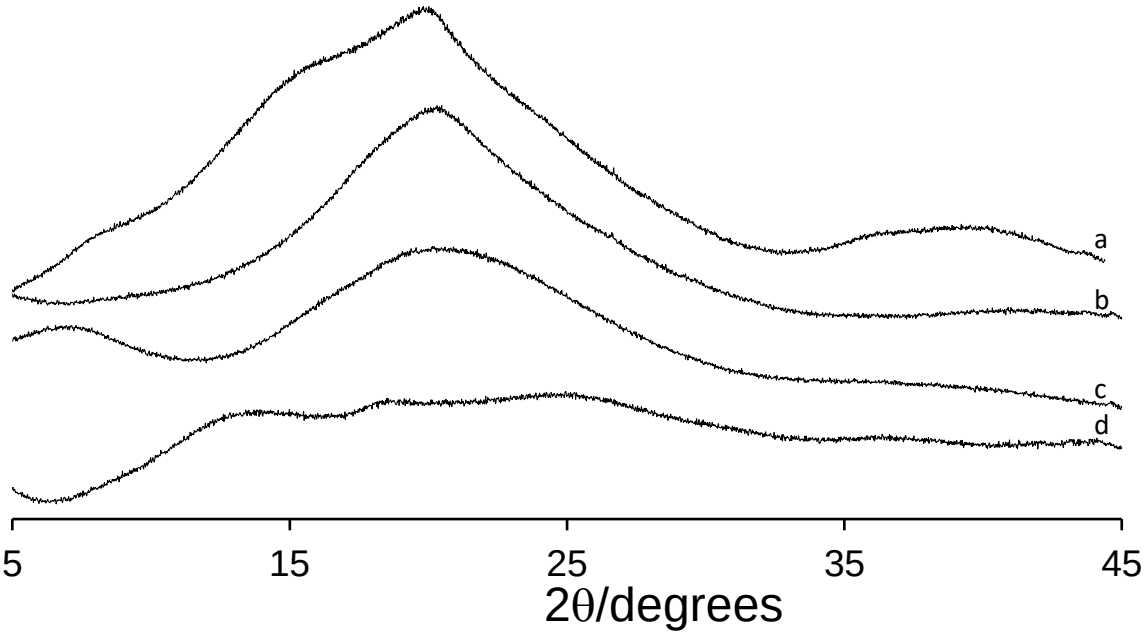


Figure 4 – XRD patterns of (a) pristine locust bean gum (LBG), (b) LBGS-M2-B2, (c) LBGA, and (d) LBGC.

3.2. Characterization of nanoparticles

The production of LBG derivatives described above endowed the polymer with charged groups, enabling the preparation of nanoparticles by polyelectrolyte complexation. This is a mild method occurring in hydrophilic medium, devoid of aggressive conditions such as organic solvents or high shear forces, and involving electrostatic interactions between oppositely charged polymers (Grenha, 2012; Prego, Torres & Alonso, 2005). Three derivatives were synthesized which were used in the production of different formulations of nanoparticles. The negatively charged sulfate and carboxylate derivatives were complexed with chitosan to produce CS/LBGS and CS/LBGC

nanoparticles, respectively. In turn, the ammonium derivative was complexed with the sulfate derivative in the innovative approach of producing LBG-only nanoparticles (LBGA/LBGS). The results regarding the physicochemical characterization of the referred nanoparticle formulations are displayed and discussed below.

3.2.1. CS/LBGS and CS/LBGC nanoparticles

The first approach towards the formulation of CS/LBGS and CS/LBGC nanoparticles involved the production of carriers having higher or at least the same amount of LBG derivative comparing to chitosan. In this regard, the starting mass ratios selected for the production of the referred formulations of nanoparticles were 1:1, 1:1.5 and 1:2. In the course of the experiments, the need to test other ratios was identified, not necessarily being coincident for each formulation, thus justifying the slight differences observed between the two formulations.

Table 1 displays the physicochemical characteristics of CS/LBGS nanoparticles. For the production of these nanoparticles, LBGS corresponding to method 1 was used. With CS/LBGS mass ratios varying between 1:1 and 1:2.5, and recalling that CS amount remains constant in all formulations, it was verified that nanoparticle size generally increased with increasing amounts of LBGS. The minimum size was 364 nm (CS/LBGS = 1:1, w/w) and the highest size was 589 nm (CS/LBGS = 1:2.5, w/w) ($P < 0.05$).

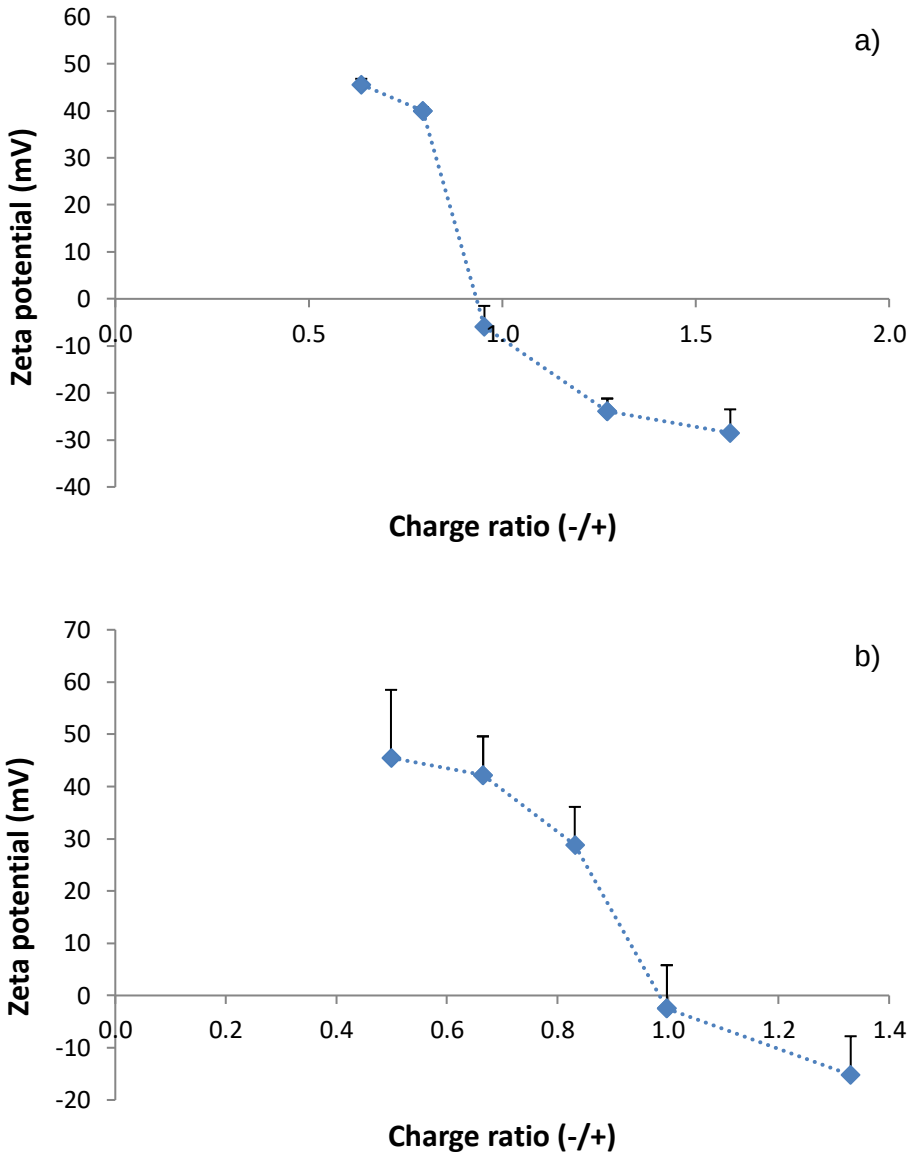
Table 1 - Physicochemical characteristics and production yield of CS/LBGS, CS/LBGC and LBGA/LBGS unloaded nanoparticles (mean ± SD; n ≥ 3). Different letters represent significant differences in each parameter and formulation ($P < 0.05$).

