Electronic Supplementary Material (ESI) for Soft Matter

Supporting Online Material for

Palmitoylation of xanthan polysaccharide for self-assembly microcapsule formation and encapsulation of cells in physiological conditions

Ana Carina Mendes , Erkan Turker Baran, Cláudia Nunes, Manuel A. Coimbra, Helena Sepúlveda Azevedo*, Rui Luís Reis

*To whom correspondence should be addressed. E-mail: hazevedo@dep.uminho.pt

This file includes:

MATERIALS AND METHODS

RESULTS

Figures S1 to S3

Tables S1 to S5

Supporting Online Material

MATERIALS AND METHODS

Characterization of native xanthan (starting material) and palmitoyl xanthan

Elemental analysis

The carbon, hydrogen, and nitrogen contents of xanthan were determined by elemental analysis using a LECO CHNS-932 elemental analyser and following the manufacturer's standard procedures. Oxygen was calculated by difference and cafein (C= 49.48%, H=5.19% N=28.85%) was used as standard material. At least three measurements were performed for each sample.

Determination of sugar composition (monosaccharide content)

Neutral sugars were determined as alditol acetates as described by Coimbra *et al.* [S1]. A pre-hydrolysis with 72% H_2SO_4 for 3 h at room temperature was performed in order to solubilise the material. Afterwards, the polysaccharide was submitted to a hydrolysis with H_2SO_4 2 M at 120 ºC during 1 h. 2-Deoxyglucose was used as internal standard. Monosaccharides were reduced with sodium borohydride and acetylated by acetic anhydride using methylimidazole as catalyst. The alditol acetate derivatives formed were analyzed by gas chromatography (GC) equipped with a 30 m column DB-225 (J&W Scientific, Folsom, CA, USA) with i.d. and film thickness of 0.25 mm and 0.15 μm, respectively and using a flame ionisation detector. The oven temperature program used was: initial temperature 200 ºC, a rise in temperature at a rate of 40 ºC/min until 220 ºC, standing for 7 min, followed by a rate of 20 ºC/min until 230 ºC and maintain this temperature 1 min. The injector and detector temperatures were, respectively, 220 and 230 ºC. The flow rate of the carrier gas (H_2) was set at 1.7 mL/min.

Uronic acids were quantified by a modification (Coimbra et al. [S1]) of the 3-phenylphenol colorimetric method (Blumenkrantz & Asboe-Hansen [S2]). Samples were prepared by hydrolysis with 72% H_2SO_4 for 3 h at room temperature followed by 1 h in 1 M H_2SO_4 at 100 ºC. A calibration curve was made with D-galacturonic acid. The hydrolysis and analysis of the samples was done in triplicate.

Methylation analysis

Linkage analysis was carried out by methylation as described by Ciucanu and Kerek [S3]. Xanthan samples (1-2 mg) were dissolved in 1 mL of anhydrous

dimethylsulfoxide (DMSO), then powdered NaOH (40 mg) was added and allowed to react during 30 min, followed by addition of CH_3I (80 μL) that was allowed to react during 20 min. The methylated polysaccharides were hydrolyzed with 2 M TFA at 121 ºC for 1 h, and then reduced and acetylated as previously described for neutral sugar analysis. The partially methylated alditol acetates (PMAA) were separated and analyzed by gas chromatography–mass spectrometry (GC-MS) on an Agilent Technologies 6890N Network. The GC was equipped with a DB-1 (J&W Scientific, Folsom, CA, USA) capillary column (30 m length, 0.25 mm of internal diameter and 0.15 μm of film thickness). The samples were injected in splitless mode (time of splitless 5 min), with the injector operating at 220 ºC, and using the following temperature program: 45 ºC for 5 min with a linear increase of 10 ºC/min up 140 ºC, and standing for 5 min at this temperature, followed by linear increase of 0.5 ºC/min up to 170 ºC, and standing for 1 min at this temperature, followed by linear increase of 15 ºC/min up to 280 ºC, with further 5 min at 280 ºC. The helium carrier gas had a flow rate of 1.7 mL/min and a column head pressure of 2.8 psi. The GC was connected to an Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 70 eV and scanning the range m/z 40–500 in a 1 s cycle in a full scan mode acquisition.

