



Studies on the cationization of agarose

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ABSTRACT

Cationized agaroses with different degrees of substitution (0.04–0.77) were synthesized, employing 3-chloro-2-hydroxypropyltrimethylammonium chloride (CHPTAC). The influence of different reaction parameters on the substitution degree and molecular weight was evaluated. The investigated parameters were concentration of reagents, temperature, time, and addition of NaBH₄. The products were characterized by means of scanning electronic microscopy, infrared spectroscopy, viscosimetry, and NMR spectroscopy. Methanolysis products were studied by electrospray ionization mass spectrometry. The higher the concentration of CHPTAC employed, a higher degree of substitution was obtained, if the optimum concentration of NaOH in each case was employed. Insufficient quantities of NaOH reduced epoxide formation and the reacting alkoxides of the polysaccharide, whereas an excess of NaOH favored degradation of the epoxide and decrease in the molecular weight of the product. A reaction time of 2 h was sufficient to obtain products with the maximum degree of substitution for each case. The addition of NaBH₄ gave products with a slightly higher molecular weight, but the extra cost involved should not justify its use for large-scale application.

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1. Introduction

Groups containing amino or ammonium functions have been introduced in a variety of polysaccharides such as starch,^{1–4} dextrans,^{5,6} cellulosic derivatives,^{7–9} hemicelluloses,^{10,11} chitosan,^{12,13} chitin,¹⁴ konjac glucomannan,^{15,16} and guar gum.^{17,18} As a consequence of their properties, low cost, biodegradability, and low toxicity, cationic polyelectrolytes derived from those natural products have found a place in many diverse areas. Thus, they are used in effluent treatment, paper, chemical, food, cosmetic, pharmaceutical, petrol and textile industries, analytical chemistry, and molecular biology among others.

Etherification reactions with 2-hydroxy-3-(*N,N,N*-trimethylammonium)propyl groups^{1–18} have been commonly used to achieve cationization over the last few years. This cationization can be obtained by the reaction of different polysaccharides with 2,3-epoxypropyltrimethylammonium chloride (EPTAC). As EPTAC is unstable, a very common strategy is to prepare the reagent in situ from 3-chloro-2-hydroxypropyltrimethylammonium chloride (CHPTAC).

Unlike other polysaccharides, there are only a few reports on the use of seaweed polysaccharides as substrates for cationization

with any group. Geresh et al. employed the extracellular polysaccharide from the red microalgae *Porphyrium* sp., using CHPTAC as reagent in aqueous alkaline medium in heterogeneous conditions.¹⁹ This polysaccharide was composed of approximately ten different monosaccharides, among which xylose (40%), galactose (18%), and glucose (15%) were the most abundant; it also contained glucuronic acid and sulfate groups. This research group investigated the effects of reaction mixture dilution on the degree of substitution (DS), and found that a higher DS was obtained when the reaction was carried out in small volumes of water in a gel-like phase. The authors also concluded that there was no degradation of the polysaccharide because they did not observe change in the phenol-sulfuric acid assay between the native and the modified polysaccharide, even though this is a very unspecific method for total sugar content and does not indicate decrease in the molecular weight.

In the search of new materials for packing antibody purification columns, Wang et al. covered glass spheres (63–106 μm of diameter) with films of agarose and performed a crosslinking reaction with epichlorhydrin.²⁰ They achieved densities of functionalization in the range 119–374 μmol mL⁻¹ of gel and reported that different densities could be obtained by modifying the reaction conditions. Unfortunately they did not state the density obtained with the only condition tested. They did not perform a characterization of the functionalized product, but focused on the permeability and

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compressibility of the formed beds and their application in the purification of monoclonal antibodies.

Herein, the cationization of a commercial agarose with CHPTAC is presented (Fig. 1) and the influence of experimental parameters on different properties will be analyzed by means of characterization of the products.

2. Results and discussion

2.1. Agarose cationization experiments

In Table 1 experiments of agarose cationization are presented. In these experiments, different parameters were modified: CHPTAC–agarose molar ratio, NaOH–CHPTAC molar ratio, temperature of the reaction (50 and 80 °C), reaction time (2 and 18 h), and the presence or absence of NaBH₄.

A reaction time of 18 h was initially selected based on a recent article where different polysaccharides were modified with CHPTAC;²¹ later, and as a result of the study for determination of the optimum reaction time described below (Section 2.2), 2 h reaction experiments were carried out.

All the experiments were performed in homogeneous phase with agarose in sol state. In almost all the experiments, an excess of NaOH with respect to CHPTAC was employed; this is because an equivalent of NaOH is necessary for generating the epoxide EP-TAC from the chlorohydrin CHPTAC. On the other hand, NaOH is also required as a catalyst in order to ionize the hydroxyl groups of the polysaccharide, which are the nucleophiles of the reaction. This excess of base is required for the cationization of any polysaccharide with CHPTAC, but not if EPTAC is employed.^{10,21}

During the cationization of native agarose, the alkaline modification is also produced through the cyclization of L-galactose

6-sulfate units to 3,6-anhydro-galactose (LA), and elimination of the sulfate group. In CAG9 a NaOH–CHPTAC molar ratio of 0.58 was employed; in this experiment it is observed that the cyclization reaction was not completed and as a result a product with cationic and anionic groups (sulfates) of amphoteric nature was obtained. This product formed lumps instead of being soluble as the other ones. Because of the aforementioned issues, the DS_{EA} (degree of substitution determined by elemental analysis) of CAG9 was low (0.09) and increased considerably when a NaOH–CHPTAC molar ratio of 2.30 was employed ($DS_{EA} = 0.58$) in CAG11. Due to the characteristics of CAG9, it was not possible to measure the M_n or the M_w of that sample. CAG9 was the only experiment that presented sulfur in its elemental analysis.

From these experiments it is evident that for each concentration of CHPTAC, there is an optimum concentration of NaOH that makes the degree of substitution (and the efficiency) to reach a maximum. The maximization of efficiency is not only necessary for green chemistry concerns, avoiding the spoilage of cationizing reagent but also for economic reasons as well. The cost of the cationizing reagent can influence considerably the price of the derivatized polysaccharide, as it is the case of starch.⁴ In general, the higher the CHPTAC–agarose molar ratio, the lower the optimum NaOH–CHPTAC molar ratio was. This can be appreciated more clearly by comparing groups of reactions with the same CHPTAC–agarose molar ratio. For example, in the experiments CAG1, CAG2, and CAG3 a CHPTAC–agarose molar ratio of 1.00 was employed; herein the higher degree of substitution ($DS_{EA} = 0.08$) for that fixed amount of cationizing reagent was achieved with a NaOH–CHPTAC molar ratio of 2.30. In contrast, in experiments CAG12, CAG14, and CAG15 where a CHPTAC–agarose molar ratio of 8.00 was used, a higher DS_{EA} (0.77) was achieved for a NaOH–CHPTAC molar ratio of 1.73 (CAG14), lower concentrations of NaOH (NaOH–CHPTAC