Formulation	Ratio (w/w)	Size (nm)	PdI	Zeta potential (mV)	Production yield (%)
CS/LBGS	1:1	364.1 ± 30.0 [a]	0.34 ± 0.09	+45.6 ± 1.2 [d]	37.3 ± 5.6 [h]
	1:1.25	403.7 ± 37.7 [ab]	0.40 ± 0.06	+40.0 ± 0.8 [e]	58.1 ± 2.7 [i]
	1:1.5	pp*	1.0 ± 0.0	-5.9 ± 4.4 [f]	n.d.
	1:2	500.3 ± 59.6 [bc]	0.47 ± 0.08	-23.9 ± 2.7 [g]	56.6 ± 7.2 [i]
	1:2.5	589.0 ± 69.5 [c]	0.54 ± 0.03	-28.5 ± 5.0 [g]	n.d.
CS/LBGC	1:0.75	489.9 ± 63.6 [a]	0.45 ± 0.04	+45.5 ± 13.0 [b]	49.0 ± 5.0 [d]
	1:1	479.1 ± 30.8 [a]	0.51 ± 0.07	+42.2 ± 7.4 [b]	54.3 ± 7.0 [d]
	1:1.25	828.8 ± 299.8 [a]	0.64 ± 0.15	+28.8 ± 7.3 [b]	n.d.
	1:1.5	pp*	1.0 ± 0.0	-2.5 ± 8.3 [c]	n.d.
	1:2	pp*	1.0 ± 0.0	-15.2 ± 7.4 [c]	n.d.
LBGA/LBGS	1:2	206.6 ± 5.0 [a]	0.13 ± 0.03	-27.8 ± 1.4 [c]	30.0 ± 8.6 [e]
	1:1	pp	-	-	-
	2:1	368.3 ± 19.3 [b]	0.38 ± 0.05	+48.1 ± 1.5 [d]	16.7 ± 3.8 [f]

n.d.: not determined; pp: precipitate; *slight precipitation compromised the measurement of this parameter

The registered increase in size as higher amount of LBGS is included in the formulations as compared with CS, might be explained by the increase of total mass of polymers that is present. This effect was also reported in other works using the same nanoparticle production method (Grenha et al., 2010; Rodrigues, da Costa & Grenha, 2012). Precipitation was found to occur for an intermediate formulation (CS/LBGS = 1:1.5, w/w), being coincident with a zeta potential close to zero (-5.9 mV) that possibly

is not sufficient to provide particle repulsion, thus leading to aggregation. A clear Tyndall effect was observed in all the other nanoparticle formulations. The formulations 1:1 and 1:1.25 (w/w) exhibited a strong positive zeta potential of more than +40 mV. The incorporation of a higher amount of LBGS, from formulation 1:1 to 1:1.25 (w/w) resulted in a corresponding decrease in the zeta potential from +46 mV to +40 mV ($P < 0.05$). The formulations 1:2 and 1:2.5 (w/w) presented a complete shift in the zeta potential as the nanoparticles became negatively charged, with zeta potential reaching -29 mV. Again, the incorporation of a higher amount of LBGS led to a nominal decrease in the zeta potential, although this is not statistically significant. This absolute shift of nanoparticle charge reflects the higher amount of LBGS that is present in the nanoparticles, but also demonstrates that both polymers have different charge density. Zeta potential results are perfectly in line with the charge ratios that were calculated for each formulation of nanoparticles, as is depicted in **Figure 5-a**. This figure shows the effect of charge ratios on the zeta potential of CS/LBGS nanoparticles prepared with varying polymeric ratios.



570

Figure 5 – Effect of charge ratio (-/+) on the zeta potential of (a) CS/LBGS nanoparticles and (b) CS/LBGC nanoparticles.

575 For each polymer, by dividing the charge of the repeating unit by its molar mass, a charge per mass ratio may be obtained. CS has higher charge per mass ratio than LBGS (4.72×10^{-3} *vs* 3.00×10^{-3} charges/g, respectively), which justifies why formulations CS/LBGS = 1:1 and 1:1.25 (w/w) have a -/+ charge ratio below 1. The strong positive zeta potential (> +40 mV) of these nanoparticles is due to the predominance of positive

580 charges. In turn, the occurrence of precipitation in the formulation 1:1.5 (w/w) was

coincident with a charge ratio around 1, justifying that the determined zeta potential was close to neutrality. In fact, although a 1:1 -/+ charge stoichiometry might not imply the occurrence of complete charge neutralization, due to steric limitations and different charge spacing in the intervenient species (Rodrigues, da Costa & Grenha, 2012), one may assume a preferential interaction between the sulfate and the ammonium groups, both weakly hydrated, instead of with the strongly hydrated counterions (Crouzier & Picart, 2009). This mainly leads to an intrinsic charge match in detriment of an extrinsic charge compensation and, thus, to a small deviation from neutrality. Finally, the continued addition of the negative polymer (formulations CS/LBGS = 1:2 and 1:2.5, w/w) produced an excess of negative charges, resulting in -/+ charge ratio above 1 and, consequently, negatively charged nanoparticles. A similar behavior concerning the charge ratios leading to either precipitation or formation of nanoparticles, was previously described (Rodrigues, da Costa & Grenha, 2012).

The polydispersity index varied between 0.3 and 0.5, which is considered high. Regarding the production yield, very reasonable values for this nanoparticle production methodology, were obtained. A yield of 37% was registered for formulation 1:1 (w/w) which increased to 58% ($P < 0.05$) for formulation 1:1.25 (w/w). This is a result of the proper mechanism of nanoparticle formation, based on the neutralization of chitosan amino groups by the sulfate groups of LBGS. The incorporation of a higher amount of LBGS provides an additional amount of sulfate groups that interacted with chitosan, thus forming a higher amount of nanoparticles (Fernández-Urrusuno, Romani, Calvo, Vila-Jato & Alonso, 1999). However, this effect occurs up to a certain limit. As observed, further increasing the amount of LBGS led to precipitation, certainly because of the demonstrated neutralization of charges, as referred above. On keeping increasing

LBGS mass, nanoparticles are again formed (CS/LBGS 1:2 and 1:2.5, w/w), this time with an opposite charge and a high yield (57% for formulation 1:2, w/w).

The results obtained for CS/LBGC nanoparticles were rather different comparing to those described above regarding CS/LBGS formulations. In this case, as shown in **Table 1**, the initially approached formulation of CS/LBGC 1:1 (w/w) resulted in a size of 479 nm, which is more than 30% higher than the corresponding CS/LBGS formulation ($P < 0.05$). The formulation 1:1.5 (w/w) already presented precipitation, similarly to 1:2 (w/w) and, therefore, the intermediate formulation 1:1.25 (w/w) was produced.