Palmitoyl content analysis

The palmitoyl content of palmitoyl xanthan was determined by transesterification with sodium methoxide and analysis by GC. The transesterification was performed using an adaptation of the methodology described by Auded-Pimentel et al. [S4]. The polysaccharide sample (10 mg) was dissolved in 1 mL of methanol and 0.5 mL of the internal standard solution (heptadecanoate methyl ester, 75 μg/mL prepared in hexane) was added. Then, 0.05 mL of a methanolic KOH solution (2 M) was added and the mixture was stirred vigorously for 30 s in a vortex. Afterwards, 0.2 mL of a saturated sodium chloride solution was added. The mixture was centrifuged at 2000 rpm during 5 min. The organic phase (0.1-0.5 μL) was used for GC analysis. The GC (Perkin Elmer Clarus 400, USA) equipped with a 30 m column DBFFAP fused silica capillary column (J&W Scientific Inc., Folsom, CA, USA) with 0.32 mm (ID), and 0.25 μm film thickness and a flame ionization detector. The oven temperature program used was: initial temperature 75 ºC, a rise in temperature at a rate of 15 ºC/min until 155 ºC, followed by rate of 3 ºC/min until 180 ºC, and a last rise in temperature at a rate of 40 ºC/min until 220 ºC and maintain this temperature 3 min. The injector and detector temperatures were, respectively, 245 and 250 ºC. The flow rate of the carrier gas (H_2) was set at 1.7 mL/min. The samples were analysed in triplicate.

Determination of acetate and pyruvate degree of substitution of native xanthan by ^1H NMR analysis

The acetate and pyruvate content was determined by ^1H NMR spectroscopy by reference to an external standard (sodium acetate) as described by Hamcerencu *et al.* [S5]. Xanthan was dissolved in D_2O (3 g/L) in presence of Na acetate ($3x10^{-3}$ M). ^1H NMR spectra were recorded at 75 °C. Three peaks were considered: two peaks at δ = 1.47 and δ = 2.16 ppm attributed to pyruvate and acetate groups bound to the Xanthan and one at δ = 1.90 ppm assigned to Na acetate (Figure S1-S2). From the integrals of ^1H signals corresponding to both substituents by reference to external standard protons (Figure S2) one can calculate the number of the substituents (N_A, N_P)/gram of polysaccharide and, finally the number of acetate and pyruvate groups (n_A, n_P)/side chain of Xan:

$$N_A = \frac{h_A}{h_E} \cdot \frac{c_E}{c_P} \qquad N_P = \frac{h_P}{h_E} \cdot \frac{c_E}{c_P}$$

$$n_A = N_A \cdot 829/\left(1 - 92N_P - 42N_A\right)$$
$$n_P = N_P \cdot 829/\left(1 - 92N_P - 42N_A\right)$$

h_A, h_P, h_E - integrals of ^1H signals corresponding to acetate (h_A) and pyruvate (h_P) group, and to external reference (h_E), respectively; c_E, c_P - concentration of Na acetate (c_E, mole/L) and polysaccharide (c_P, g/L); 829 - molar weight of repeating unit of xanthan without acetate or pyruvate; 42 - molar weight of acetate group; 92 - molar weight of pyruvate group.

Determination of molecular weight of native xanthan and palmitoyl xanthan by size-exclusion chromatography (SEC)

For determination of the average molecular weight (Mw) by SEC, the samples were dissolved in an aqueous 0.1 M $NaNO_3$ solution (0.4% w/v). A PL-GPC 110 chromatograph was equipped with a pre-column PLaquagel-OH 15 µm and two SEC columns in series (PLaquagel-OH40 15 µm, 300× 7.0 mm and PLaquagel-OH60 15 µm, 300× 7. 0 mm). The pre-column, the SEC columns, the injection system, and the refractive index detector were maintained at 36 ºC. The eluent (aqueous 0.1 M $NaNO_3$ solution) was pumped at a flow rate of 0.9 mL min^{-1}. The analytical columns were calibrated with pullulan standards (Polymer Laboratories, UK) in the range of 5.8-1600 kDa.

