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New cationic hydrophilic and amphiphilic polysaccharides synthesized by one pot procedure

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ABSTRACT

Synthesis of cationic polysaccharides carrying quaternary ammonium groups of various chemical structures was performed by one pot procedure involving the chemical modification of a neutral polysaccharide (dextran, pullulan) with an equimolar mixture epichlorohydrin/tertiary amine, in aqueous media. Study of the reaction mechanism showed that the quaternization reagent was the glycidyl derivative of the tertiary amine formed *in situ*. Formation of cationic polysaccharides and their chemical structure were confirmed by elemental analysis, potentiometric titration, FT-IR and NMR spectroscopy. The reaction efficiency of the one pot procedure was similar to other methods using preformed clorohydroxypropyl or glycidyl quaternary ammonium reagents, but gave better results for the introduction of quaternary ammonium groups with more hydrophobic substituents. The procedure allowed the synthesis of a large variety of hydrophilic and amphiphilic cationic polysaccharides with potential application as hipolipemic drugs, flocculants or antibacterial agents.

1. Introduction

Polysaccharides containing quaternary ammonium groups are hydrophilic, biodegradable, bacteriostatic, and can support some components of the skin and the hair (Brode, Goddard, Harris, & Salensky, 1991). These polymers have numerous applications in a variety of fields, including the paper and textile, food, cosmetics, chemical, and pharmaceutical industries (Belalia, Grelier, Benaissa, & Coma, 2008). Due to their ammonium groups, cationic polysaccharides can control the surface charge of particles in aqueous suspensions, and have a great potential in clarification of raw and industrial wastewater by separation of iron ore (Brostow, Lobland, Pal, & Singh, 2008), coal (Pal, Sen, Karmakar, Mal, & Singh, 2008), dye contaminants from textile industry (Klimaviciute, Riauka, & Zemaitaitis, 2007; Pal, Ghosh, Sen, Jha, & Singh, 2009), contaminating metals (Tseng, Wu, & Juang, 1999) or bentonites (Levy, Garti, & Magdassi, 1995). Cationic cellulose (Celquat^{R}), cationic guar, and amino-modified starches are large-scale commercial products playing an important role in papermaking processes, as retention aids, strength agents, or antimicrobial agents for packaging.

Synthesis of quaternary ammonium groups containing polysaccharides have been performed by using commercially available reagents such as N-(3-chloro-2-hydroxypropyl)-N,N,N-trimethylammonium chloride (CHPTAC) ((QUAB®188)) or N-(glycidyl)-N,N,N-trimethylammonium chloride (GTAC) (QUAB®151), and modified polysaccharides were cellulose (Abbott, Bell, Handa, & Stoddart, 2006; Song, Sun, Zhang, Zhou, & Zhang, 2008; Yan, Tao, & Bangal, 2009), microcrystalline cellulose (Antal & Micko, 1992), hemicellulose (Ren, Sun, Liu, Lin, & He, 2007), starch (Haack, Heinze, Oelmeyer, & Kulicke, 2002; Kavaliauskaite, Klimaviciute, & Zemaitaitis, 2008; Pal, Mal, & Singh, 2005; Wang & Xie, 2010), corn cob meal (Simkovic, Mlynar, & Alfoldi, 1992), glycogen (Pal, Mal, & Singh, 2006), arabinoxylan (Köhnke, Brelid, & Westman, 2009), tamarind kernel polysaccharide (Pal et al., 2009), guar gum (Huang, Yu, & Xiao, 2007), chitosan (Sajomsang, Gonil, & Tantayanon, 2009; Sajomsang, Tantayanon, Tangpasuthadol, & Daly, 2009), konjak glucomannan (Yu, Huang, Ying, & Xiao, 2007), maltodextrin (Loiseau, Imbertie, Bories, Betbeder, & De Miguel, 2002), or dextran (Thomas, Rekha, & Sharma, 2010). The modification can proceed in homogeneous (solutions) or heterogeneous media (suspensions). These reactions require alkaline solutions (NaOH) which accelerate the etherification reaction, but determine also an intensification of reagent inactivation by hydrolysis when the amount of NaOH exceeded 0.4 mol/anhydroglucose unit (AGU) (Kavaliauskaite et al., 2008), and in some cases can damage the polysaccharides' supramolecular structure or degrade the polymer.

Amphiphilic cationic polysaccharides share properties and applications with their hydrophilic analogues, but have additional properties due to their surface activity and ability to self-organize in micelle like aggregates. There is only one com-

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mercial product, Quatrisoft LM-200 (Polyquaternium-24), based on hydroxyethyl cellulose with 5 mol% N-(2-hydroxypropyl)-N-dodecyl-N,N-dimethylammonium chloride groups, largely used as additive for personal care products (Zhang et al., 2008) or as thickening agent (Zhang, 2001). Brode, Kreeger, and Salensky (1995) and Brode, Kawakami, Doncel, and Kemnitzer (1998) reported the synthesis of double cationic cellulose derivatives containing both hydrophilic and amphiphilic pendant cationic groups. Other amphiphilic cationic polysaccharides were obtained by the quaternization of tertiary amino groups containing polysaccharides with alkyl halides (Souguir, Roudesli, About-Jaudet, Picton, & Le Cerf, 2010).

The present work describes the synthesis of polysaccharides with quaternary ammonium pendant groups by one pot procedure involving the reaction of a neutral polysaccharide (dextran, pullulan, hydroxyethyl cellulose) with a mixture of epichlorohydrin (ECH) and a tertiary amine ($R^1R^2R^3N$), where R^1, R^2, R^3, are identical or different, and at least one of the R substituents is an alkyl chain with 2–16 carbons or a benzyl group. Heterocyclic tertiary amines can be also used. The procedure can be applied to various water soluble or water swellable polysaccharides in linear or crosslinked forms. The reaction mechanism for dextran quaternization, cationic polymer chemical structure, and optimal reaction conditions are presented. Several potential applications of some new cationic amphiphilic polysaccharides prepared by this procedure are highlighted as well.

2. Experimental

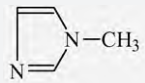
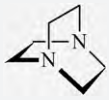
2.1. Materials

Dextran (Dex-40) with molar mass 40 000 was from S.C. Sicomed S.A. (Bucharest, Romania). Pullulan (Pull-200) with a molar mass of 200 000 was supplied by Hayashibara Lab, Okoyama, Japan. All the other reagents were from Aldrich and were used as received. The solvents were of analytical grade and were purified and/or dried by standard procedures. Crosslinked dextran gels (CDex-w, as 0.2 mm spherical particles) were obtained as previously described (Nichifor, Cristea, Mocanu, & Carpov, 1998) and are differentiated by their capacity for water retention (w), in gram water/10 g gel. Thus, CDex-25 indicates a crosslinked dextran able to retain 25 g water/10 g dry gel at equilibrium.

2.2. Synthesis of quaternization reagents

Synthesis of N-(3-chloro-2-hydroxypropyl)-N,N,N-trialkylammonium chloride ($CHPR^1R^2R^3A$) was performed according to the method described in Houben Weyl (1967), by reaction

Table 1
Tertiary amines and their derivatives used for polysaccharide quaternization.