The registered size revealed a strong increase to 829 nm, although this is not statistically significant as is accompanied by an extremely high standard deviation, which indicates reproducibility issues. This formulation also presented a high polydispersity index and, thus, was not characterized for production yield. An attempt was also performed to produce nanoparticles at CS/LBGC ratio of 1:0.75 (w/w), but the characteristics were very similar, under all aspects, to those of ratio 1:1 (w/w). The polydispersity index was around 0.5 – 0.6, which is even higher than those registered for CS/LBGS nanoparticles, reinforcing the difficulty in producing suitable nanoparticles with the LBGC derivative. The zeta potentials were highly positive (around +45 mV), which probably contributes to the system stability. The determination of the charge ratios involved in each formulation of nanoparticles is depicted in **Figure 5-b**.

As observed, formulations 1:0.75 and 1:1 (w/w) have a -/+ charge ratio between 0.5 and 0.7 which does not translate into significant differences in the zeta potential. Nanoparticles 1:1.25 (w/w) displayed a -/+ charge ratio of 0.85 which induced a nominal decrease of the zeta potential to +29 mV, although not to a statistically significant level. As observed above for CS/LBGS nanoparticles, reaching a -/+ charge

ratio around 1 (formulation 1:1.5, w/w) resulted in precipitation. However, in this case the continued addition of the negative polymer to formulate CS/LBGC = 1:2 (w/w) nanoparticles still resulted in precipitation, despite the -/+ charge ratio of 1.4. It is important to highlight that, while the resulting zeta potential for this formulation was of -15 mV, in the CS/LBGS corresponding formulation was -24 mV, which possibly permitted enough repulsion to stabilize the formed nanoparticles.

The determined production yields were satisfactory for this methodology, as referred above, being around 50%. When comparing the zeta potentials of these nanoparticles with those obtained for CS/LBGS nanoparticles (**Table 1**), a similar trend was observed. In this regard, increasing the amount of LBGC present in the formulation reflected in a decrease of the surface charge, owing to the higher amount of negative groups being incorporated. Similarly to CS/LBGS nanoparticles, the formulation 1:1.5 was the one showing neutrality (zeta potential of -2.5 mV) and the further incorporation of LBGC led to a decrease in the surface charge. The precipitation verified for the latter was possibly due to the fact that the existing surface charges were not sufficient to ensure particle repulsion. The resemblance of the trend, particularly regarding the shift of the zeta potential (occurring for mass ratio of 1:1.5), suggests the similarity of charge density in both derivatives. In fact, LBGS has a charge per mass ratio of 3.00×10^{-3} charges/g, as stated before, and LBGC has 3.14×10^{-3} charges/g.

3.2.2. LBGA/LBGS nanoparticles

One of the great novelties of producing LBG charged derivatives was the possibility of using these to produce, for the first time, LBG-only nanoparticles. Given the difficulties in producing nanoparticles with the LBGC derivative, as stated above, it was decided to produce the LBG-only nanoparticles using just LBGS as negative counterpart. The

nanoparticles were produced by complexation of this derivative (method 2 – 50/50 mixtures of batches 2 and 3) with the ammonium derivative (LBGA) by the same methodology reported in the other cases (polyelectrolyte complexation).

After observing the precipitation of the formulation LBGA/LBGS 1:1 (w/w), possibly resulting from a (-/+) charge ratio of 1.09, formulations 1:2 (w/w) and 2:1 (w/w) were developed, which results are depicted in **Table 1**.

The formulation containing the highest amount of LBGS registered size of 207 nm and low polydispersity index of 0.13. Naturally, the zeta potential was negative (-28 mV), reflecting the higher content of negatively charged derivative, which translated into a (-/+) charge ratio of 2.17. As expected, the formulation having more LBGA exhibited a strongly positive zeta potential (+48 mV; $P < 0.05$), as a result of the (-/+) charge ratio of 0.54. However, this particular formulation presented higher size (368 nm) along with higher polydispersity index ($P < 0.05$). At a first evaluation, the size differences could be considered unexpected. In fact, for the preparation of these nanoparticles, LBGA is kept constant at 0.5 mg/mL and LBGS concentration is adapted to meet the desired ratio. Therefore, formulation 1:2 (w/w) accounts with a total polymeric mass of 1.5 mg, while formulation 2:1 (w/w) accounts with 0.75 mg. In line with this, formulation 1:2 (w/w) was perhaps expected to have larger size. However, if one considers the molecular weight of the derivatives, reported in section 3.3.1, LBGA has much higher M_n than LBGS (500 600 *vs* 21 380). In this regard, it becomes justifiable that nanoparticles having double amount of LBGA comparing with LBGS are those displaying the highest size.

Regarding the production yield, this was very different between the two formulations. While formulation 1:2 (w/w) resulted in 30%, formulation 2:1 (w/w) presented 17% ($P < 0.05$). This difference is probably due to variances in the molecular weight of the two

derivatives. In formulation 1:2 (w/w), there is a determined amount of a high molecular weight polymeric chain and a double amount of a shorter macromolecule that possibly presents higher diffusion. On the contrary, in formulation 2:1 (w/w) the amount of the polymer with higher molecular weight is double comparing with that of the smaller polymer, thus resulting in a lower number of interactions and limiting the amount of nanoparticles formed.

LBG-only nanoparticles were morphologically characterized by TEM and the specific formulation LBGA/LBGS 1:2 (w/w) was considered representative for this end. As shown in **Figure 6**, nanoparticles present a spherical shape and have compact structure.

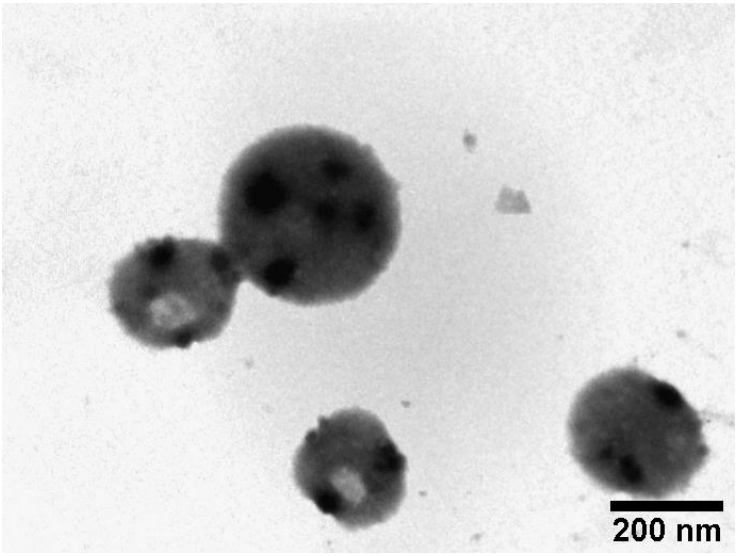


Figure 6 – TEM microphotograph of LBGA/LBGS = 1:2 (w/w) nanoparticles.