Determination of degree of substitution (DS) of palmitoyl xanthan

The DS (fraction of hydroxyl groups modified per average of repeating unit) of PX(X=1.7P) was calculated on basis of %C from elemental analysis results (Table S1). Considering that each repeating unit has 11 hydroxyl groups (Figure 1), the maximum possible DS value would be 11 (100%). The DS values for Palmitoyl Xanthan were determined as follows:

	Average carbon percentage	Mol number	Degree of substitution (per repeating subunit)
Xanthan (35 Carbon/molecule)	35.66[a]	35.66/(35x12)= 0.0849	0.0118/0.0849x100= 13.9%
Palmitoyl Xanthan	37.93[a]	-	
Palmitic acid (16 carbon/molecule)	2.275[b]	2.275/(16x12)= 0.0118	

[a] From elemental analysis (Table S1)

[b] Difference between Xanthan-Palmitoyl Xanthan

Differential scanning calorimetry (DSC) of palmitoyl xanthan

To prove that the endothermic peak observed in DSC thermograms of palmitoyl xanthans is due the conjugation of palmitic groups on xanthan, conjugate sample (PX(X=1.7P)) was extracted with excess chloroform three times. After evaporation of the pooled solvent residue was recovered and analysed by DSC between -40 and 200 ºC.

RESULTS

Elemental analysis

The chemical structure of xanthan gum repeating unit ($C_{35}H_{49}O_{29,}$ molecular weight 933) is depicted in Figure 1. Considering that each inner and terminal mannose units bear an acetyl and a pyruvic acid group, respectively, the theoretical values for xanthan elemental analysis are 45.02% C, 5.25% H, 49.73% O. Table S1 display the elemental analysis of xanthan gum and palmitoyl xanthan obtained experimentally. For the calculation of the theoretical values, it was considered that each inner and terminal mannose units bear an acetyl and a pyruvic acid group. However, the acetyl and pyruvate substituents are linked in variable amounts to the side chains and this may explain the difference between the results obtained experimentally and the ones expected theoretically. The presence of nitrogen in xanthan samples might be due to the impurities (e.g. proteins) from xanthan production (fermentation process). The increase in the carbon content in palmitoyl xanthan was expected and confirms the conjugation palmitic groups to xanthan.

Table S1 Elemental composition of native xanthan gum (starting material) and palmitoyl xanthan (PX(X=1.7P). The values represent the mean of two replicates with standard deviation.

Sample/material	%C	%H	%N	%O[a]
Native xanthan	35.660 ± 0.099	5.710 ± 0.098	0.560 ± 0.003	58.070 ± 0.194
Palmitoyl xanthan	37.935 ± 0.148	5.895 ± 0.134	0.554 ± 0.019	55.617 ± 0.04

[a] by difference

Sugar composition

Sugar analysis showed that the xanthan and palmitoyl xanthan samples were composed by glucose, mannose, and uronic acid (Table S2). According to the Palaniraj and Jayaraman [S6] xanthan gum is composed by a cellulose-like backbone and trisaccharide side chains composed of two residues of mannose (Man) intermediated by a glucuronic acid residue (GlcA). This justifies the attribution to GlcA the uronic acids quantified.

The amount of sugars quantified in xanthan was 85% whereas in palmitoyl xanthan the amount was 76%. Taking into account that xanthan gum is pyruvated at 50% of all terminal Man residues, and also some residues are acetylated, this sugar content allows inferring that the polysaccharide is in a pure form. Concerning the palmitoyl xanthan, palmitoyl residues were quantified as 17.5 mg/g (Table S2), contributing to the lower content of sugars estimated in this sample.

For xanthan gum the ratio between Glc and GlcA content is 4, which allows inferring that for each 4 residues of Glc one is branched, assuming that each

branching point contains one GlcA residue. The content of Man is twice that of GlcA, which is in accordance with literature, whereas each side chain is reported to be constituted by two Man residues and one GlcA residue.