$R^1R^2R^3N$ or $R^1R^2R^3R^4N^+Cl^-$						
Code amine	R^1	R^2	R^3	R^4	Method	Maximum *DS* (*a*/AGU)[a]
DMEA	CH_3	CH_3	C_2H_5	–	iii	90 (10)
CHPDMEA	CH_3	CH_3	C_2H_5	$CH_2-CH(Cl)-CH_2-OH$	i	50 (10)
GDMEA	CH_3	CH_3	C_2H_5	$CH_2-CH(O)-CH_2-$	ii	90 (10)
TEA	C_2H_5	C_2H_5	C_2H_5	–		80 (10)
CHPTEA	C_2H_5	C_2H_5	C_2H_5	$CH_2-CH(Cl)-CH_2-OH$		40 (10)
GTEA	C_2H_5	C_2H_5	C_2H_5	$CH_2-CH(O)-CH_2-$	ii	75 (10)
DMBzA	CH_3	CH_3		$C_6H_5-CH_2-$	iii	70 (9)
DMBuA	CH_3	CH_3	C_4H_9	–	iii	70 (9)
MDBuA	CH_3	C_4H_9	C_4H_9	–	iii	10 (8)
TBuA	C_4H_9	C_4H_9	C_4H_9	–	iii	6 (8)
DMOctA	CH_3	CH_3	C_8H_{17}	–	iii	70 (10)
MDOctA	CH_3	C_8H_{17}	C_8H_{17}	–	iii	10 (10)
DMDodA	CH_3	CH_3	$C_{12}H_{25}$	–	iii	40 (4)
DMCetA	CH_3	CH_3	$C_{16}H_{33}$	–	iii	30 (6)
GDMCetA	CH_3	CH_3	$C_{16}H_{33}$	$CH_2-CH(O)-CH_2-$	ii	5 (6)
MeIm				–	iii	70 (9)
DABCO				–	iii	70 (8)

[a] Maximum degree of substitution, in mol% (molar ratio of reagent mixture, *a*/AGU, where *a* refers to as amine reagents, necessary for obtaining this maximum *DS*). Reaction conditions: 70 °C, 6 h, 1 g linear dextran/10 ml water.

between $R^1R^2R^3N$ and ECH in water medium, with subsequent addition of concentrated HCl till pH 3. The reagents were obtained as 50% (w/w) water solutions. For analysis, the solid products were separated by freeze drying.

N-(3-chloro-2-hydroxypropyl)-N,N,N-triethylammonium chloride (CHPTEA): Analysis, found (calculated), %: C 49.2 (47.2); H 9.71 (9.1); N 6.6 (6.1); total chlorine 24.0 (30.6); chloride ion 16.4 (15.3). Active compound (calculated from covalent chlorine content, and related to the solid product): 58%.

N-(3-chloro-2-hydroxypropyl)-N,N-dimethyl-N-ethylammonium chloride (CHPDMEA): Found (calculated), %: C 43.7 (41.8); H 9.0 (8.4); N 7.6 (6.9); total chlorine 29.8 (34.8); chloride ion 19.3 (17.4). Active compund: 60%.

N-glycidyl-N,N,N-trialkylammonium chloride ($GR^1R^2R^3A$) were prepared from the corresponding tertiary amines and ECH by the method of McClure and Williams (1969), using anhydrous acetone as a solvent. The gel-like products were separated by solvent evaporation, trituration with diethyl ether (for removal of unreacted amine and ECH) and dried under vacuum.

N-glycidyl-N,N,N-triethylammonium chloride (GTEA): Yield, related to tertiary amine: 40%. Analysis, found (calculated), %: C 54.8 (55.8); H 10.4 (10.3); N 6.9 (7.3); total chlorine 18.2 (18.3), chloride ion 17.4 (18.3); epoxy group (EP) 0.34 (0.52). Active compound (calculated from EP content): 65.8%.

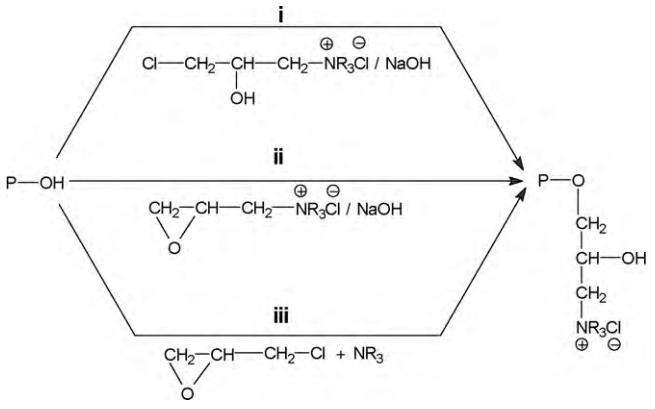
N-glycidyl-N,N-dimethyl-N-ethylammonium chloride (GDMEA): Yield: 45%. Analysis, found (calculated), %: C 49.8 (50.7); H 9.7 (9.6); N 8.7 (8.8); total chlorine 20.0 (21.4), chloride ion 19.5 (21.4); epoxy group (EP) 0.46 (0.6). Active compound: 75.5%.

N-glycidy-N,N-dimethyl-N-cetylammonium chloride (GDMCetA): Yield: 10%. Analysis, found (calculated), %: C 70.1 (69.7); H 12.5 (12.2); N 3.7 (3.9); total chlorine 8.7 (9.8), chloride ion 8.1 (9.8); epoxy group (EP) 0.17 (0.27). Active compound: 61.5%.

In the following, the reagent content in the quaternization mixtures was related to the active compounds. The reagents used in the present paper are included in Table 1.

2.3. Polysaccharide quaternization

Quaternization reactions were performed in water medium (ratio polysaccharide/deionized water = 1 g/10 ml), under constant stirring. Different quaternization reagents and catalysts, according to procedures (i)–(iii) (Scheme 1) were applied. The amounts of NaOH used in the procedures (i) (1.5 mol/AGU) and (ii) (0.5 mol/AGU) correspond to optimal conditions previously reported for polysaccharides quaternization by these procedures (Kavaliauskaite et al., 2008; Wang and Xie, 2010).



Scheme 1. Procedures for the synthesis of polysaccharides with N-(2-hydroxypropyl)-N,N,N-trialkylammonium chloride groups.

(i) The reagent was $CHPR^1R^2R^3A$ (according to Table 1) (as 50 wt% water solution). Molar ratios AGU:reagent (active compound):NaOH = 1:*a*:1.5*a* (here and in the following *a* refers to as the amine reagents).
(ii) The reagent was the glycidyl derivative of a tertiary amine ($GR^1R^2R^3A$). Molar ratios AGU:reagent (active compound):NaOH = 1:*a*:0.5.
(iii) The reagent was an equimolar mixture of epichlorohydrin and a tertiary amine (the molar ratio AGU:reagent mixture = 1:*a*). When an excess of tertiary amine was used as a catalyst, the molar ratios AGU:tertiary amine:ECH was 1:(*a* + 1):*a*.

Purification of quaternized products was performed as follows: crosslinked dextran gels were filtered and sequentially rinsed on the filter with water, 0.1N NaOH, 0.1N HCl, water and methanol. The final products were dried under vacuum. Aqueous solutions of linear polysaccharides were precipitated in mixtures methanol/acetone, then redissolved in deionized water and dialyzed against 0.1N HCl using tubings with MW cut-off of 12–14 kDa (Aldrich) then against water in an Amicon ultrafiltration cell (Millipore) fitted with an Ultracel-PL-membrane with a nominal molecular weight limit (NMWL) of 1 kDa, till the conductivity of the dialyzate was close to that of deionized water. After concentration in the same ultrafiltration cell, the solutions were freeze dried.