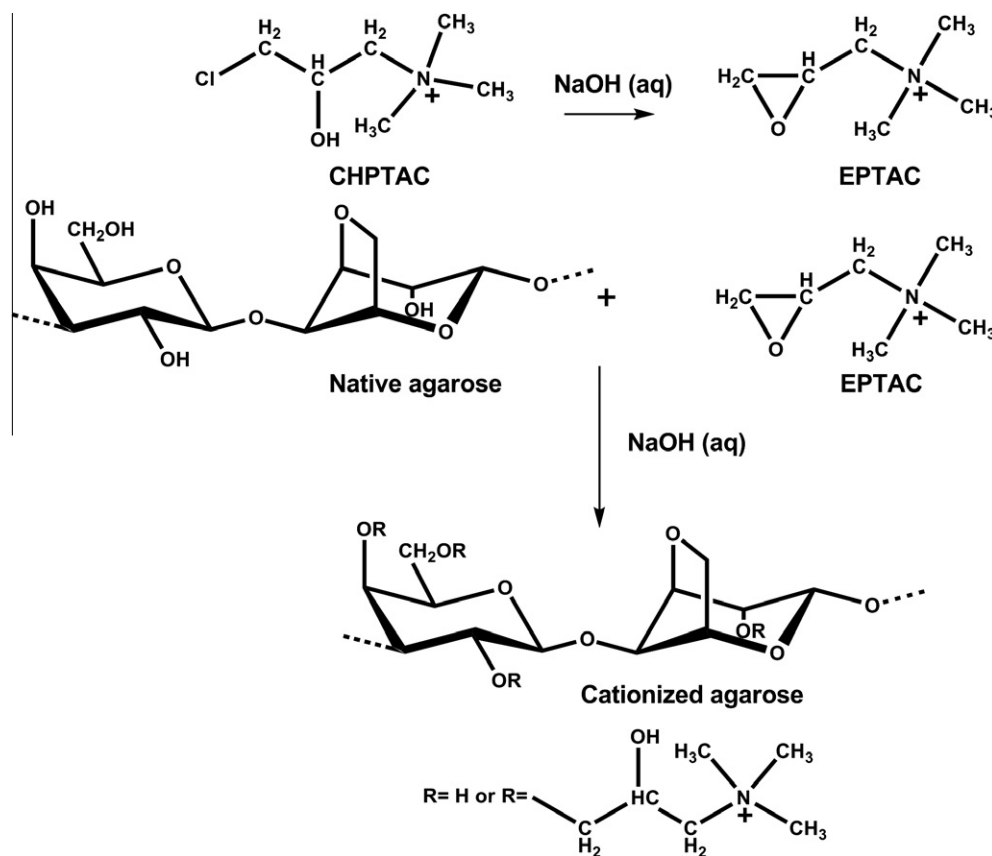


Figure 1. Cationization reaction of agarose with CHPTAC.

Table 1

Molar ratios of the reagents employed, degree of substitution, and molecular weight obtained

Experiment	CHPTAC:agarose molar ratio ^a	NaOH:CHPTAC molar ratio	Temp of reaction (°C)	Time (h)	NaBH ₄	%N	DS _{EA}	Yield (%)	Reaction Efficiency	Molecular weight ^b	
										M _n (kDa)	M _w (kDa)
CAG1	1.00	1.15	50	18	No	0.39	0.04	92	4.47	113.2	192.4
CAG2	1.00	1.73	50	18	No	0.50	0.06	93	5.82	112.7	214.1
CAG3	1.00	2.30	50	18	No	0.67	0.08	93	7.93	110.3	198.5
CAG4	2.00	2.30	50	2	No	1.45	0.19	95	9.44	134.5	242.0
CAG5	2.00	2.30	50	18	No	1.45	0.19	91	9.44	113.8	182.0
CAG6	2.00	2.30	50	2	Yes	1.45	0.19	92	9.44	— ^c	276.3
CAG7	2.00	2.30	50	18	Yes	1.45	0.19	89	9.44	— ^c	235.4
CAG8	2.00	2.30	80	18	No	1.01	0.12	92	6.24	60.1	114.3
CAG9 ^d	4.00	0.58	50	18	No	0.76	0.09	92	2.27	— ^e	— ^e
CAG10	4.00	2.30	50	2	No	3.37	0.58	94	14.58	180.4	306.7
CAG11	4.00	2.30	50	18	No	3.37	0.58	94	14.58	144.3	245.2
CAG12	8.00	1.15	50	18	No	0.88	0.11	96	1.34	114.3	205.8
CAG13	8.00	1.73	50	2	No	3.99	0.77	94	9.66	196.6	354.0
CAG14	8.00	1.73	50	18	No	3.99	0.77	91	9.66	152.6	305.1
CAG15	8.00	2.30	50	18	No	3.25	0.55	93	6.91	96.1	163.4

^a 1 g of agarose was employed in all cases (corresponding to 6.54 mmol of the average unit) and the final volume of reaction was 100 mL.^b Native agarose had a M_w of 202.3 kDa (determined at 35 °C).^c For samples treated with NaBH₄ Park & Johnson method was not applied.^d This sample contained S = 0.29 % in the elemental analysis (native agarose contained S = 0.41 % and the rest of the samples contained S = 0%).^e The molecular weight of this sample was not determined due to the presence of lumps.

molar ratio of 1.15) in experiment CAG12 produced an important decrease in the DS_{EA} to 0.11. Higher concentrations of NaOH, such as in CAG15 (NaOH–CHPTAC molar ratio of 2.3), also produced a lower DS_{EA} (0.55), due to degradation of the epoxide EPTAC generated in situ, to the undesirable diol. This diol does not react with agarose but can react with CHPTAC leading to the formation of dimers and trimers of the cationizing reagent.^{2,4} At the optimum concentration of NaOH, the increase in the concentration of CHPTAC increased the degree of substitution. For example, CAG3, with a CHPTAC–agarose molar ratio of 1, presented a DS_{EA} of 0.08, CAG14, with a CHPTAC–agarose molar ratio of 8, reached a DS_{EA} of 0.77. Intermediate CHPTAC–agarose molar ratios, such as 2 and 4, led to products with intermediate DS_{EA}.

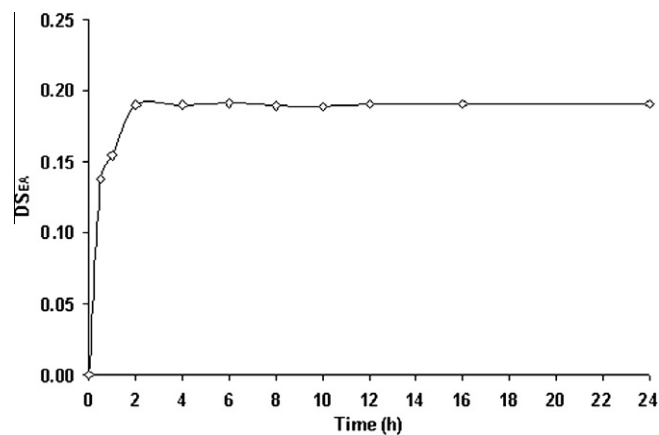
From the comparison of CAG5 and CAG8, it arises that the increase in temperature from 50 to 80 °C produced only a slight increase in the DS_{EA}, and favored, on the other hand, an important decrease in the molecular weight of the products. For the cationization of agarose in homogeneous media, the lower temperature of reaction is restricted by the gelling temperature of the native agarose (31 °C). Higher temperatures, besides the aforementioned degradation, constitute an unnecessary energetic expense, especially considering a potential synthesis at industrial scale.