3.3. Safety evaluation

Caco-2 cells were used to evaluate the toxicological profile of LBG and the synthesized derivatives. Cell viability was determined after exposure to the mentioned materials at different concentrations, for a period of 3 h (**Figure S3**) and 24 h (**Figure 7**). Cell viability values were calculated in relation to the 100% cell viability considered for the incubation with DMEM (negative control of cell death). The evaluation of LBG-based samples generally evidenced a mild effect on cell viability, considered to be devoid of

biological relevance. In fact, with the exception of LBGA, all the other samples resulted in viabilities above 70% after 3 h or 24 h of exposure, when tested at concentrations varying within 0.1 and 1.0 mg/mL. While at 3 h values remained above 88% in all conditions, the prolonged exposure until 24 h induced slight alterations. However, these were in most cases devoid of physiological relevance and the only remarkable effect resides in the decrease of the viability induced by the contact with LBGC at the highest concentration tested (1.0 mg/mL) ($P < 0.05$) to a value around 70%. Importantly, this is the value considered by ISO 10993-5 (ISO, 2009) as the level below which a toxic effect is assumed to occur.

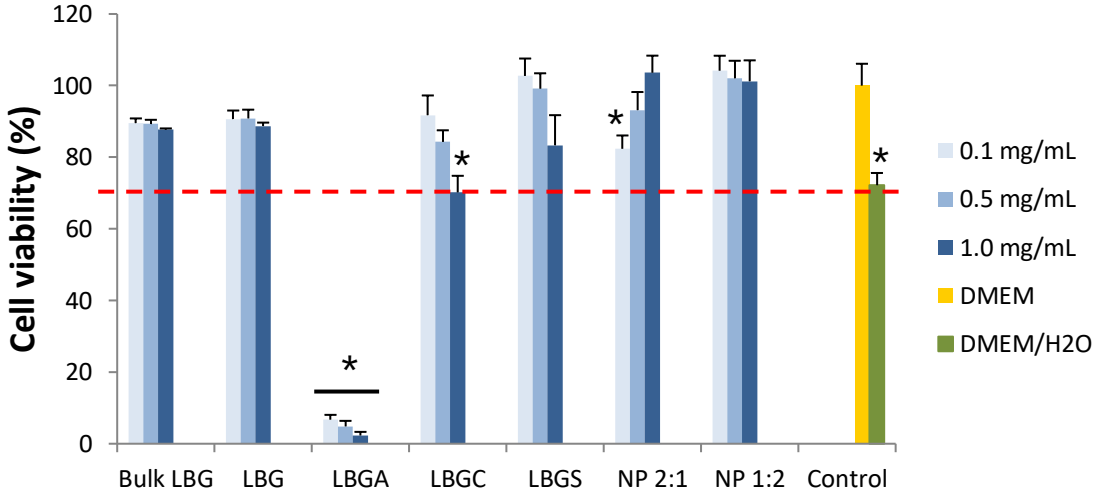


Figure 7 – Caco-2 cell viability measured by the MTT assay after 24 h exposure to increasing concentrations of bulk Locust Bean Gum, purified Locust Bean Gum (LBG) and its ammonium (LBGA), carboxylate (LBGC) and sulfate (LBGS) derivatives; and LBGA/LBGS nanoparticles (NP). Data represent mean ± SEM ($n \geq 3$, six replicates per experiment at each concentration). Dashed line indicates 70%. * $P < 0.05$ compared with DMEM.

Although not directly proposed herein as matrix material *per se*, unmodified LBG was also tested, because its application in drug delivery has been reported, in many occasions addressing oral delivery strategies (Colombo et al., 1990; Conte & Maggi, 1996; Coviello, Alhaique, Dorigo, Matricardi & Grassi, 2007; Dey, Sa & Maiti, 2015;

Jana, Gandhi, Sheet & Sen, 2015; Malik, Arora & Singh, 2011a; Malik, Arora & Singh, 2011b; Ngwuluka, Choonara, Kumar, du Toit, Modi & Pillay, 2015; Sandolo, Coviello, Matricardi & Alhaique, 2007; Sujja-areevath, Munday, Cox & Khan, 1998; Syed, Mangamoori & Rao, 2010; Tobyn, Staniforth, Baichwal & McCall, 1996), but data on its effect on epithelial cells are not available in the literature. Moreover, a comparison between bulk LBG and purified LBG was performed, revealing no significant differences, which indicates an absence of effect of the purification process in the cytotoxic profile of the material. It is important to mention that the results shown for LBGS sample correspond to the derivative obtained by the second method of synthesis (method 2 – batch 1), which were similar to those registered for the derivative obtained in the first method (method 1; data not shown).

As mentioned before, LBGA is the material that presents the most distinct behavior, appearing as the exception to the mild effect observed for the tested materials. In fact, a strong decrease of cell viability to approximately 30% was obtained for all the tested concentrations even upon 3 h exposure (**Figure S3**). The effect was even more drastic after 24 h (**Figure 7**), when a very low level of cell survival was registered ($P < 0.05$). Regarding concentration, there are no evidences of statistically significant concentration-dependent effect. The influence of surface charge on cytotoxicity remains largely unresolved and sometimes the literature reports contradictory results. This is possibly due to different characteristics of basic materials being used and also to dissimilar assay conditions, which are frequently not described in sufficient detail. Nevertheless, there are many indications suggesting that surface charge has a role on cellular uptake (Fröhlich, 2012; Zhao, Zhao, Liu, Chang, Chen & Zhao, 2011) and on the toxicological effect of substances. In this context, positively charged materials have been frequently found to be more cytotoxic than neutral or negatively charged

counterparts, because positive charges provide a means for stronger interaction with cell surfaces, in many cases associated with internalization of the material (Bhattacharjee et al., 2010; Fröhlich, 2012; Ilinskaya, Dreyer, Mitkevich, Shaw, Pace & Makarov, 2002; Platel, Carpentier, Becart, Mordacq, Betbeder & Nesslany, 2016; Turcotte, Lavis & Raines, 2009). These statements are coincident with the results of our work, since the neutral (bulk LBG and LBG) and negatively charged materials (LBGC and LBGS) were devoid of toxicity. Another parameter that could be indicated as playing a significant role on toxicity consists on the molecular weight of the polymers. In this regard, although it could be suggested that smaller sizes have higher probability to be internalized by the cells, the literature has been reporting no correlation (Huang, Khor & Lim, 2004). In this work, the molecular weight of the polymers also seems to not be driving the cytotoxic behaviour, as LBGS is the smallest molecule and shows no toxic effect.

Comparing to LBGA, a very similar toxicological profile was observed for an ammonium derivative of another polysaccharide, pullulan, which was synthesized using the same methodology (Dionísio, Braz, Corvo, Lourenço, Grenha & da Costa, 2016; Dionísio, Cordeiro, Remuñán-López, Seijo, Rosa da Costa & Grenha, 2013). In that case, the assessment was performed in Calu-3 cells (bronchial cell line) and cell viabilities around 50-60% were observed after 3 h, decreasing to 40% at 24 h. Although a time-dependent effect is also clearly observed, the effect on cell viability is not as strong as for LBGA. The first consideration to take into account is the fact that the assessment was performed in different cell lines, which may translate into different sensitivity. Additionally, different charge density of the polymers might be indicated as possible justification. In this regard, LBGA has a DS of 4.24, while the corresponding pullulan derivative (ammonium pullulan) has a DS of 2 (Dionísio, Braz, Corvo,

Lourenço, Grenha & da Costa). A higher number of positive charges results in stronger interactions and, thus, in lower cell viability. Complementing this idea, a work reporting the cytotoxic effect of cationic pullulan microparticles on human leukemic K562(S) cells, has established that toxicity increased with the increase molar concentration of amino groups (Constantin, Fundueanu, Cortesi, Esposito & Nastruzzi, 2003). In the work reporting the cytotoxic evaluation of pullulan derivatives, a sulfate derivative of that polysaccharide was also assessed. Similarly to what was observed for LBGS, the registered cell viability was well above 80% (Dionísio, Cordeiro, Remuñán-López, Seijo, Rosa da Costa & Grenha, 2013).