2.4. Characterization and analysis

2.4.1. Analysis of the reaction mixtures

The reaction mechanism was studied by analysis of two sets of reaction mixtures, A and B, consisting of deionized water (30 ml), DMBuA (5.62 g, 55.5 mmol) and ECH (5.13 g, 55.5 mmol), without (A) or with dextran (3 g, 18.5 meq AGU) (B). At given time intervals aliquots were withdrawn from both reaction mixtures, accurately weighed (0.4–0.5 g) and analyzed for chloride ion (Cl_i) and epoxy group content. The obtained values were related to the initial content of chlorine and epoxy groups corresponding to ECH amount in the reaction mixture. Aliquots of about 10 ml were also taken from the reaction mixture B, at similar time intervals, and precipitated in methanol/acetone. Precipitated polymer samples (products B_t, where *t* indicate the time interval at which the sample was withdrawn) were purified once more by the same procedure, and after drying under vacuum they were analyzed for the content in nitrogen, Cl_i, and total chlorine. After 24 h, the reaction mixture A was freeze dried, giving product A, and the quaternized polymer from reaction mixture B was purified by dialysis and recovered by freeze drying (product B). Both products (A and B) were analyzed by FT-IR and NMR.

2.4.2. Analysis methods

Elemental analysis (C, H, N) was carried out by means of a CHNS 2400 II Perkin Elmer analyzer. The total chlorine content was determined by Schoninger's method followed by potentiometric titration with $AgNO_3$, using an automatic TitraLab Radiometer 840. Chloride ions were quantified by potentiometric titration. Epoxy group content was determined using the procedure described by Jay (1964), by titration of exactly weighed aliquots from the reaction mixtures A or B (about 0.5 g) with perchloric acid, in the presence of tetrabutylammonium iodide, in acetic acid as solvent. The method was validated by titration of known amounts of ECH in the presence of water (0.1–0.5 ml) and dextran (0.2–0.3 g) amounts corresponding to their concentrations in the reaction mixture's aliquots.

Degree of substitutions determined from epoxy group analysis (DS_{Ep}) of the reaction mixtures A and B, and elemental analysis (N or Cli) (DS_{El}) of the products B_t, were calculated with the following

equations:

$$DS_{Ep} = a \cdot \{[Ep]_A - [Ep]_B\}, \quad \text{mol/100 AGU} \tag{1}$$

$$DS_{El} = \frac{162 \cdot El}{100 \cdot M_{El} - El \cdot M_S} 100, \quad \text{mol/100 AGU} \tag{2}$$

where a is the molar ratio ECH/AGU, $[Ep]_A$ and $[Ep]_B$ are the residual contents in epoxy groups, as % of the initial ECH content in the reaction mixture of experiment A and B, respectively; El is the element amount, in %, determined by elemental analysis (N) or potentiometric titration (Cl_i), M_{El} is elemental mass (14 for N, 35.5 for Cl_i), and M_S is the molecular weight of N-(2-hydroxypropyl)-N,N,N-trialkylammonium chloride group. There was a good agreement between DS determined from N and Cl_i content, therefore we used in the following only DS_{Cli}. The DS is expressed as mol% in order to avoid too many digits. For example, a DS value of 30 mol% corresponds to 0.3 value used in the conventional notation of polysaccharide substitution.

^{1}H NMR and ^{13}C NMR spectra were acquired on a Bruker Avance DRX 400 spectrometer in D_2O or DMSO-d6.

FT-IR spectra were recorded on a Bruker Vertex 70 spectrometer using KBr pellets.

2.5. Antimicrobial activity assessments

In vitro antimicrobial activity of cationic dextran derivatives obtained by procedure (iii) was assessed against three strains of bacteria (*Escherichia coli* ATCC 22923, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27835) and one fungal strain (*Candida albincas* 90028). Before testing, pure cultures were realized with all the strains in Mueller Hinton Agar. Sterile Petri dishes (d = 10 cm) were prepared with a base layer of Mueller Hinton Agar (Difco). Bacteria at density of 10^6–10^8 cfu were inoculated on solid agar, and the Petri dishes were incubated at 37 °C for 24 h. The inocula were prepared by adjusting the turbidity of the suspension to match the Mc Ferland standard 0.5 for bacteria and 2 for fungus. The diluted microorganism cultures on Petri dishes were divided in 4 zones and different amounts of polymer stock solution (concentration 200 μg/ml) were added to each zone, then incubated at 37 °C for 14 h. After this period, the results were visually assessed by inhibition area.

3. Results and discussion

Scheme 1 presents the main procedures reported for the synthesis of quaternary ammonium group containing polysaccharides. Procedures (i) and (ii) commonly employ CHPTAC and GTAC reagents, respectively, and the *DS*s depend on reaction conditions and type of polysaccharide, ranging form 0.1 to 1.5 (10–150 mol%). The use of a mixture tertiary amine/ECH (iii) for the surface quaternization of cellulose was first reported by Gruber and Ott (1996) who used two basic catalysts, 1-methyl-imidazol (MeIm) and 1,4-diazabicyclo [2,2,2]octan (DABCO), and mentioned the lack of reactivity of the mixture in the absence of these catalysts. The *DS*s obtained by this procedure were very low (0.1–0.12 mmol/g, or 1.5–2 mol%).

When we first attempt to apply the procedure (iii) for polysaccharide quaternization in homogeneous medium, we found that the tertiary amines proposed as catalysts react themselves, at significant levels, with polysaccharides, as ^{1}H NMR spectra taken for dextran modified with equimolar mixtures MeIm/ECH or DABCO/ECH indicated the presence of specific signals for quaternized MeIm (Fig. 1a) or DABCO (Fig. 1b). Consequently, the use of these "catalysts" together with another tertiary amine will result in a cationic polysaccharide with a mixed and uncontrolled chemical composition.

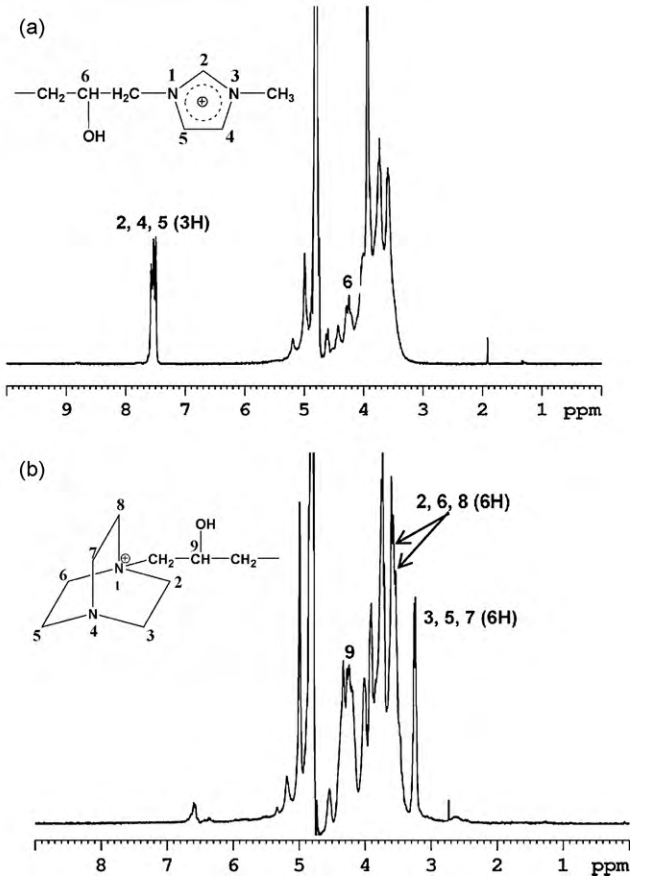


Fig. 1. ^{1}H NMR spectra (D_2O) of Dex-40 modified with mixtures ECH/N-methyl imidazol (a) and ECH/DABCO (b). Reaction conditions: 1 g polymer/10 ml water, molar ratio reagents/AGU = 3/1, 70 °C, 6 h.