As peeling processes of polysaccharides in alkaline media are initiated at the reducing end,²² a common strategy is the use of a reducing agent to reduce the aldehyde in the reducing end to alcohol. With that purpose in mind, NaBH₄ was employed, partially avoiding the decrease of the M_w of the products and maintaining the same DS_{EA} (compare CAG6, M_w = 276.3 kDa with CAG4, M_w = 242.0 kDa and CAG7 M_w = 235.4 kDa with CAG5 M_w = 182.0 kDa). Even so, the molecular weight of the products was not the maximum achievable because of nonspecific degradation. The presence of a reduced reducing end in CAG6 and CAG7 did not allow the determination of M_n by the Park and Johnson method.²³ However, it was possible to determine the M_w by static light scattering. It is important to take into account that the variation in the molecular weight of the products is determined by two conflicting factors. On one hand, the progress of the reaction of substitution with the cationic groups produces an increase in the molecular weight. On the other hand, the processes of degradation in the alkaline medium lead to a decrease in the molecular weight. Despite the fact that the addition of NaBH₄ was favorable for the molecular weight, it was not applied in a general way, as this

reagent would make the production of cationized agarose more expensive on an industrial scale.

2.2. Optimum reaction time determination

A necessary parameter to optimize this reaction is the reaction time. It was determined by successive sampling at different times and determination of DS_{EA}. Figure 2 shows the change of DS_{EA} for a CHPTAC–agarose molar ratio of 2.00 and a NaOH–CHPTAC molar ratio of 2.30, at 50 °C. The data obtained at 2 and 18 h correspond to those of CAG4 and CAG5, respectively (Table 1). It is evident that the maximum degree of substitution is reached after 2 h of reaction. It is possible to compare other pairs of experiments from Table 1 that differ from each other only in the reaction time: CAG6 with CAG7, both with CHPTAC–agarose molar ratio of 2.00 and NaOH–CHPTAC molar ratio of 2.30, CAG10 with CAG11, with CHPTAC–agarose molar ratio of 4.00 and NaOH–CHPTAC molar ratio of 2.30 and CAG13 with CAG14, with CHPTAC–agarose molar ratio of 8.00 and NaOH–CHPTAC molar ratio of 1.73. From these comparisons, it can be deduced that results are analogous for other DS_{EA}. An increment in reaction time beyond 2 h does not improve DS_{EA} and only favors degradation of the polysaccharide lowering the molecular weight.

**Figure 2.** Evolution of the DS_{EA} as a function of the time of reaction.

2.3. Scanning electronic microscopy

Figure 3 shows scanning electronic microscopy (SEM) images of native agarose and of agaroses cationized in different experimental conditions. At low magnifications, it can be appreciated that cationized agarose CAG1 ($DS_{EA} = 0.04$) has similar characteristics to native agarose. However, at higher magnifications, a branched reticular structure is observed in CAG1, in contrast to native agarose where a smooth surface is shown. The characteristics were maintained in repeated syntheses in the same conditions and became more remarkable in CAG4 ($DS_{EA} = 0.19$). Cationized agarose CAG10 ($DS_{EA} = 0.58$) again presents a change in appearance, the shape of the particles becoming irregular and smooth. This is also true for CAG13 ($DS_{EA} = 0.77$). An interesting fact is that native agarose is more difficult to observe by SEM as electrostatic charges rapidly develop on the surface of the sample, turning the images blurred. This phenomenon does not occur in cationized agaroses, possibly due to the presence of the substituting groups.

2.4. Fourier transform infrared spectroscopy

In the spectrum of native agarose characteristic bands appear at: 776 cm^{-1} (β -galactose skeleton bending), 892 cm^{-1} (anomeric C–H from the β -galactopyranosyl residue) and 930 cm^{-1} (presence of 3,6-anhydro-galactose).²⁴ In the spectra of cationized agaroses, the band at 1482 cm^{-1} , which corresponds to C–H bending of the methyl groups in the quaternary ammonium of the substituent, increases its intensity with increasing DS_{EA} . In a similar fashion but with less intensity, the band at 1414 cm^{-1} , due to C–N stretching, becomes more evident with increasing DS_{EA} . Another band that shows an increment is the one that appears at approximately 1080 cm^{-1} and corresponds to the ether linkage. Also, the broad band at 3450 cm^{-1} , due to the O–H stretching is modified becoming narrower. The bands assigned to the substituent are consistent with those reported for the reaction of starch,³ amylose, amylopectin, glycogen, guar gum,²¹ hemicelluloses,¹⁰ cellulose,^{7,8} carboxymethylcellulose,⁹ and chitosan with the same group.^{12,13}

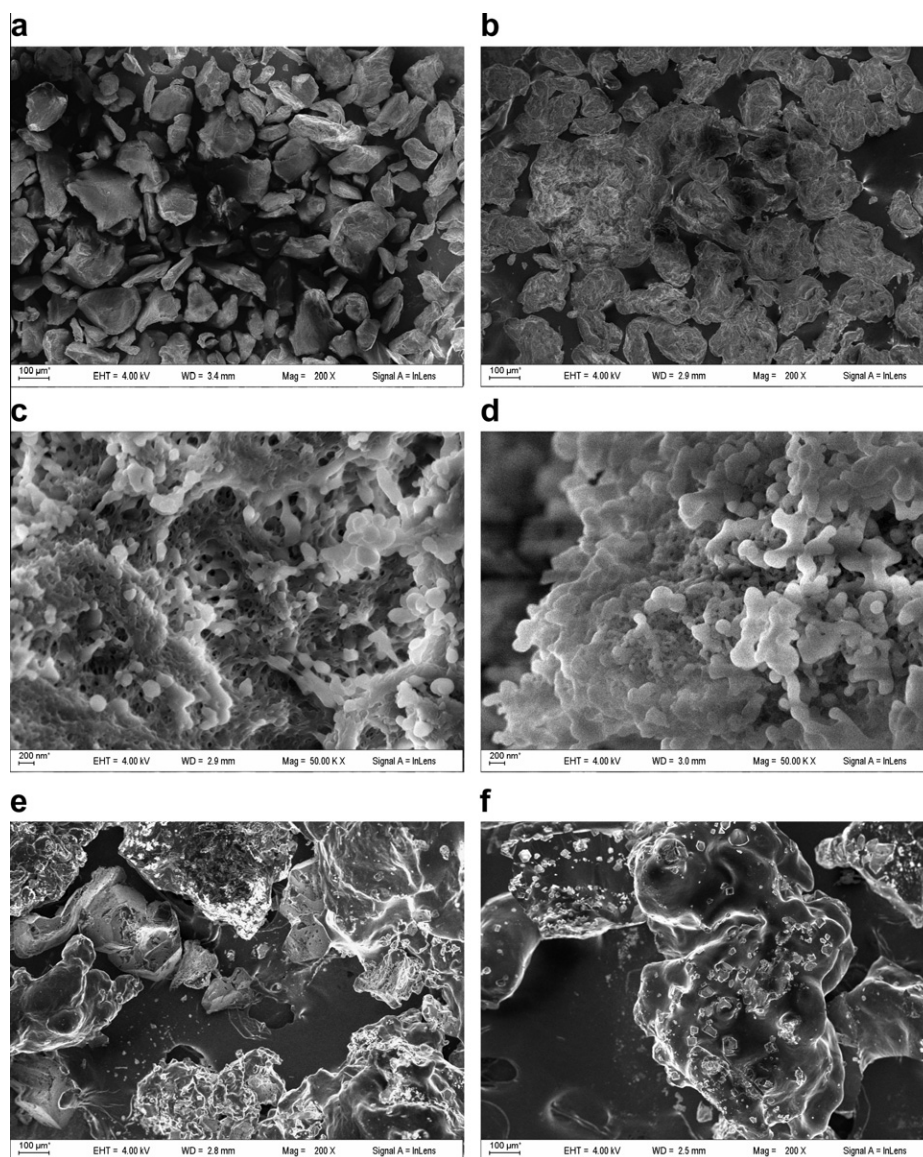


Figure 3. Scanning electron micrographs of native agarose 200 \times (a); CAG1 ($DS_{EA} = 0.04$) 200 \times (b) and 50,000 \times (c); CAG4 ($DS_{EA} = 0.19$) 50,000 \times (d); CAG10 ($DS_{EA} = 0.58$) 200 \times (e), and CAG13 ($DS_{EA} = 0.77$) 200 \times (f).