Considering that polymer samples were solubilized in water and diluted with cell culture medium prior to incubation with the cells, an additional control was performed consisting in a mixture of DMEM and H_2O in the same ratio used for the samples. This enables a real evaluation of the contribution of the polymers to the final cell viability. The cell viability induced by this control varied between 72% and 80%. Upon 3 h of contact there is a statistically significant difference between the control (DMEM + H_2O) and all samples, but with LBGS (**Figure S3**). In fact, higher cell viability is observed upon exposure to bulk LBG, LBG and LBGC, suggesting a positive effect of the presence of the polymers. Interestingly, after 24 h exposure, a shift is observed in the effect induced by LBGC and LBGS (**Figure 7**). In the former, the prolonged contact with the cells at the two highest concentrations reverts the positive effect on cell viability observed at 3 h. For LBGS, the results demonstrate that at the two lowest concentrations, the more prolonged contact improves cell viability, which was not registered at 3 h.

One of the most important information provided by the evaluation performed with the MTT assay, is that only the more prolonged exposure to the highest concentration tested

(1.0 mg/mL; 24 h) induced a relevant decrease of Caco-2 cell viability (exception for LBGA). Therefore, it was deemed important to complement the results at these conditions by means of the quantification of the amount of LDH released by Caco-2 cells. To perform this assay, DMEM was used as negative control of LDH release and a lysis buffer was used as positive control. Thus, the negative control (DMEM) corresponds to a normal cell death, while the positive control (lysis buffer) represents 100% cell death.

The results of LDH release after 24 h exposure to the materials at the concentration of 1.0 mg/mL (**Figure S4**) showed no statistically significant differences between the negative control (DMEM), bulk LBG, LBG, LBGC and LBGS. This means that these materials do not compromise Caco-2 cell membrane integrity, as LDH release was not increased when compared with that observed upon incubation with cell culture medium (DMEM). On the contrary, the contact with LBGA resulted in 90% LDH release, which is considered comparable to that induced by the lysis buffer, thus indicating a high cytotoxic effect that results in cell membrane disruption. The results obtained in this assay reinforce those found in the MTT tests, confirming the high cytotoxicity of LBGA.

Overall, the results obtained with these complementary cytotoxicity assays indicate that, with the exception of LBGA, LBG and negatively charged derivatives, present no cytotoxicity towards this *in vitro* intestinal model. This was observed even for the highest concentration tested (1.0 mg/mL) and for prolonged contact (24 h), suggesting their relative safety for an application as matrix materials of oral drug delivery systems. Complementarily, the effect on cell viability provided by LBGS (method 2 – batch 1) was assessed in Calu-3 and A549 cells (respiratory epithelial cells) and the results are in line with those observed for Caco-2 cells (**Figures S5 and S6**).

Proposing materials for drug delivery applications requires testing the developed carriers and not only assume the apparent absence of cytotoxicity of the polymers. In this regard, it is consensual that carriers exhibit new and unique properties, thus generating potential different risks as compared to the raw materials of the same chemistry (Aillon, Xie, El-Gendy, Berkland & Forrest, 2009), as observed in other works (Dionísio, Braz, Corvo, Lourenço, Grenha & da Costa, 2016; Dionísio, Cordeiro, Remuñán-López, Seijo, Rosa da Costa & Grenha, 2013). In this regard, in addition to the evaluation of the polymer and the synthesized derivatives, a preliminary evaluation of LBG-based nanoparticles was further performed using the MTT assay. Although several formulations were proposed and developed herein, that corresponding to LBG-only nanoparticles was selected for this step due to the novelty of the polymer in nanoparticle production.

The viability of Caco-2 cells upon exposure to LBGA/LBGS nanoparticles is shown in **Figure S7** (3 h) and **Figure 7** (24 h). The two formulations LBGA/LBGS 2:1 and 1:2 (w/w) were assessed. For formulation 2:1 (w/w) the comparison of results obtained for each tested time revealed a statistically significant difference between concentrations 0.1 and 1.0 mg/mL ($P < 0.05$). Formulation 1:2 (w/w) did not evidence significant differences between all concentrations at the two tested times. A similar observation was made after comparing the same concentrations for different times (3 h and 24 h). The most remarkable result is that no significant effect on cell viability is observed for both formulations at all concentrations, up to 24 h. Actually, the registered viability was over 80% in all cases, which, as said before, is considered very acceptable according to the ISO10993-5 (ISO, 2009).

Curiously, the exposure of the cells to the formulation LBGA/LBGS 2:1 (w/w) resulted in an increase of cell viability with the increase of nanoparticle concentration at 3 h and

24 h ($P < 0.05$). This was unexpected and may be due to the fact that LBG is a polysaccharide with capacity to promote cell proliferation in some cell lines, as reported in the literature (Perestrelo, Grenha, Rosa da Costa & Belo, 2014). Despite the formulation LBGA/LBGS 2:1 (w/w) could improve cell proliferation with increasing concentrations, formulation LBGA/LBGS 1:2 (w/w), generally induced constant cell viability near 100%, irrespective of the concentration.

Comparing with the control (DMEM + H_2O) it is observed that the nanoparticles generally elicit higher cell viability, varying between 82% and 100% ($P < 0.05$). The most remarkable observation in the whole set of cell viability assessment is that, in spite of the strong decrease in cell viability induced by the contact with LBGA, this effect was completely reverted when the cells were exposed to a nanoparticulate form of the derivative. This was also observed in works using an ammonium derivative of pullulan, in which the derivative elicited around 40% cell viability upon 24 h of exposure, while nanoparticles produced with the polymer registered increased cell viabilities to values of 70% - 80% (Dionísio, Braz, Corvo, Lourenço, Grenha & da Costa; Dionísio, Cordeiro, Remuñán-López, Seijo, Rosa da Costa & Grenha, 2013). The different impact on cell viability generated by LBGA in form of polymer and of nanoparticles is possibly explained by a differential contact of each of the materials with the cells. While the polymer in form of a solute is presented as an extended chain and, thus, has a higher surface of contact with the cells, nanoparticles have comparatively a lower contact. Additionally, the number of positive charges available for interaction with the negatively charged cells upon complexation with LBGS is significantly decreased, thus decreasing the potential toxicity (Huang, Khor & Lim, 2004). This reinforces the need to evaluate separately the carriers and the raw materials, as the former may exhibit

different properties, that may encompass different risks (Aillon, Xie, El-Gendy, Berkland & Forrest, 2009).