Table S2 Sugar composition of native xanthan gum (starting material) and palmitoyl xanthan (PX(X=1.7P). The values represent the mean of three replicates with standard deviation.

Sample/material	Man (mol%)	Glc (mol%)	GlcA (mol%)	Total sugars (mg/g)	Palmitoyl (mg/g)
Native xanthan gum	29.1 ± 0.5	56.7 ± 0.8	14.2 ± 0.8	851.3 ± 15.8	-
Palmitoyl xanthan	32.8 ± 0.6	46.5 ± 1.9	20.7 ± 1.8	757.7 ± 31.9	17.5 ± 0.7

In palmitoyl xanthan the ratios Glc:GlcA and Glc:Man are lower than that observed for xanthan gum. This means that the polysaccharide in this sample is more branched than the xanthan gum. Also, the ratio between Man and GlcA (1.6) is lower than obtained for xanthan gum. Based on the amount of sugar and palmitoyl residues, the degree of substitution of sugar residues with palmitoyl can be estimated as 1 in 60 sugar residues (1.67%).

In order to obtain more information about the structural features of these polysaccharides, a methylation analysis was performed. Methylation analysis (Table S3) revealed the presence in both polysaccharide samples of terminally-, (1→4)-, and (1→3,4) -linked Glc*p* residues and terminally- and (1→2) -linked Man*p* residues. These linkages are, in accordance with literature, those expected to occur in xanthan gum polysaccharides. Also, it was observed the occurrence of (1→4,6) -Man*p* possibly derived from pyruvated Man not hydrolysed under the conditions used. In fact, the 2 M TFA hydrolysis at 121 ºC during 1 h, described for the non-specific hydrolysis of methylated polysaccharides (Ciucanu and Kerek [S3]), seems that did not hydrolysed quantitatively all glycosidic linkages. This is stated due to the occurrence of lower amount of (1→3,4) -Glc*p* and (1→2) -Man*p* and even (1→4) -Glc*p* when compared to the reported in literature xanthan gum structures. The higher content of terminally-linked Man would suggest higher amount of these sugar residues. However, the linkage between uronic acid and a neutral sugar (aldobiouronic acid), as the one observed between GlcA and Man, is very difficult to quantitatively hydrolyse (Ferreira et al. [S7]). Also, the β-(1→4)-linked Glc residues may be not easily hydrolysed under dilute acid conditions. These results are in accordance with the resistance of these glycosidic linkages to acid hydrolysis. The Seaman hydrolysis usually performed for hydrolysis of polysaccharides using 1 M sulphuric acid at 100 ºC during 2.5 h did not release all the sugars, the reason why a strongest hydrolysis (2 M sulphuric acid at 120 ºC, 1 h) was applied to completely and quantitatively hydrolyse these polysaccharides (see Materials and Methods).

Table S3 Glycosidic-linkage analysis (mol%)

Linkage (mol%)	Xanthan	Palmitoyl Xanthan
T-Glc*p*	15.9	2.5
T-Man*p*	30.8	28.9
(1→2)-Man*p*	12.0	21.9
(1→4)-Glc*p*	37.4	37.3
(1→3,4)-Glc*p*	3.8	4.6
(1→4,6)-Man*p*	t	4.8

t- trace amounts

Acetate and pyruvate degree of substitution of native xanthan

We have characterized the acetate and pyruvate content of xanthan gum by proton NMR spectroscopy and using sodium acetate as external standard. According with Hamcerencu *et al.* [S5], xanthan spectra resolution is better at higher temperatures. Therefore, the ^{1}H NMR spectra were recorded at 75 ºC (Figure S1).