Table 2
Decomposition temperatures of different cationized agaroses

Sample	DS_{EA}	Decomposition temperature (°C)
Native agarose	0	259.09
CAG4	0.19	258.85
CAG10	0.58	249.16
CAG13	0.77	240.15

2.5. Thermal analysis

Thermal analysis of native agarose and cationized agaroses CAG4, CAG10, and CAG13, were studied (Table 2). The initial weight losses detected by thermogravimetry (TG) can be attributed to desorption of water from the structure of the polysaccharide. The decomposition temperature, calculated as the maximum of the decomposition isotherm in the differential scanning calorimetry (DSC) curve, was lower the higher the DS_{EA} was. At that temperature an abrupt weight loss was detected by TG. The intensity of the decomposition exotherm also decreased with the increment in DS_{EA} . Because the decomposition exotherm is related to the cleavage of intra and intermolecular interactions and the decomposition of the polysaccharide,¹⁰ it could be concluded that the etherification produced by cationization significantly breaks hydrogen bonds in polysaccharide chains.⁸ A reduction in decomposition temperature with increasing substitution was also reported for cellulose⁸ and hemicelluloses¹⁰ modified with the same group. Despite the decrease in decomposition temperature, the values are still adequate for most of the potential applications of these cationized products.

2.6. Viscosimetry

Cationized agaroses CAG4, CAG10, and CAG13, presented anomalous behavior in solution: an increase in reduced viscosity (η_{red}) was observed at low concentrations, as a consequence of intra and intermolecular electrostatic repulsions. This behavior has been reported for solutions of other polysaccharides cationized with the same group (dextran⁶ and cellulose⁷), and causes the graph to describe more or less pronounced curves. As a result, it was not possible to extrapolate reduced viscosity to zero concentration in order to determine intrinsic viscosity $[\eta]$.

This problem could be overcome by the addition of low molecular weight polyelectrolytes, or by employing empirical equations

such as those proposed by Fuoss and Strauss,²⁵ or by Fedors²⁶ modified by Rao,²⁷ applicable to describe the viscosity of diluted or moderately concentrated polymer solutions.

The equation of Rao is as follows:

$$\frac{1}{2(\eta_{rel}^{1/2} - 1)} = \frac{1}{[\eta]c} - \frac{a-1}{2.5}$$

where η_{rel} is the relative viscosity and a is a constant for a specific polymer solvent system.

As it can be appreciated in Rao's equation graph (Fig. 4) and from the adjustment coefficient R^2 , the equation is adequate to describe the rheological behavior of these samples. The values of $[\eta]$ obtained from the inverse of the slope of these straight lines, can be useful as a semi quantitative comparison of the behavior of salt-free polyelectrolyte solutions. The applicability of Rao's equation was also verified for cationized dextrans with the same group.⁴

From Figure 4 and Table 3 it can be deduced that $[\eta]$ values increase with increasing DS_{EA} for agaroses of similar molecular weight; an analogous behavior was observed in cationized glycogen with the same group.²⁸ This increase in viscosity could be explained by the increase in the electrostatic repulsion among cationic groups linked to the polysaccharide forcing cationized agarose to adopt a more extended conformation.

2.7. Nuclear magnetic resonance

Nuclear magnetic resonance was employed in an attempt to determine the position and degree of substitution of agarose with the cationic group.

Starting from the definition of degree of substitution as the ratio between the number of substituting groups and the number of monosaccharide units, the formula shown in the experimental section was deduced using ¹H NMR spectroscopy (Fig. 5) in the following way. The number of substituents was estimated by the area of the peak at 3.21 ppm, corresponding to the methyl groups linked to the quaternary nitrogen in the substituent, as this peak integrates for nine protons, it is divided by that number in order to normalize its area. The number of monosaccharide units can be estimated as the sum of the area at 5.14 ppm, corresponding to the anomeric proton of the 3,6-anhydro-galactose moiety, and the area of the peak at 5.17 ppm, originated by the anomeric proton of 3,6-anhydro-galactose shifted to lower fields by the presence of a substituent at C-2. As agarose is formed by equal quantities of galactose and 3,6-anhydro-galactose, multiplication

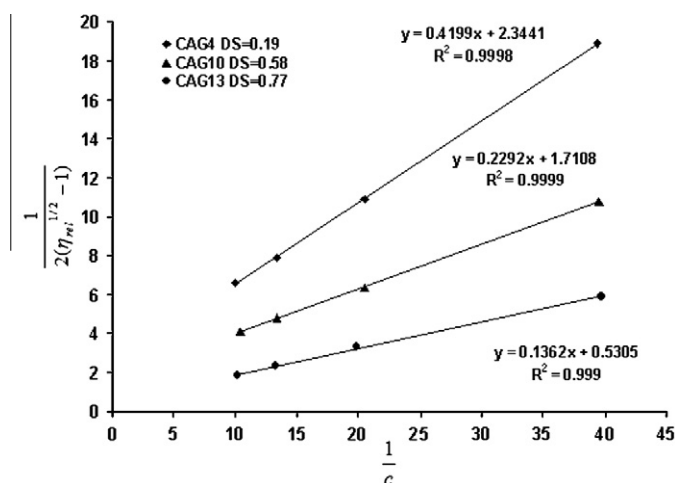


Figure 4. Representation of Rao's equation for cationized agaroses CAG4 ($DS_{EA} = 0.19$), CAG10 ($DS_{EA} = 0.58$) and CAG13 ($DS_{EA} = 0.77$).

Table 3
Intrinsic viscosity of cationized agaroses with different DS_{EA}

Sample	DS_{EA}	$[\eta]$	R^2
CAG4	0.19	2.38	0.998
CAG10	0.58	4.36	0.999
CAG13	0.77	7.34	0.999

of the number of 3,6-anhydro-galactose by two gives the number of monosaccharide units. The area of the anomeric proton of galactose was not used because it appears in a region that presents multiple superpositions with other signals.

From the spectra of different cationized agaroses the DS_{NMR} was calculated (Table 4). Thus, there is a good correlation between the degree of substitution determined by elemental analysis and that determined by 1H NMR spectroscopy. This determination was useful in the range 0.04–0.58. A variation in the area ratio between the peaks at 5.14 and 5.17 ppm was determined. An increase in the relative area of the peak at 5.17 is evident with increasing DS , until a value of approximately 1:1 is reached (Table 5).