We reinvestigated the potential of the quaternization pathway (iii) by using only one tertiary amine (the reagent) in the reaction mixture, together with ECH, and tried to establish the possible mechanism for polysaccharide substitution and to optimize reaction conditions in order to obtain a *DS* as high as possible. The main goal of the present study was to apply this procedure in the synthesis of cationic amphiphilic polysaccharides with a well controlled chemical structure. The polysaccharide of choice was dextran, and we used either water soluble (linear) polymer for the study of the reaction mechanism and confirmation of the chemical structure, or crosslinked polymers for the comparative studies and reaction parameters' investigation.

3.1. Mechanism of quaternization reaction by one pot procedure

In order to investigate the quaternization mechanism for procedure (iii), experiments A and B were performed as described in Section 2. Experiment A was performed in the absence of dextran and quantification of the chloride ions and epoxy groups at different time intervals (Fig. 2a) indicated that, after 30 min at 40 °C, 80% of the covalent chlorine (found in the initial amount of ECH) was converted to chloride ions, but epoxy groups remain unchanged (100% from initial), showing that the reaction between ECH and the tertiary amine (NR_3) leads mainly to the formation of the glycidyl derivative **3** (Scheme 2a). At longer periods (>30 min), the chloride ion content increases, but with a lower rate, and reaches 94% after 3 h. The occurrence of chloride ions in the reaction mixture is mainly the result of reaction (a), and not to the ECH hydrolysis, as an experiment performed under the same conditions but only

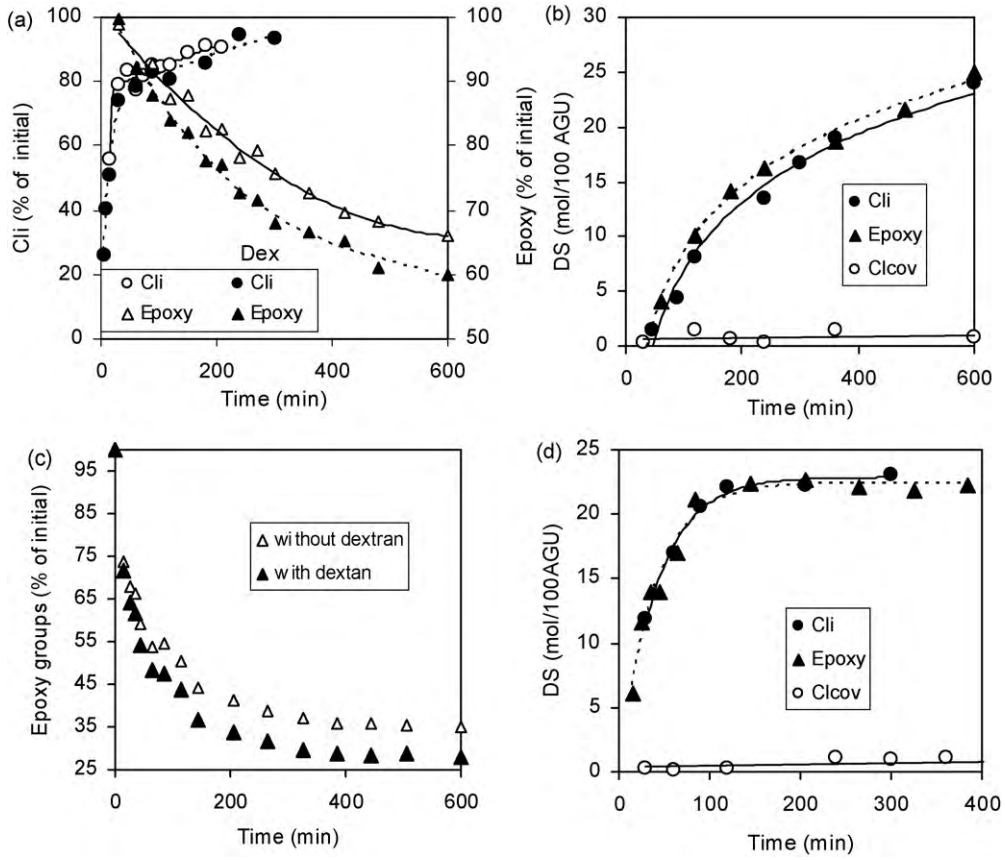


Fig. 2. Time dependence of chloride ions and epoxy group contents in the reaction mixtures ECH/DMBuA/water (a) and ECH/DMBuA/Dex-40/water (c); and (b and d) degree of substitution of Dex-40 with N-(2-hydroxypropyl)-N,N-dimethyl-N-butyl ammonium chloride groups as determined from epoxy group content in the reaction mixtures or chloride ions in the separated and purified polymer samples (B_t). Content in covalently bound chlorine is also shown. Reaction conditions: 1 g polymer/10 ml water, molar ratio reagents/AGU = 3/1, 40 °C (a and b) or 70 °C (c and d).