As also observed for another formulation of LBG-based nanoparticles (CS/LBGS), which already shown to be promising for oral immunization (Braz, Grenha, Ferreira, Rosa da Costa, Gamazo & Sarmento, 2017), these preliminary results suggest an absence of overt toxicity of LBG-only nanoparticles, thus potentiating possible applications. Nevertheless, it is recognized that further studies need to be performed to reach a more accurate conclusion in this regard.

Conclusions

LBG demonstrated to be a good substrate for the production of charged derivatives, permitting the synthesis of ammonium, sulfated and carboxylated LBG. Several characterization techniques were used to confirm the presence of the new chemical groups introduced in each new derivative.

Using a method of polyelectrolyte complexation, the produced derivatives were applied in the preparation of different formulations of LBG-based nanoparticles, reported herein for the first time. When the negatively charged derivatives (sulfated and carboxylated LBG) were used, chitosan was the applied positively charged polyelectrolyte. In turn, ammonium LBG was complexed with sulfated LBG to obtain LBG-only nanoparticles. The physicochemical characteristics of nanoparticles were highly dependent on their composition and on the charge ratios applied in each complexation being performed. Generally, the observed characteristics, with sizes around 200-400 nm in certain cases, and tailorable zeta potential according to setup conditions, are suggested as adequate for drug delivery applications.

A preliminary toxicological evaluation of LBG derivatives and the produced nanoparticles was performed, assessing both the metabolic activity and the cell membrane integrity of representative intestinal cells (Caco-2) after an exposure of up to 24 h to concentrations as high as 1 mg/mL. Severe cytotoxicity was found for the ammonium derivative of LBG, but this was clearly reverted after the assembly of nanoparticles, which evidenced a very mild effect on Caco-2 cell viability. The results as a whole indicate the possibility to use the synthesized LBG derivatives to produce nanoparticles for drug delivery applications.

Acknowledgements

This work was supported by national Portuguese funding through FCT - Fundação para a Ciência e a Tecnologia, project ref. PTDC/SAU-FCF/100291/2008, PEst-OE/EQB/LA0023/2011, UID/QUI/00100/2013, UID/Multi/04326/2013, UID/BIM/04773/2013, UID/CTM/50025/2013 and PEst-OE/QUI/UI4023/2014. This work was also financed by FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia/ Ministério da Ciência, Tecnologia e Inovação in the framework of the project "Institute for Research and Innovation in Health Sciences" (POCI-01-0145-FEDER-007274), POCI-01-0145-FEDER-007688 and PTNMR 22161.

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Supplementary material

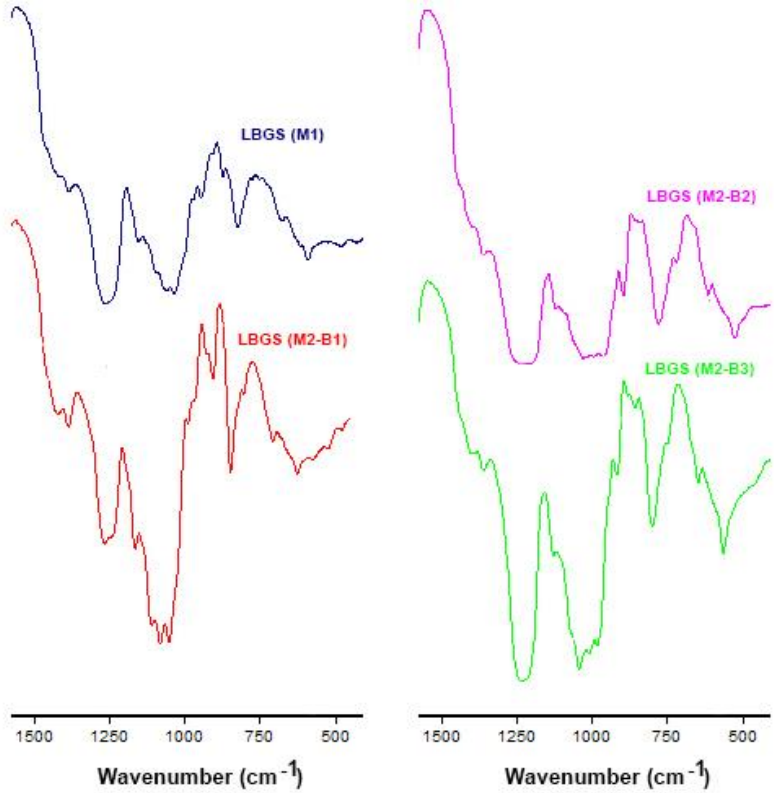

Figure S1 – FTIR spectra of Locust Bean Gum sulfate derivatives (LBGS) obtained in method 1 (M1) and method 2 (M2). B1, B2 and B3 refer to batch 1, 2 and 3, respectively.