Despite the fact that 1H NMR area determinations are subject to error, the data obtained would indicate that the substitution starts preferentially on the 2-position of 3,6-anhydro-galactose residues, reaches a maximum, and the proportion of substituted galactose begins to increase. For example, in the case of CAG4 ($DS_{EA} = 0.19$), it was determined by 1H NMR spectroscopy that there are 46 units of modified 3,6-anhydro-galactose for every 146 units of total 3,6-anhydro-galactose. If total substitution in agarose would correspond only to the mentioned proportion, the DS would be 0.15. As DS_{EA} is 0.19, the difference could be attributed to substitution in galactose. Similarly, according to the 1H NMR spectroscopic results, the proportion in the substitution of 3,6-anhydro-galactose of CAG10 ($DS_{EA} = 0.58$), would give a degree of substitution of 0.25, from which it follows by difference (0.33) a greater cationization of galactose for that sample.

Cationized agaroses were also analyzed by ^{13}C NMR spectroscopy. In Figure 6 it is observed that signals in CAG1 ($DS_{EA} = 0.04$) spectra are in agreement with those described in the literature for agarose,³² in addition to those corresponding to the cationic

Table 4
Degrees of substitution estimated by 1H NMR

Sample	DS_{EA}	DS_{NMR}
CAG1	0.04	0.06
CAG4	0.19	0.21
CAG6	0.19	0.23
CAG10	0.58	0.61
CAG13	0.77	n.d. ^a

^a n.d.: this value was not determined because the spectrum of CAG13 was very complex.

Table 5
Ratio between LA: LA substituted in C-2 signals in the 1H NMR spectra of cationized agaroses of different DS_{EA}

Sample	DS_{EA}	Ratio LA: LA subst. in C-2
CAG1	0.04	1.00:0.23
CAG4	0.19	1.00:0.46
CAG6	0.19	1.00:0.46
CAG10	0.58	1.00:1.00
CAG13	0.77	n.d. ^a

^a n.d.: this value was not determined because it was not possible to integrate signals.

substituent (C-7, 73.9 and 74.2 ppm; C-8, 66.0 ppm, C-9, 68.4 ppm, C-10, 55.1 ppm) (Fig. 7). The signal of C-7 seems to be split by different environment (different position of substitution).

As the DS increased, the signals corresponding to the substituent also increased, and those belonging to the repeating unit of agarose decreased. On the other hand, new resonances appeared, belonging to the substituted repeating units. Therefore, in the spectrum of CAG4 ($DS_{EA} = 0.19$) a broadening toward higher fields of the signal of β -D-galactose at 102.7 ppm was observed in the anomeric region, compatible with substitution on C-2 of this unit; a new peak at 99.4 ppm, next to the signal at 98.5 ppm of 3,6-anhydro-galactose was also detected. The appearance of a new signal shifted 2–3 ppm toward higher fields was observed in starch,¹ whereas the deformation of the original anomeric signal was observed in xylans¹¹ and cellulose.⁸

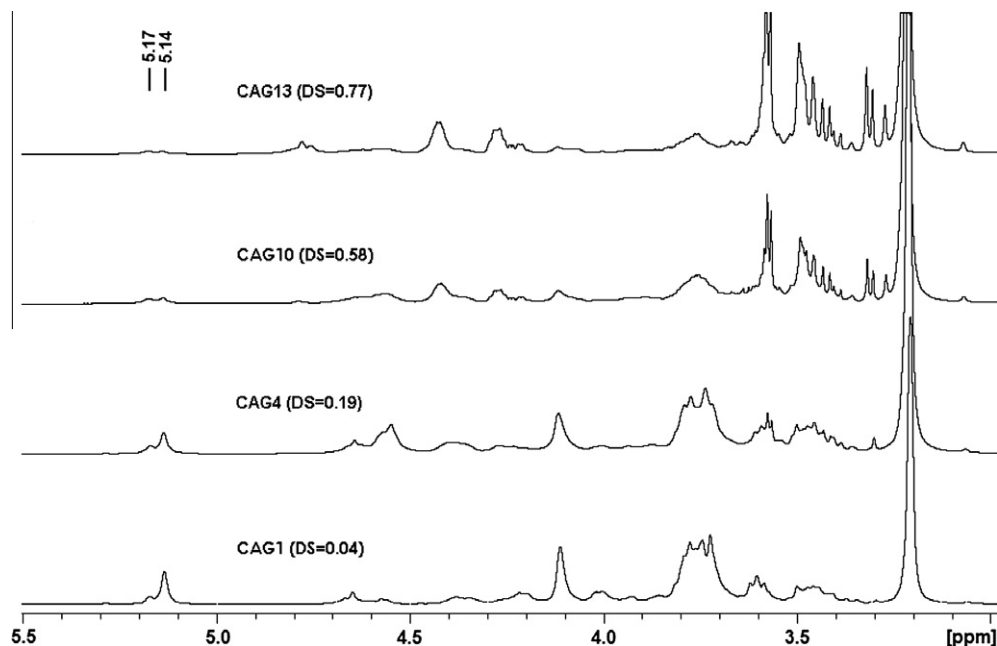


Figure 5. 1H NMR spectra of cationized agaroses with different degree of substitution.

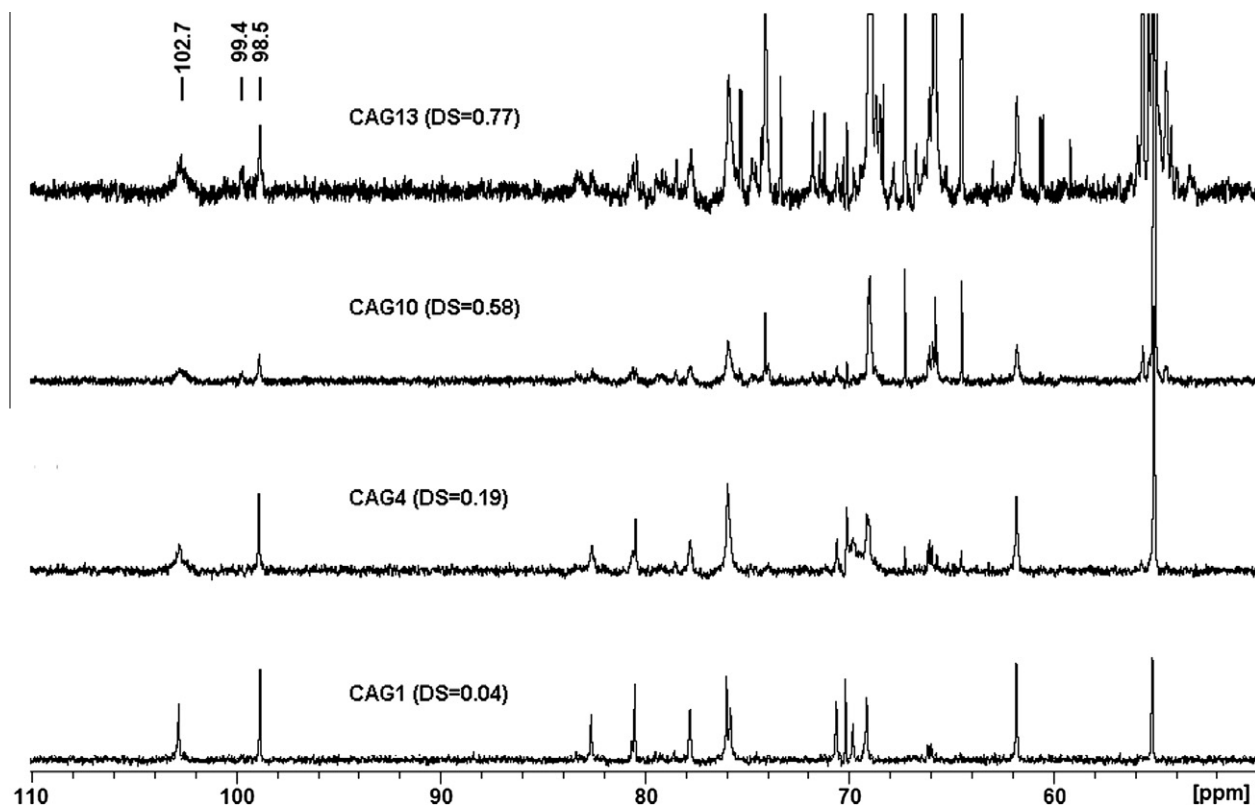


Figure 6. ^{13}C NMR spectra of cationized agaroses with different degrees of substitution.