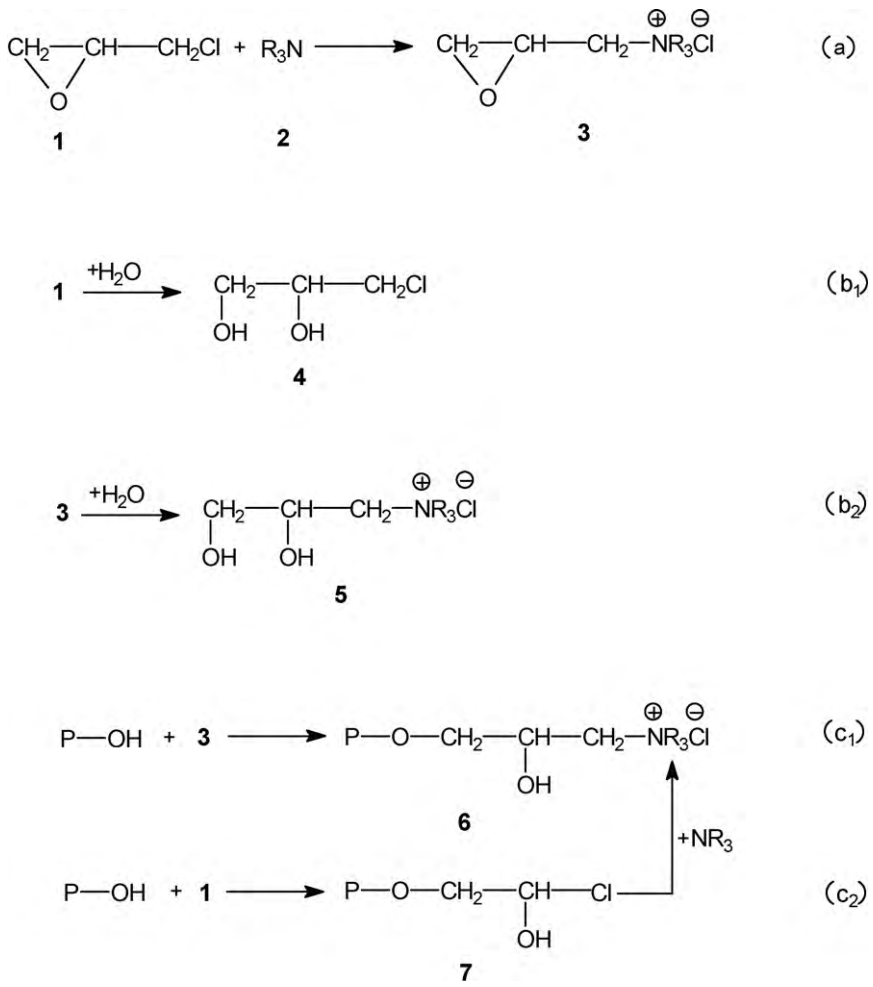
with ECH in the reaction medium did not reveal a significant conversion of chlorine to chloride ions, which was less than 2% after 24 h at 40 °C. In the same time, epoxy content starts to decrease slowly, perhaps by hydrolysis according to the reactions (b1) and (b2) (Scheme 2), with formation of inactive glycidol derivatives. The formation of glycidyl derivative **3** and its hydrolysis to glycidol derivative **5** is supported by the ^{1}H NMR spectrum of the product (A), obtained by freeze drying of reaction mixture A, after 24 h at 40 °C. The spectrum (Fig. 3) indicates the presence of both glycidyl and glycidol derivative specific peaks, and the content in glycidyl derivative **3**, calculated from the ratio of integrals for peaks assigned to CH_2 protons of the epoxy group (δ = 2.71 and 2.93 ppm) and those for butyl chain protons CH_3–CH_2–CH_2– (δ = 0.937, 1.306 and 1.68 ppm, respectively), was about 60%. This value fairly agrees with the epoxy group content (67%) determined directly from the reaction mixture A analysis, before freeze drying.

In the presence of dextran, the evolution of chloride ion content with time is very close to that found in the absence of dextran, indicating that the reaction (a) proceeds with a similar rate with formation of glycidyl derivative **3**. However, epoxy group content decreases faster in the presence of dextran, and we can assume that, besides reaction (b), reactions (c) take place, with final formation of polysaccharide derivative **6**. If we assume that the reactions (b) proceed with the same rate irrespective of the dextran presence, the difference between the epoxy group content in the presence and the absence of dextran should represent the degree of substitution in the product **6**. Therefore, we compared DS_{Ep} calculated from the difference in epoxy groups between experiments A and B (Eq. (1)) and DS_{Cli} determined from chloride analysis performed on products B_t (Eq. (2)). As Fig. 2b shows, the two *DS* values are very close, with DS_{Ep} exceeding DS_{Cli} by about 1–2 mol%, and this difference could arise from a lesser rate of **6** formation from **7**. This hypothesis was confirmed by the presence of covalent chlorine (1–2 mol%) in the quaternized samples B_t separated from the reaction mixture by precipitation. This chlorine disappeared at longer reaction times (24 h), indicating either its hydrolysis or the reaction with tertiary amine according to reaction (c2).

Similar experiments carried out at 70 °C (Fig. 2c and d) showed an identical evolution of chloride ion content (data not shown) (rapid formation of compound **3**), and a faster rate of both quaternization reactions (c) and secondary reactions (b). A *DS* = 20 mol% was obtained after 1.5 h at 70 °C and only after 8 h at 40 °C (Fig. 2b and d). After 10 h in the absence of the polysaccharide, epoxy group residual content was about 67% at 40 °C and 35% at 70 °C. It is worth observing the lack of a difference between DS_{Ep} and DS_{Cli} (Fig. 2d) meaning that the product **7** is not formed, or reaction (c2) goes to completion, and this is supported by lower content in covalent chlorine found in the polymer samples separated from the reaction mixture when the reaction was performed at 70 °C (<0.5 mol%, which falls into the experimental errors). The faster rate of secondary reactions (b) leads to a rapid leveling of the *DS*, which did not overcome 25 mol% after 24 h, comparing with 36 mol% in case of reaction performed at 40 °C.

3.2. Chemical structure of the quaternized products obtained by one pot synthesis

A sample obtained by modification of dextran D-40 with a mixture DMBuA/ECH, having a DS = 36 mol% (product B), was analyzed by FT-IR, ^{1}H NMR and ^{13}C NMR. FT-IR spectrum showed, besides the



Scheme 2. Mechanism of polysaccharide quaternization in the presence of equimolar mixtures epichlorohydrin/tertiary amine.

bands characteristic for a polysaccharide, a clear band at 1486 cm^{-1}, which was attributed to the methyl groups bound to quaternary nitrogen, and the band at 1420 cm^{-1} was referenced as the C–N stretching vibration (Pal, Sen, et al., 2008; Song et al., 2008).

The NMR spectra of the product B clearly indicate the presence of the signals characteristic to protons (Fig. 4b) and carbons (Fig. 4b) of both saccharide rings (Bajgai et al., 2009; van Dijk-Wolthuis, Kettenes-van den Bosch, van der Kerk-van Hoof, & Hennink, 1997) and quaternary ammonium groups (Haack et al., 2002; Song et al., 2008), a proof for chemical structure depicted in Fig. 4 and reaction mechanism given in Scheme 2. The ^{13}C NMR spectrum highlights the presence of two new signals for saccharide ring carbons (not found for unsubstituted dextran), at 96.3 ppm, assigned to the C^1 adjacent to a substituted C^2 (1′S), and at 80 ppm for substituted C^2 (2S) (Heinze, Haack, & Rensing, 2004; van Dijk-Wolthuis et al., 1997). No clear evidence for signals attributed to substituted C^3 or C^4 could be found, suggesting that, at the moderate *DS* of that sample, the substitution takes place preferentially at C^2–OH. For much higher *DS* (about 90 mol%), the intensities of the signals corresponding to 1′S and 2S (inset to Fig. 4b) are similar, indicating that only about a half of ammonium groups are bound to C^2, the other being, most probably, located at C^3 of dextran saccharide ring.

The ratio of the integrals of the signals assigned to CH_3–$(CH_2)_3$– (0.923 ppm) and anomeric glucopyranosidic protons (4.68 ppm) (Fig. 4a) gives a *DS* of 38 mol%, close to the value determined from elemental (N) and potentiometric (Cl_i) analysis (36 mol%).

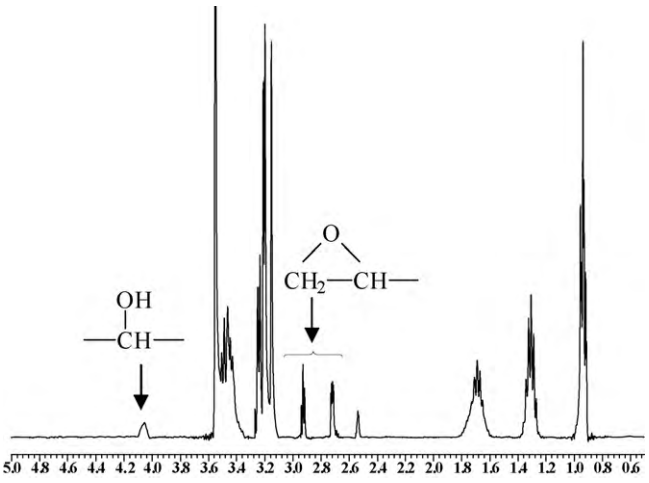


Fig. 3. ^1H NMR spectrum (DMSO-d6) of reaction mixture ECH/DMBuA/water freeze dried after 24 h at 40 °C (product A).