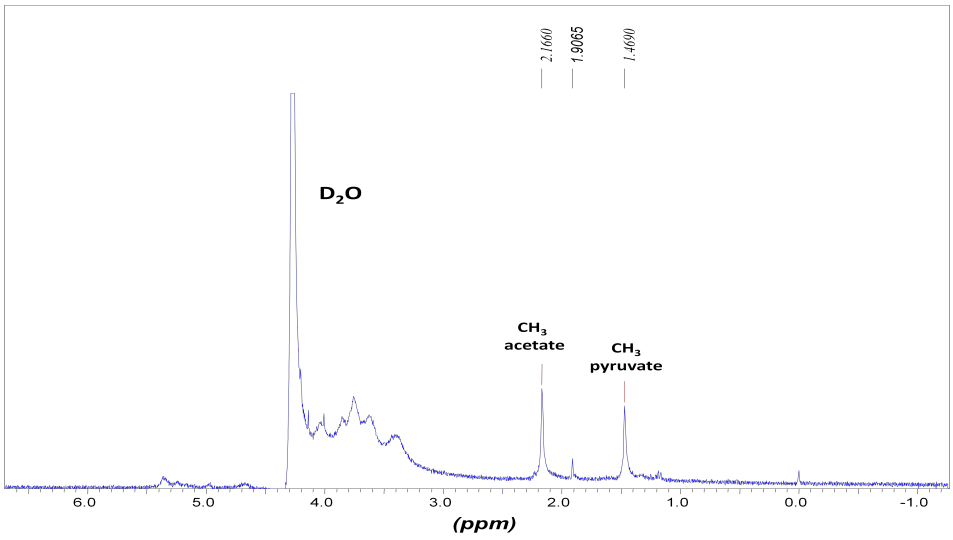

Figure S1 ^{1}H NMR spectrum of native xanthan gum (5 g/L in D_2O) at 75 ºC.

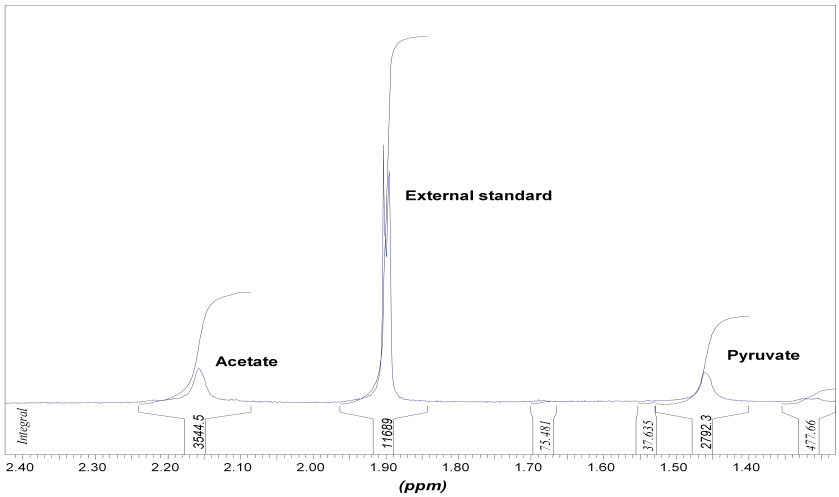

Figure S2 Integrals of ^{1}H signals (Figure S1) of acetate and pyruvate groups of xanthan (3 g/L) and of external standard (sodium acetate) for calculation of acetate and pyruvate degree of substitution.

The acetylation and pyruvate degree was calculated using the integrals obtained from Figure S2 and using the equations described in materials and methods. The mean of three determinations was found to be 44% (Table S4)

The substitution degree of Palmitoyl Xanthan (PX(X=1.7P)) was found to be 0.139 or 13.9% per repeating unit (2.8% per sugar residue). This value is not very different from the one calculated by sugar composition analysis (Table S2) which estimated a 1.67% of degree of substitution of palmitoyl per sugar residue. Considering hydroxyl groups (11 in the repeating unit) the degree of substitution per hydroxyl group is 1.26%.

Table S4 Acetate and pyruvate degree of native xanthan gum (starting material) per side chain and degree of substitution (DS) of palmitoyl xanthan (PX(X=1.7P)) per repeating unit. The values represent the mean of three replicates with standard deviation.