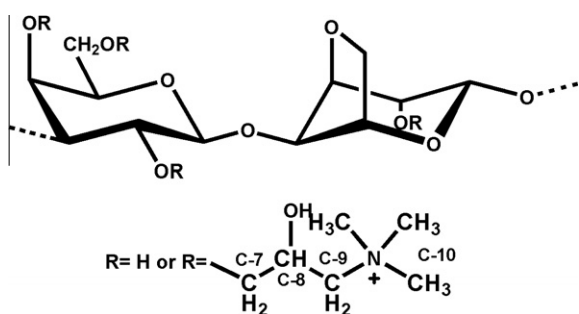


Figure 7. Structure of cationized agaroses; the numbering of carbons in the substituent is indicated.

For higher degrees of substitution (CAG10, $DS_{EA} = 0.58$, and CAG13 $DS_{EA} = 0.77$), the complexity of the spectrum increased, as expected. Signals corresponding to the reducing ends caused by chain degradation were not observed in these spectra (approximately 91 ppm, for the hydrated aldehyde of the reducing end of 3,6-anhydro-galactose, together with signals at 95 and 97 ppm, originated by α - and β - galactose).

It is noteworthy that the signal at 61.7 ppm (C-6 from the β -D-galactose unit) was maintained with increasing degree of substitution. This would suggest a higher substitution in position 2 over the other positions; this reactivity may be attributed to the higher acidity of HO-2 enhanced by its proximity to the anomeric center.²⁹ Similar trends were informed for a monosubstituted fraction of starch modified with the same group.^{30,31}

When the spectrum of CAG6 ($DS_{EA} = 0.19$) was performed at 80 °C, a considerable enhancement in the signal to noise ratio was observed (Fig. 8); as was expected, the main signals corresponded to agarose and the cationizing group. In addition, small signals from the substituted polysaccharide units were also found.

From COSY and HSQC experiments it was found that signals at 5.17 and 3.81 ppm corresponded to H-1 and H-2 of LA monosubstituted in the 2 position. In turn, the resonances at 99.4 and 79.0 were assigned to the C-1 and C-2 of the same unit. On the other hand, the shoulder at 102.1 ppm of the signal at 102.7 ppm due to C-1 of the β -D-galactose substituted in the 2 position, correlated with a signal at 4.63 ppm (H-1) and the signal of H-2 at 3.94 ppm did the same with C-2 at 78.5 ppm. Analysis could not be continued because correlations led to regions where the signals of unsubstituted agarose appeared (mainly for a $DS_{EA} = 0.19$).

2.8. ESI mass spectrometry

Derivatization of cationized agaroses CAG4 ($DS_{EA} = 0.19$) and CAG13 ($DS_{EA} = 0.77$) by methanolysis was performed. Native agarose was subjected to the same treatment for comparison. The ESI spectrum of CAG4 is shown as an example (Fig. 9). The peaks indicating substitution are: m/z 310 [monosubstituted methyl galactoside]⁺, m/z 292 [monosubstituted methyl 3,6-anhydro-galactoside]⁺ (or m/z 310-H₂O) and m/z 324 [monosubstituted dimethylacetal of 3,6-anhydro-galactose]⁺. The structures of the main monosaccharides present after the methanolysis of cationized agaroses are shown in Figure 10.

3. Conclusions

Cationized agaroses of different degree of substitution in the range 0.04–0.77 were successfully synthesized. The effect of different reaction parameters such as reagent concentration, temperature, and time and the addition of NaBH₄ on the substitution degree and molecular weight were evaluated. The higher the concentration of CHPTAC employed, the higher the degree of substitution obtained, but only if the optimum concentration of NaOH in each case was employed. Insufficient quantities of NaOH reduced

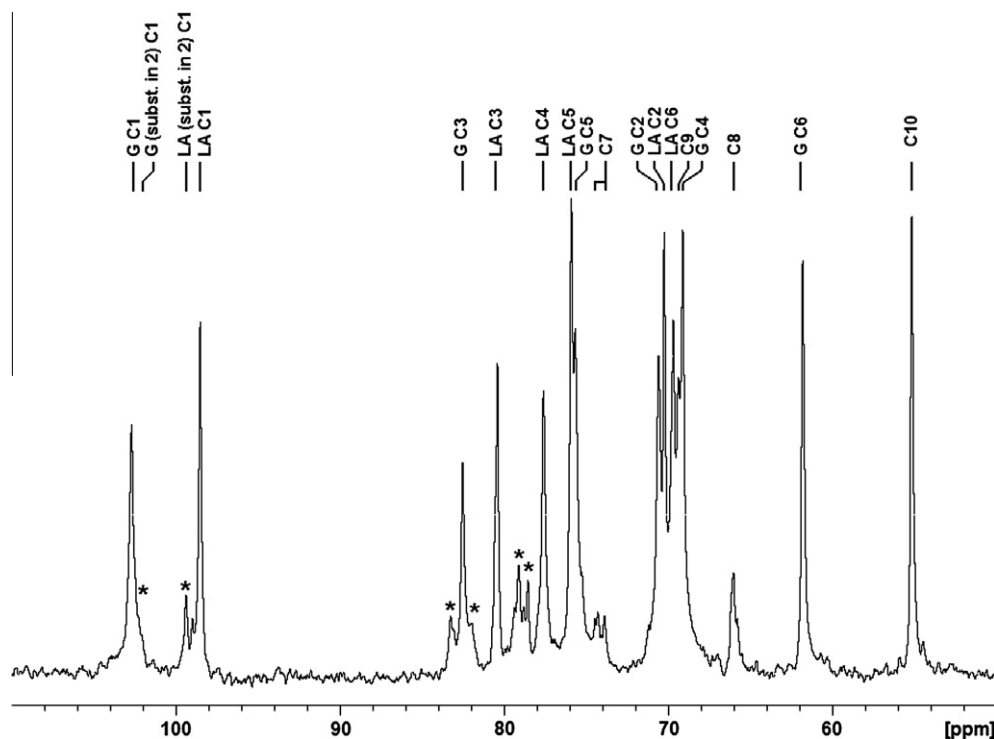


Figure 8. ^{13}C NMR spectrum of CAG6 ($D_{\text{SEA}} = 0.19$) at 80 °C.