3.3. Comparison of quaternization procedures

It is well established that the modification of polysaccharides with CHPTAC in the presence of NaOH (procedure i) takes place actually via GTAC, which is formed *in situ* from CHPTAC and NaOH (Heinze et al., 2004; Kavaliauskaite et al., 2008). We have already shown that the glycidyl derivatives are the reagents in the procedure (iii) too. The comparative efficiency of the three procedures

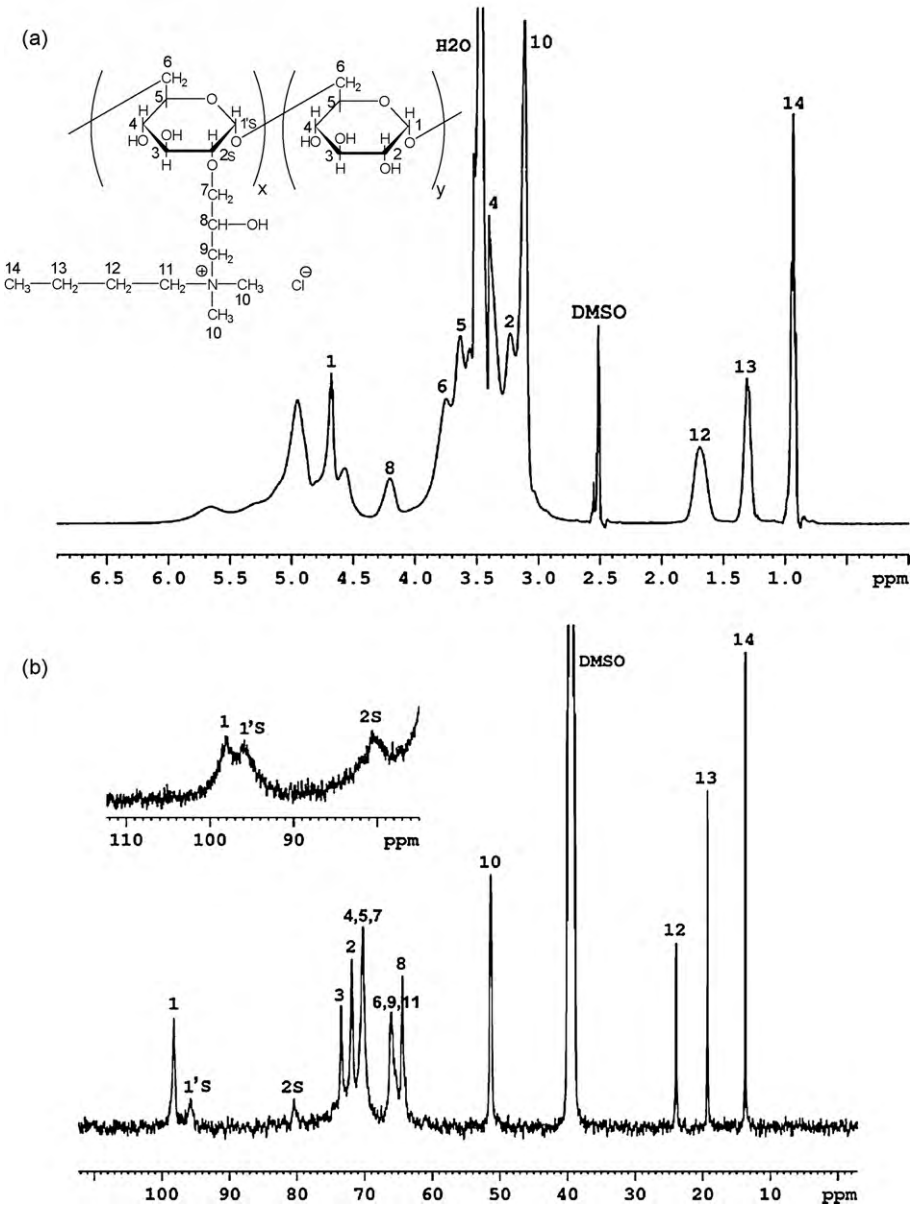


Fig. 4. NMR spectra (DMSO-d6) of Dex-40 with N-(2-hydroxypropyl)-N,N-dimethyl-N-butyl ammonium chloride groups. DS = 36 mol%. Inset to (b) DS = 90 mol%.

is illustrated in Fig. 5, using as starting reagents triethylamine (TEA)/ECH mixture and clorohydroxypropyl or glycidyl derivatives of TEA. In all cases, the *DS* increases with increase in the ratio *a*/AGU, but the efficiency decreases in the order: (ii) > (iii) > (i). The higher efficiency of GTAC versus CHPTAC was previously shown for the starch quaternization (Bendoraitiene, Kavaliauskaite, Klimaviciute, & Zemaitaitis, 2006; Haack et al., 2002), and was explained by the competition between the main quaternization reaction and the CHPTAC enhanced inactivation due to the higher content in NaOH required for the procedure (i). The better performances of the procedure (iii) in comparison with (i) can be a supplementary proof of glycidyl derivative formation *in situ*, in the absence of NaOH.

The quaternization efficiency proved to be better for (ii) procedure only for tertiary amines with short substituents (mainly CH_3). When amines with longer alkyl substituents were used (for instance TEA of DMCetA), the better results obtained by procedure (iii) are obvious (Fig. 5b). The explanation could be either the contribution of reaction (c2) (Scheme 2) to the final substitution efficiency or a better reagent compatibility/solubilization of the step-wise formed glycidyl derivatives of more hydrophobic tertiary amines.

3.4. Factors influencing the efficiency of quaternization reaction

Several factors are responsible for quaternization efficiency by procedure (iii): temperature, catalyst, solvent, hydrophobicity or bulkyness of the amine substituents, and polysaccharide support characteristics.

The influence of the temperature on the reactions (c) efficiency was presented above. Higher reaction temperatures decrease the reaction time but diminish the final *DS*, therefore the proper choice of experimental temperature will depend on the desired cationic polymer characteristics and reagent properties (tertiary amine boiling point, polysaccharide thermal resistance).