Sample/material	Acetate Degree (%)	Pyruvate Degree (%)	DS (%)
Native xanthan	44.4 ± 5.1	35.2 ± 2.6	-
Palmitoyl xanthan	-	-	13.8

Molecular weight of native xanthan and palmitoyl xanthan

The average molecular weight of xanthan gum and palmitoyl xanthan was estimated by SEC as 152 and 60 kDa, respectively (Table S5). The palmitoyl xanthan revealed a value of molecular weight 2.5 times lower than that observed for xanthan gum.

Table S5 Molecular weight of native xanthan gum (starting material) and palmitoyl xanthan (PX(X=1.7P))

Sample/material	Mw (KDa)	Mn (KDa)	Mw/Mn
Native xanthan	152	174	0.87
Palmitoyl xanthan	60	52	1.15

Differential scanning calorimetry (DSC) of palmitoyl xanthan

Figure S3 shows a small peak at 62.50 ºC and broadening of major a peak until 71.99 ºC. This peak was not observed in native xanthan (Figure 4). This result confirms that the endotherm observed at that range of temperature is due to the conjugation. We could not see Tg value on the DSC thermogram. We could use the DSC equipment until -40 ºC maximum.

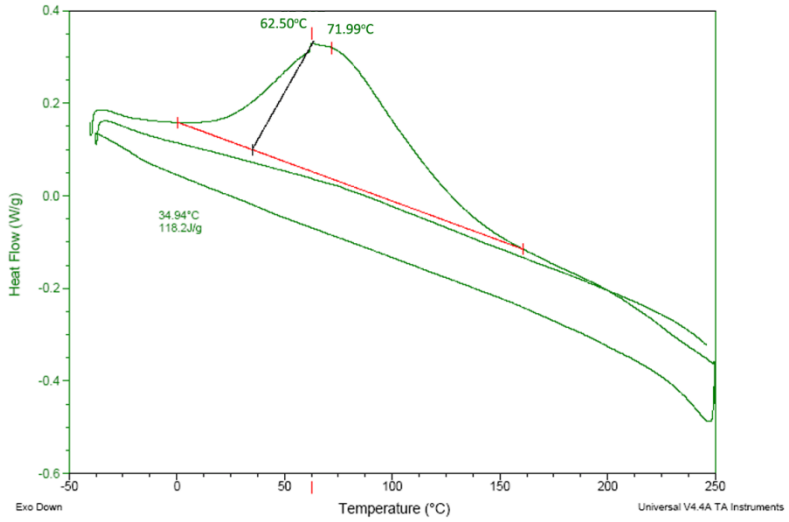

Figure S3 DSC thermogram of palmitoyl xanthan (PX(X=1.7P)) after extraction with chloroform.

References

[S1] M.A. Coimbra, I. Delgadillo, K. W. Waldron, R. R. Selvendran, Isolation and Analysis of Cell Wall Polymers from Olive Pulp. In H.-F. Linskens, & J. F. Jackson. Modern Methods of Plant Analysis, 1996, Berlin: Springer-Verlag, pp. 19-44.

[S2] N. Blumenkrantz, G. Asboe-Hansen, *Analytical Biochemistry*, 1973, **54**(2): 484-489.

[S3] I. Ciucanu, F. Kerek, *Carbohydrate Research*, 1984, **131**(2): 209-217.

[S4] S. Aued-Pimentel; J.H.G. Lago, M.H. Chaves, E.E. Kumagai, *Journal of Chromatography* A, 2004, **1054**, 235–239.

[S5] M. Hamcerencu, J. Desbrieres, M. Popa, A. Khoukh, and G. Riess, *Polymer*, 2007, **48**(7), 1921-1929.

[S6] A. Palaniraj, V.Jayaraman, *Journal of Food Engineering*, 2011, **106**, 1-12.

[S7] J.A. Ferreira, M.R.M. Domingues, A. Reis, M.C. Figueiredo, M.A. Monteiro, M.A. Coimbra, *Carbohydrate Research*, 2011, **346**, 638-643.