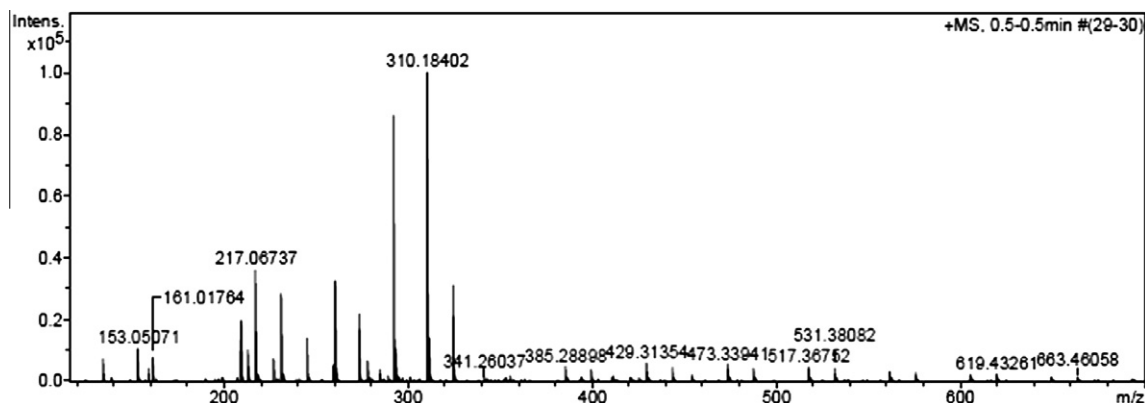


Figure 9. ESI spectrum of CAG4 ($D_{\text{SEA}} = 0.19$) after methanolysis.

epoxide formation and the reacting alcoxides of the polysaccharide, whereas an excess of NaOH favored degradation of the epoxide and the decrease in the molecular weight of the product. A reaction time of 2 h was sufficient to obtain products with the maximum degree of substitution for each case. The addition of NaBH_4 generated products with a slightly higher molecular weight, but the extra cost involved should not justify its use in industrial scale. The products were characterized by scanning electronic microscopy, thermal analysis, viscosimetry, and NMR. Monosaccharides derived from cationized agaroses were obtained and studied by ESI mass spectrometry.

4. Experimental

4.1. Materials

The agarose employed for the reactions was Agar Bacteriological (Agar No. 1), (Oxoid Ltd). The cationizing reagent was

(3-chloro-2-hydroxypropyl)trimethylammonium, 60% w/w aqueous solution (Sigma–Aldrich, Inc.). All the other reagents were of analytical grade.

4.2. Methods

4.2.1. Synthesis of cationized agaroses

One gram of agarose was employed in all the experiments (corresponding to 6.54 mmol of the average unit, 153 Da) and the final volume of reaction was 100 mL; as a result the concentration of the polysaccharide was 1% w/v. The concentration of the reagents NaOH and CHPTAC is expressed as molar ratios with regard to the mean unit of the polysaccharide.

Agarose was dispersed in water in a round bottomed flask, and heated at 90 °C for 15 min. The flask was immersed in an oil bath over a hot plate provided with magnetic stirring, at the temperature of reaction. The solution of CHPTAC and the solution of NaOH of the required molarity were added. The reaction was allowed to

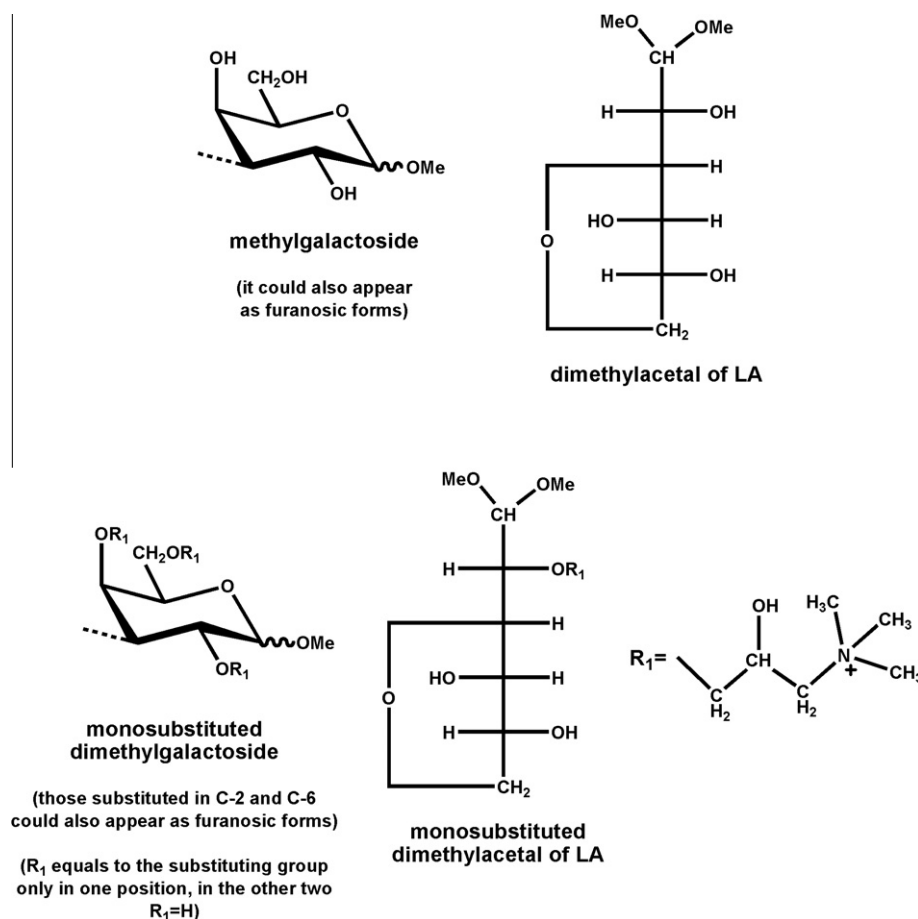


Figure 10. Structures of the main monosaccharides present after methanolysis for the cationized agaroses.

evolve for a predefined time. Different parameters were assayed: CHPTAC–monosaccharide unit molar ratio (between 1.00 and 8.00), NaOH–CHPTAC molar ratios (between 0.58 and 2.30), temperatures of 50 and 80 °C, time between 2 and 24 h. The effect of the addition of NaBH₄ to the dissolved agarose before the addition of other reagents was also studied.

After the predefined time, the solution was neutralized with 1 M HCl and poured over four volumes of 2-propanol generating the precipitation of the polysaccharide. The sample was centrifuged, the supernatant was removed, and the precipitate was resuspended in water and 2-propanol (4:1 v/v) and centrifuged ($\times 2$). Finally, the material was dried on a rotary evaporator at 40 °C to give a white yellowish powder.

4.2.2. Determination of the DS by elemental analysis

The DS was determined by elemental analysis of nitrogen (DS_{EA}) in a Carlo Erba EA 1108 CHNS analyzer. No nitrogen was detected in native agarose. The formula used¹ (adapted to agarose) was:

$$DS_{EA} = \frac{(153 \times \%N)}{1400 - (152 \times \%N)}$$

where 153 is the molecular weight of the average unit of agarose $(162 + 144)/2 = 153$; %N is the percentage of nitrogen on dry basis; 14 is the atomic weight of nitrogen and 152 is the molecular weight of the cationic substituting group.