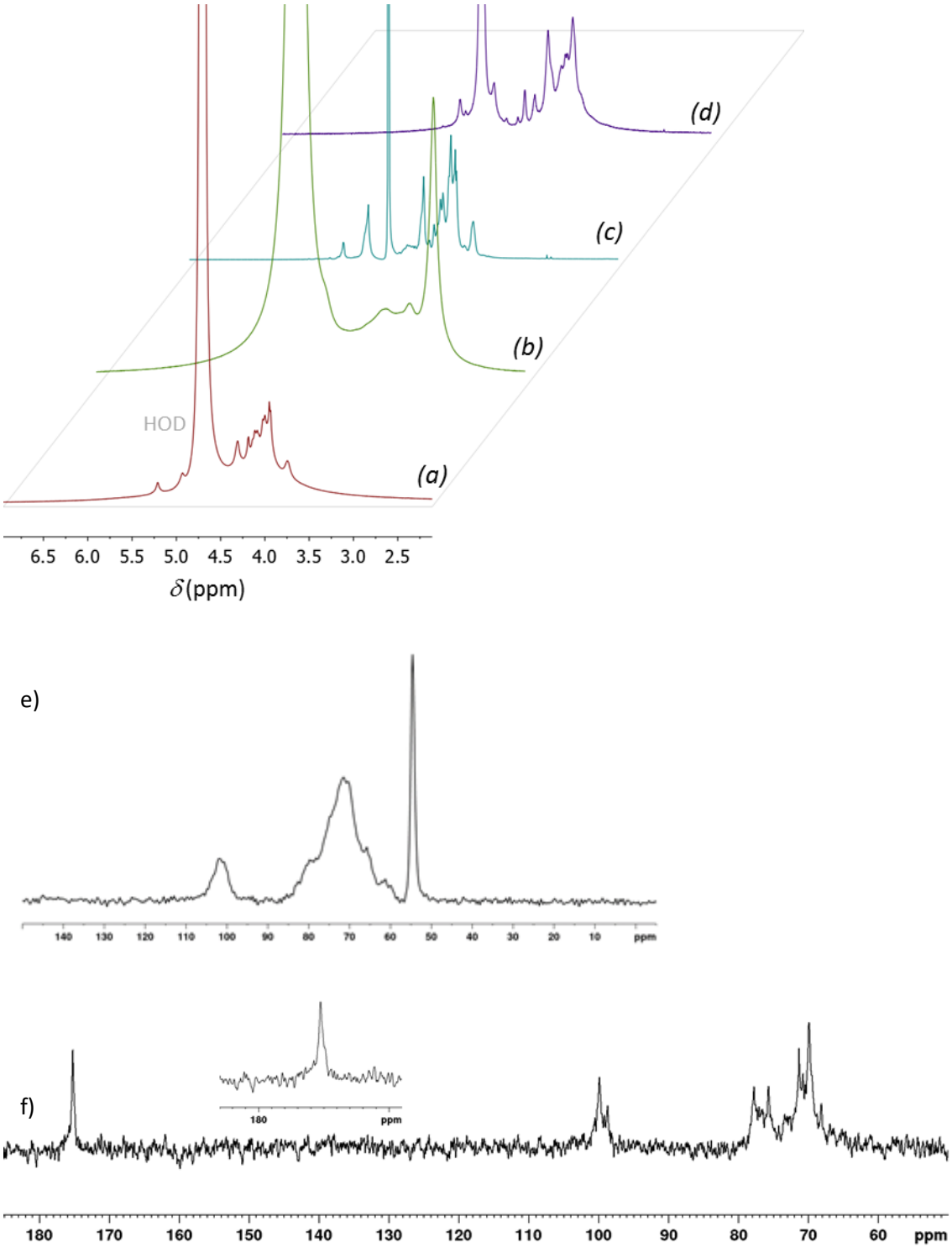

Figure S2 – ^{1}H NMR spectra of (a) LBG, (b) LBGS-M2-B1, (c) LBGC, and (d) LBGA; (e) ^{13}C CPMAS spectrum of LBGA; (f) ^{13}C NMR spectrum of LBGC; the big singlet centered at 4.7 ppm in the ^{1}H spectra is due to HOD (identified in grey).

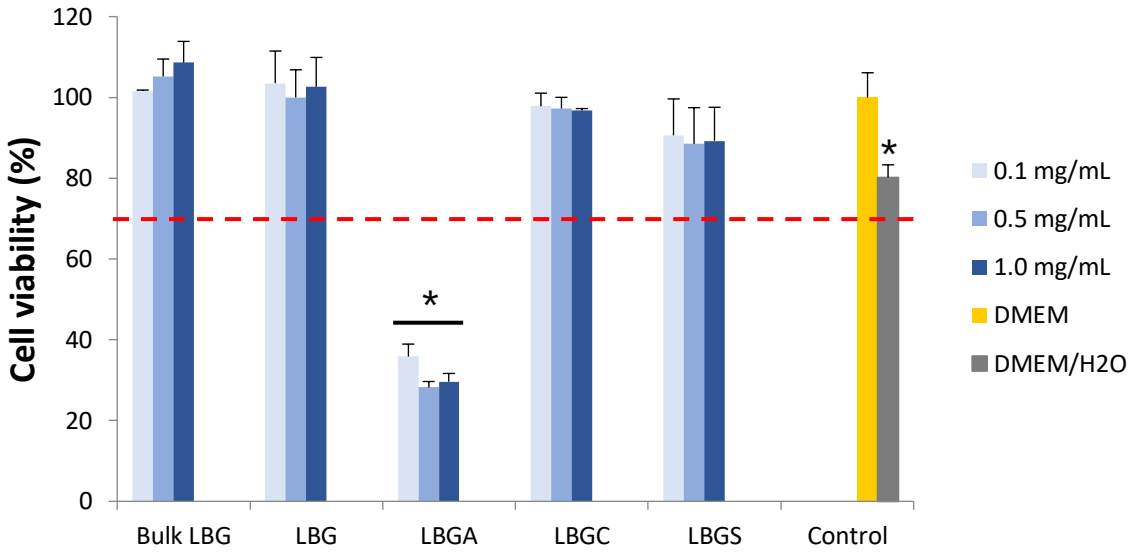

Figure S3 - Caco-2 cell viability measured by the MTT assay after 3 h exposure to increasing concentrations of bulk Locust Bean Gum, purified Locust Bean Gum (LBG) and its ammonium (LBGA), carboxylate (LBGC) and sulfate (LBGS) derivatives. Data represent mean ± SEM (n ≥ 3, six replicates per experiment at each concentration). Dashed line indicates 70%. * $P < 0.05$ compared with DMEM.

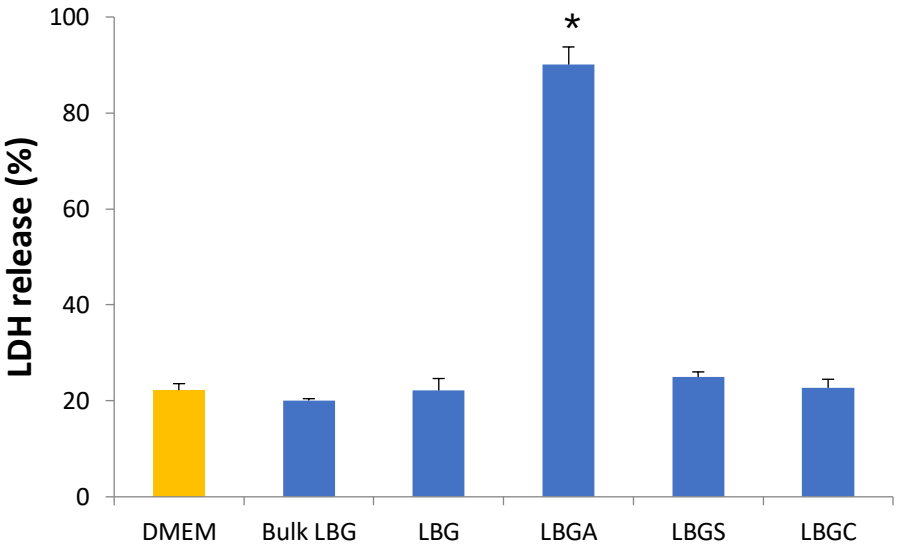

Figure S4 – Caco-2 cell viability measured by the LDH release assay after 24 h exposure to 1 mg/mL solutions of bulk Locust Bean Gum, purified Locust Bean Gum (LBG) and its ammonium (LBGA), carboxylate (LBGC) and sulfate (LBGS) derivatives. Data represent mean ± SEM (n ≥ 3, three replicates per experiment). * $P < 0.05$ compared with DMEM.