In the absence of any catalysts and at equimolar ratio tertiary amine/ECH, satisfactory *DS* were obtained by procedure iii, comparable with those of the other procedures. The presence of NaOH in

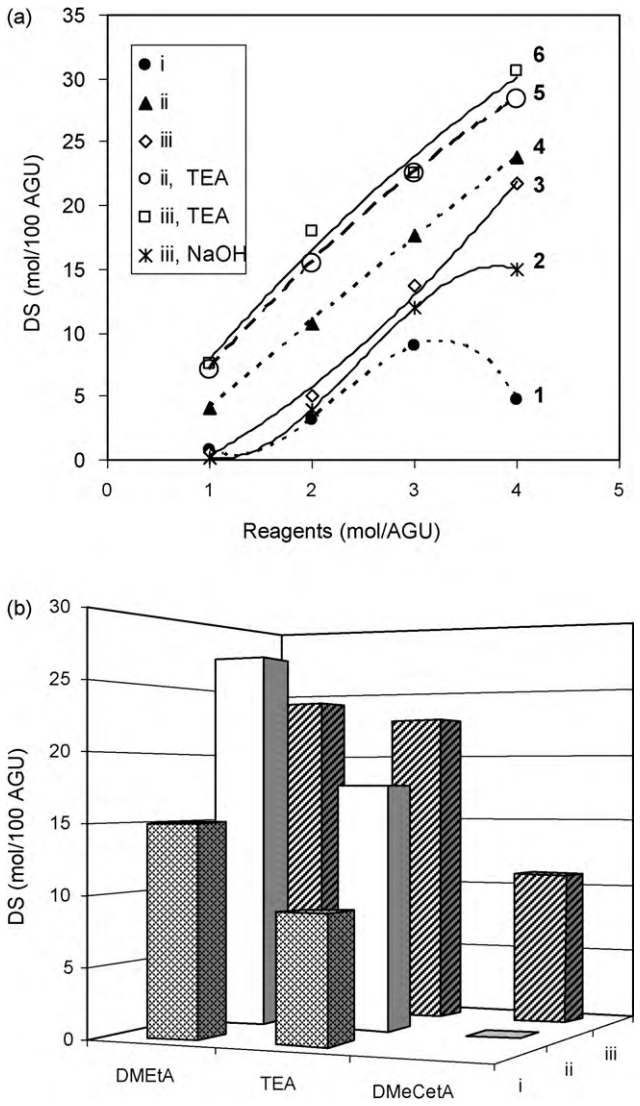


Fig. 5. Comparison of the efficacy of quaternization procedures, for the quaternization of crosslinked dextran (CDex-25) as a function of (a) molar ratio reagents/AGU and catalyst; (b) the length of amine substituents (molar ratio reagents/AGU = 3/1). Reaction conditions: 70 °C, 6 h, 1 g polymer/10 ml water. (iii) NaOH (or TEA) indicates that the reaction was performed with 1 mol catalyst/AGU, where the catalyst was NaOH (or TEA). *DS* was calculated with Eq. (2).

the reaction mixture (1 mol/AGU) did reduce the reaction efficiency (Fig. 5a, curve 2). However, an excess of the tertiary amine in the reaction mixture (molar ratio amine/ECH = $(a+1)/a$), significantly improves the reaction efficiency (Fig. 5a, curve 6). A similar behavior was noticed for the procedure (ii) when instead NaOH, TEA was used (curve 5 in Fig. 4). This suggests that the tertiary amine play the role of the catalyst in the reaction (c1).

The best solvent for the polysaccharide quaternization by procedure (iii) was water. Very low *DS*s were obtained when other solvents (DMSO, N-methylformamide, DMF) or their mixtures with water were used. The main reason for this solvent specificity might be the low rate of the reaction (a) in these solvents, as compared with water.

The length of the alkyl substituents at the nitrogen of tertiary amine used as a reagent also influences *DS*, as Fig. 6a shows. The reaction efficiency decreases with increasing R^3 length when tertiary amines DMR^3A were used as reagents. Introduction of two or three longer alkyl substituents determines a further decrease in *DS*, clearly due to the steric hindrance in the reactions (a) and (c) (Scheme 2) completion. Actually, experiments performed with mixtures of MDBuA or MDOctA and ECH in water solution, in the absence of polysaccharide, have shown that, after 6 h at 70 °C, large amounts of unreacted amines (1/4 of MDBuA and 1/2 of MDOctA used in experiments) were separated from the reaction mixtures. The maximum *DS*, i.e. the highest possible *DS* for each tertiary amine used as reagent, is indicated in Table 1 (last column). The highest *DS*s given for more hydrophobic amine reagents are leveled by the requirement of water solubility (or swellability) preservation for quaternized polysaccharides.

The reactivity of cyclic amines MeIm and DABCO was similar to acyclic amines with shorter alkyl substituents (DMBuA). It is worth mentioning that the quaternized polysaccharide obtained in the presence of DABCO as reagent (*DS* = 40 mol%) was water soluble, showing that only one tertiary nitrogen is involved in the preparation of glycidyl derivative **3** (Scheme 2) what was supported by ^{1}H NMR spectrum (Fig. 1b) where the protons of both CH_2 groups bound to tertiary nitrogen (δ = 3.25 ppm) and quaternary nitrogen (δ = 3.4–3.6 ppm) are present.

Fig. 6b illustrates the influence of the polysaccharide support properties on *DS*. The *DS* of a linear dextran was higher than that of crosslinked dextran, and a higher crosslinking degree (or a lower water retention capacity) led to a lower *DS*. This effect is obviously the result of a reduced diffusion of reagents inside the polysaccharide gels, which decreases with increasing crosslinking degree. As for the polysaccharide type, the quaternization efficacy was higher for pullulan than dextran (both as linear polymers), what is clearly due to the higher reactivity of the primary OH groups present in the pullulan structure. Molecular mass of the linear polysaccharide had no significant influence on the *DS* (data not shown).

3.5. Potential applications of amphiphilic quaternary ammonium group containing polysaccharides

Using procedure (iii), we have prepared a large variety of cationic hydrophilic or amphiphilic polysaccharides, with a well controlled and reproducible chemical structure, and their amphiphilicity could be easily varied by changing the length of a single substituent at nitrogen (R^3, according to Table 1). Therefore, we synthesized cationic amphiphilic dextran derivatives (crosslinked or as water soluble products) with different R^3 substituents at nitrogen ($R^3 = C_2$, C_4, C_8, C_{12}, C_{16}) and studied their properties and potential for different applications. The chemical composition of the dextran derivatives discussed in the following is indicated by the tertiary amine used for their preparation (according to Table 1).

Cationic amphiphilic derivatives of crosslinked dextran were tested *in vitro* for the sorption of sodium salts of bile acids (Nichifor, Zhu, Baille, Cristea, & Carpov, 2001; Nichifor, Zhu, Cristea, & Carpov, 2001), in the attempt to find the best chemical structure for their use as bile acid sequestrants, a well known class of anticholesterolemic drugs. The interaction of the steroid nucleus with one amphiphilic gel (DMDodA) was stronger in comparison with the gels having aromatic rings either as a part of polymer backbone (Cholestyramine®, a classic anticholesteremic drug) or as a substituent of the functional group (DMBzA). No improvement was noticed in the bile acid affinity for gels obtained from cyclic amines (MeIm or DABCO). The better performances of the amphiphilic cationic dextran gel (DMDodA) by comparison with Cholestyramine were latter confirmed by *in vivo* experiments performed on normolipemic rats, indicating such gels as promising hipolipemic drugs, with better therapeutic activity and better tolerance than Cholestyramine (Trinca, Nichifor, Volf, Ivas, & Stanciu, 2007).