Elemental analysis of the samples was carried out by triplicate and the mean values are reported. All the determinations showed RSD (relative standard deviations) below 2%. From the DS_{EA} the reaction yield¹ was calculated:

$$\text{Yield}\% = \frac{W_{\text{exp}}}{W_{\text{theo}}} \times 100$$

where W_{exp} is the weight of the polysaccharide obtained after the washing and drying process, and W_{theo} is the theoretical weight of cationic agarose according to DS_{EA} .

The reaction efficiency (RE)¹ was calculated as:

$$\text{RE}\% = \frac{DS_{EA}}{DS_{\text{theo}}} \times 100$$

where DS_{theo} is the theoretical DS that would have been obtained if all the CHPTAC had reacted with the polysaccharide.

4.2.3. Determination of the optimum reaction time

The evolution of the cationization reaction was studied as a function of time by means of successive sampling and determination of DS_{EA} . In order to evaluate the degradation of the polysaccharide, the number-average molecular weight was determined by the method of Park and Johnson²³ (see below), for samples of time equal 2 and 18 h (CAG4 and CAG5, respectively).

4.2.4. Determination of the molecular weight

Number-average molecular weight (M_n), was determined by the Park and Johnson method.²³ For each DS_{EA} , the average anhydrous monosaccharide unit (MW_{anhyd}) was calculated.

$$MW_{\text{anhyd}} = 153 + (DS_{EA} \times 152) - (DS_{EA} \times 1)$$

The mass-average molecular weight (M_w) was determined employing a Zetasizer Nano-Zs (Malvern Instruments Ltd) provided with a 4 mW He–Ne (633 nm) laser and a ZEN3600 digital

correlator. This instrument performs measurements using static light scattering techniques. The Rayleigh equation was used to generate a Debye plot, where the ordinate intercept is equal to the inverse of M_w . Toluene was employed to calculate the Rayleigh ratio and a $d\tilde{n}/dC$ value of 0.152 mL g^{-1} for agarose was used.³³

Four different concentrations for each sample (2, 4, 6 and 8 mg mL^{-1}) were prepared and filtered through $0.02 \mu\text{m}$ membranes. All the assays were performed at $25 \text{ }^\circ\text{C}$, except for the native agarose that was measured at $35 \text{ }^\circ\text{C}$ to avoid gelation. Glass cuvettes were employed.

4.2.5. FT-IR spectroscopy

FT-IR spectroscopy was performed in a 510P Nicolet FTIR spectrophotometer (Thermo Fisher Scientific, Inc.) employing the KBr disc method; the range measured was $4000\text{--}500 \text{ cm}^{-1}$, and 32–64 scans were taken with a resolution of $2\text{--}4 \text{ cm}^{-1}$.

4.2.6. Thermal analysis

Prior to thermal analysis the samples were dried overnight under vacuum in a desiccator with CaCl_2 . A TG-DSC SDT Q600 (TA Instruments) simultaneous thermal analyzer was employed. Nitrogen was used as purge at a flow rate of 100 mL min^{-1} , and indium was used to calibrate enthalpy and temperature values. Experiments were carried out with 7–8 mg of samples in open aluminum oxide crucibles. The heating rate was $10 \text{ }^\circ\text{C min}^{-1}$ from 0 to $300 \text{ }^\circ\text{C}$.

4.2.7. Scanning electronic microscopy

Scanning electronic microscopy (SEM) was performed in a Zeiss DSM 982 Gemini microscope (Carl Zeiss) equipped with a field emission gun (FEG) and an in-lens secondary electrons detector (SE). Acceleration voltage was 4 kV . Magnification ranges were between $200\times$ and $50,000\times$.

4.2.8. Viscosimetry

Viscosity was determined with a Cannon–Fenske type viscosimeter number 100, with a $0.015 \text{ mm}^2 \text{ s}^{-2}$ viscosimeter constant, recommended for viscosities between 3 and 15 cSt ($\text{mm}^2 \text{ s}$). Four concentrations for each sample were prepared and measured $\times 3$. Determinations were performed at $20.0 \pm 0.5 \text{ }^\circ\text{C}$ with 10.0 mL of solution. Samples were prepared in distilled water and filtered through a $0.22 \mu\text{m}$ membrane before the tests.

4.2.9. Nuclear magnetic resonance spectroscopy

Samples ($10\text{--}30 \text{ mg}$), previously exchanged with deuterium by repeated evaporations in D_2O , were dissolved in D_2O (0.5 mL) and 5 mm tubes were used. Spectra were recorded at room temperature on a Bruker Avance II 500 MHz spectrometer. For $500 \text{ MHz } ^1\text{H}$ NMR experiments, the parameters were: a spectral width of 6.1 kHz , a 90° pulse, an acquisition time of 2.7 s , and a presaturation time for solvent suppression of 7.0 s ; for $16\text{--}32$ scans. For $125 \text{ MHz } ^{13}\text{C}$ NMR experiments, the parameters were: a spectral width 29.8 kHz , a $60/90^\circ$ pulse, an acquisition time of 1.1 s , and a relaxation delay of $0.1/0.2 \text{ s}$; for $1024\text{--}3200$ scans. In all cases, signals were referenced to internal acetone at 2.21 ppm for ^1H NMR and 31.1 ppm for ^{13}C NMR experiments, respectively.

The ^{13}C NMR spectrum of CAG6 was also performed at $80 \text{ }^\circ\text{C}$ in a Bruker Avance DRX400 NMR spectrometer. For $400 \text{ MHz } ^1\text{H}$ NMR experiments the parameters were: a spectral width of 8.0 kHz , a 90° pulse, an acquisition time of 2.0 s and a relaxation delay of 1.0 s , for 32 scans. The water signal was suppressed by means of low-power irradiation during relaxation. For $100 \text{ MHz } ^{13}\text{C}$ NMR experiments the parameters were a spectral width 31.0 kHz , a 90° pulse, an acquisition time of 0.59 s and a relaxation delay of 0.1 s ; for $2000\text{--}10,000$ scans. Pulse sequences for $^1\text{H}^1\text{H}$ COSY and $^1\text{H}^{13}\text{C}$ HSQC techniques were supplied by the spectrometer manufacturer.

The degree of substitution was also determined by means of NMR (DS_{NMR}):

$$DS_{\text{NMR}} = \frac{\frac{A_{3.21}}{9}}{(A_{5.14} + A_{5.17}) \times 2} = \frac{A_{3.21}}{(A_{5.14} + A_{5.17}) \times 18}$$

where $A_{3.21}$, $A_{5.14}$, and $A_{5.17}$ are the areas at 3.21 , 5.14 , and 5.17 ppm , respectively.

4.2.10. Analysis of monosaccharides derived from cationized agaroses

This method is based on the derivatization of the analyte prior to its analysis by ESI mass spectrometry.^{20,21}

Methanolysis: Samples (20 mg) of CAG4 and CAG13 were treated with 3 mL of methanol–HCl (1.5 M) at $100 \text{ }^\circ\text{C}$ for 3 h . The acid was neutralized with 2 M NaOH . The solvent was removed in a rotary evaporator at $40 \text{ }^\circ\text{C}$ and the products were left in a vacuum desiccator overnight in order to assure its dryness.

ESI mass spectra were obtained in the positive-ion mode using a Bruker Daltonics micrOTOF-Q II ESI-Qq-TOF mass spectrometer. N_2 was used as both drying and nebulizing gas and the electrospray capillary was at 4.5 kV . The mass scan was in the range of m/z $50\text{--}950$.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2010.12.004.

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