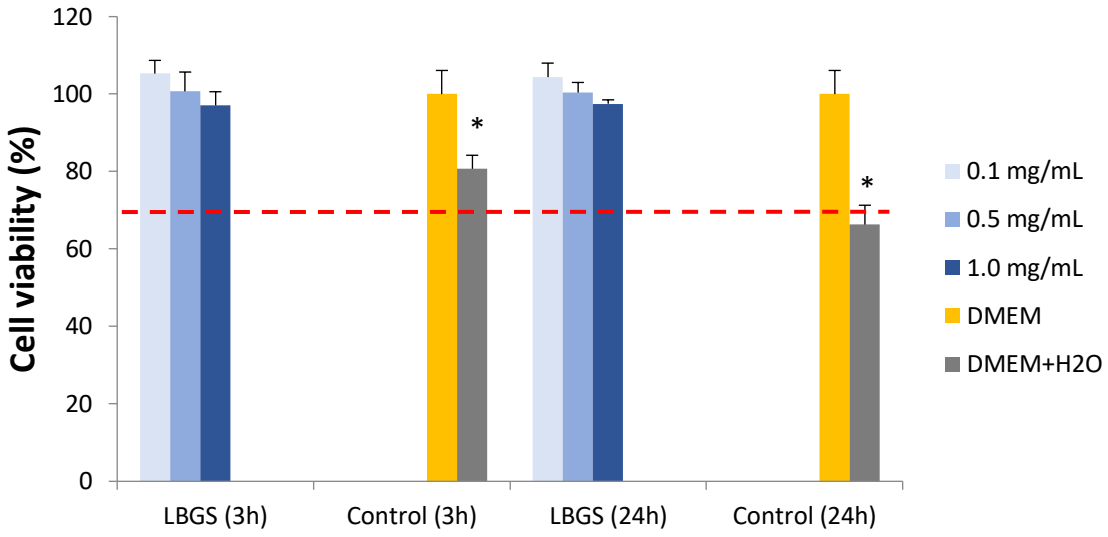

Figure S5 – A549 cell viability measured by the MTT assay after 3 h and 24 h exposure to increasing concentrations of sulfate locust bean gum (LBGS) derivative. Data represent mean ± SEM (n ≥ 3, six replicates per experiment at each concentration). Dashed line indicates 70%. * $P < 0.05$ compared with respective control (DMEM).

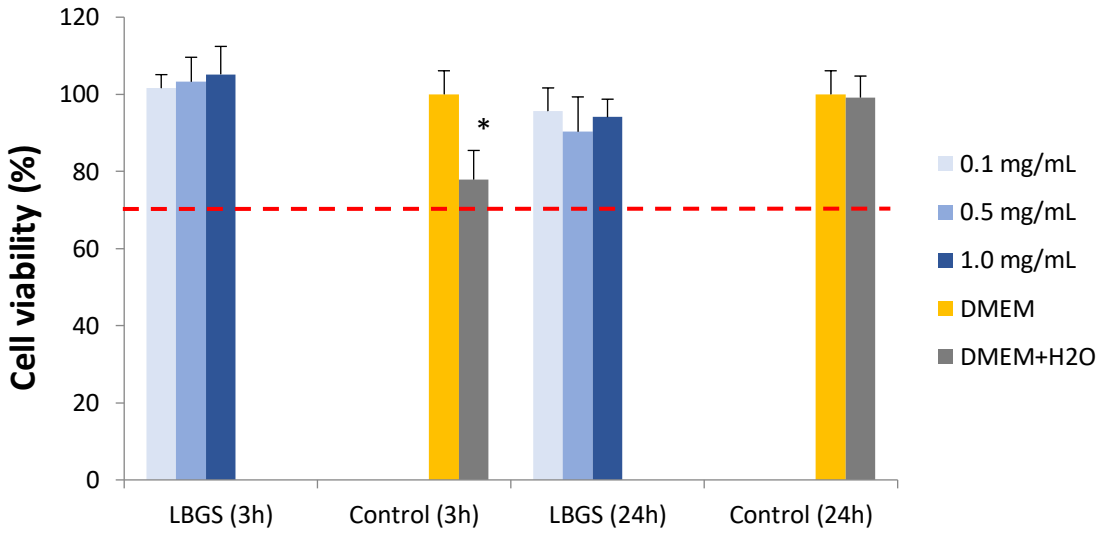

Figure S6 – Calu-3 cell viability measured by the MTT assay after 3 h and 24 h exposure to increasing concentrations of sulfate locust bean gum (LBGS) derivative. Data represent mean ± SEM (n ≥ 3, six replicates per experiment at each concentration). Dashed line indicates 70%. * $P < 0.05$ compared with respective control (DMEM).

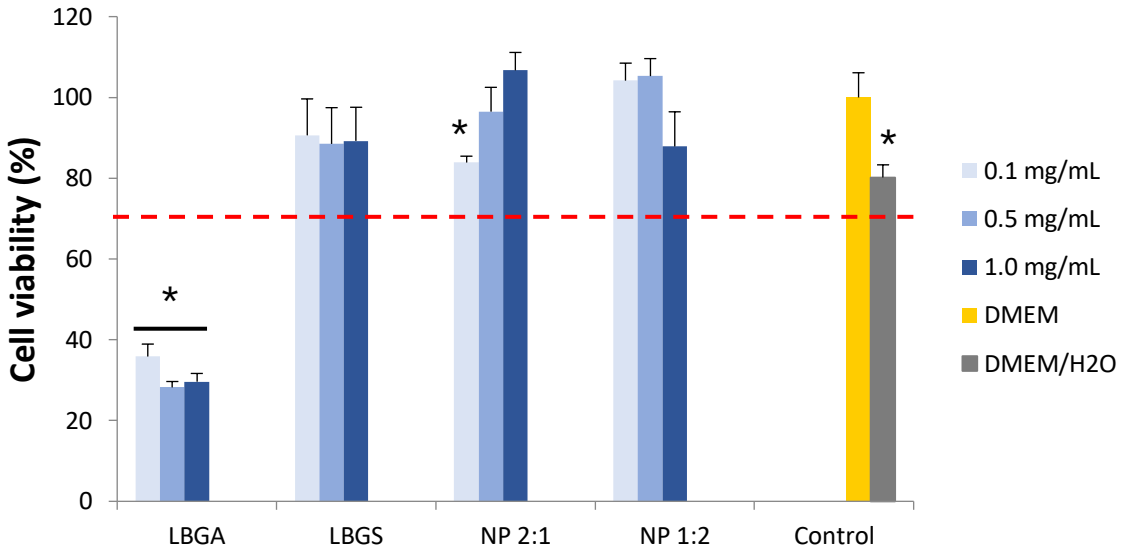

Figure S7 – Caco-2 cell viability measured by the MTT assay after 3 h exposure to increasing concentrations of ammonium Locust Bean Gum (LBGA) derivative, sulfate Locust Bean Gum (LBGS) derivative and LBGA/LBGS nanoparticles (NP). Data represent mean ± SEM ($n \geq 3$, six replicates per experiment at each concentration). Dashed line indicates 70%. * $P < 0.05$ compared with DMEM.

Table S1 – Elemental analysis data from the sulfate (LBGS), carboxylate (LBGC) and ammonium (LBGA) derivatives of locust bean gum (LBG).

Element (%)	Polymer					
	LBGS (M1)*	LBGS (M2-B1)*	LBGS (M2-B2)*	LBGS (M2-B3)*	LBGC	LBGA
N	---	---	---	---	---	3.84
C	25.55	35.06	23.94	28.42	37.39	43.39
S	7.77	3.50	9.78	7.41	---	---
O	---	---	---	---	48.96	---

*B1, B2 and B3 refer to LBGS derivatives from batches 1, 2 and 3, respectively; M1 and M2 refer to LBGS derivatives synthesized with methods 1 and 2, respectively

Table S2 – GPC analysis of purified Locust Bean Gum (LBG), and its ammonium (LBGA), carboxylate (LBGC) and sulfate (LBGS-M1) derivatives.

Polymer	M_n (Da)	M_w (Da)	PdI	R_g (nm)
LBG	327 300	589 100	1.80	71.61
LBGA	500 600	871 000	1.74	86.05
LBGC	73 790	119 500	1.62	28.19
LBGS-M1	21 380	26 510	1.24	14.21

M_n: number average molecular weight; M_w: weight average molecular weight; PdI: polydispersity index; R_g: radius of gyration