The ability of water soluble cationic amphiphilic dextrans to self-aggregate in water solutions with formation of intra- or inter-molecular hydrophobic microdomains was demonstrated

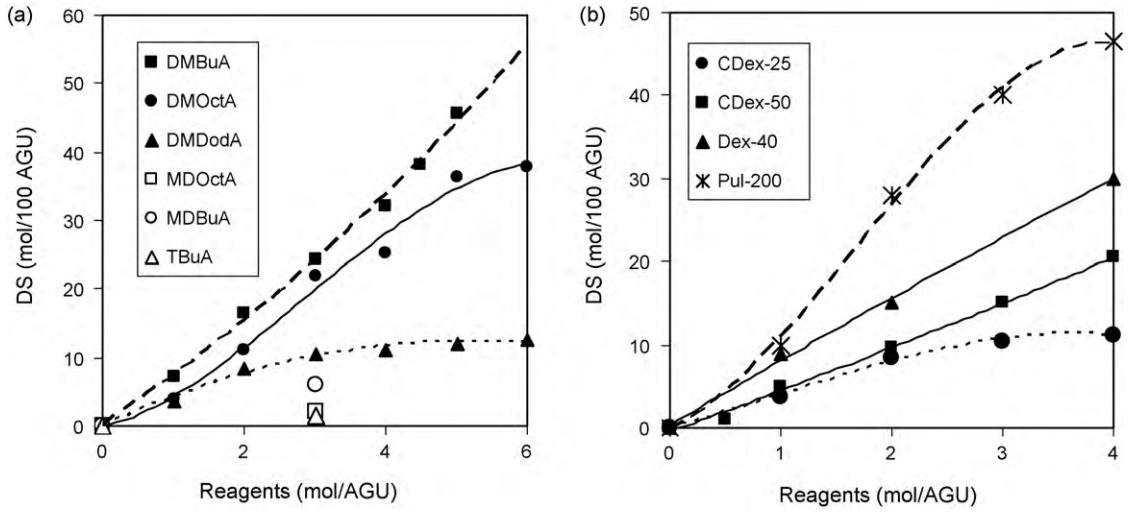


Fig. 6. Quaternization efficacy performed with procedure (iii) as a function of the tertiary amine substituent length (for polymer = CDex-25) (a); and polysaccharide support (for DMDodA) (b). Reaction conditions 1 g polymer/10 ml water, 70 °C, 6 h. *DS* was calculated with Eq. (2).

by an extensive fluorescence study (Nichifor, Lopes, Bastos, & Lopes, 2004), and by isothermal titration calorimetry (Bai, Nichifor, Lopes, & Bastos, 2005b). These amphiphilic polymers interact with surfactant of opposite charge (Bai, Santos, Nichifor, Lopes, & Bastos, 2004; Bai, Nichifor, Lopes, & Bastos, 2005a; Nichifor, Lopes, Bastos, & Lopes, 2008) or of the same charge (Bai, Catita, Nichifor, & Bastos, 2007) with formation of mixed aggregates polymer-surfactant. Both polymer self-aggregates and mixed polymer-surfactant aggregates are able to solubilize hydrophobic compounds and, therefore, can be applied as hydrophobic drug delivery systems for oral or systemic administration.

Water soluble cationic dextran derivatives, both hydrophilic and amphiphilic, proved themselves as good flocculants for clay suspensions (Ghimici, Morariu, & Nichifor, 2009). The optimum flocculation dose and the width of the flocculation window were strongly dependent on *DS* and length of substituent R^3.

Another potential application of cationic amphiphilic polysaccharides is as antibacterial agents. Quaternary ammonium compounds are well known antibacterial agents used for disinfection, and antibacterial properties of several quaternary ammonium group containing polysaccharides have been reported (Sajomsang, Gonil, et al., 2009; Sajomsang, Tantayanon, et al., 2009; Yu et al., 2007). A first trial performed on *E. coli* showed that only polymers DMBzA and DMOctA were active, meaning that only polymers carrying groups with a medium length of substituent R^3 (benzyl and octyl) are able to interact with the bacteria cell membrane. All other polymers having short (ethyl, butyl) and long (dodecyl, cetyl) alkyl substituents at quaternary ammonium groups did not display any antibacterial activity (Table 2). The DMBzA and DMOctA polymers were further tested for their activity against other bacteria (*P. aeruginosa* and *S. aureus*) and a fungus (*C. albicans*). No activity against fungus was found, but the tested polymers were active against bacteria strains studied, and inhibition activity increased in the order: DMBzA 30 < DMOctA 30 < DMOctA 70 (Fig. 7), suggest-

Table 2
Antibacterial and antifungal properties of quaternary ammonium group containing dextran.

Polymer[a]	Microorganism[b]			
	E. coli	*S. aureus*	*P. aeuruginosa*	*C. albicans*
DMEA 30	–	n.d.	n.d.	n.d.
DMEA 70	–	n.d.	n.d.	n.d.
DMBuA 30	–	n.d.	n.d.	n.d.
DMBuA 70	–	n.d.	n.d.	n.d.
DMBzA 30	+	+	+	–
DMOctA 30	+	+	++	–
DMOctA 70	++	++	++	–
DMDodA 30	–	n.d.	n.d.	n.d.
DMCetA 30	–	n.d.	n.d.	n.d.

[a] Polymers were identified after the tertiary amine used in the synthesis (according to Table 1) and the *DS*, as mol quaternary ammonium group/100 AGU.
[b] '–' no inhibition; '+' inhibition; '++' strong inhibition; 'n. d.' not determined.

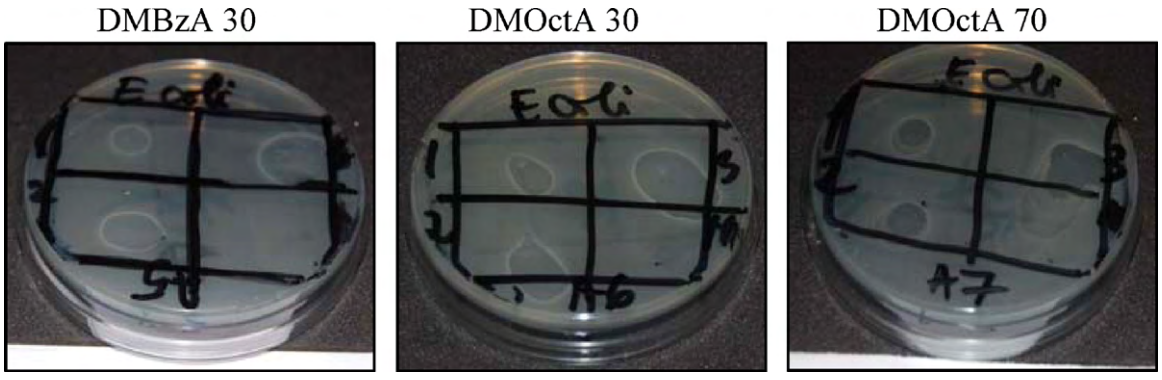


Fig. 7. Images of the *E. coli* plates incubated with cationic dextran derivatives (polymer names as in Table 2). The amount of polymer stock solution was 10 μl (zone 1); 20 μl (zone 2); μl (zone 3); 0 (zone 4).

ing a better performance of polymers carrying an octyl substituent and an increase in activity with increasing DS. A study focused on elucidation of the reason for different activity decided by only one substituent at quaternary ammonium groups and also on quantification of this activity (minimum inhibitory concentration-MIC determination) is in progress.

4. Conclusion

A new versatile procedure for synthesis of quaternary ammonium group containing polysaccharides was described. Instead of preformed quaternary ammonium group containing reagents, the procedure uses a mixture of a tertiary amine and epichlohydrin which give rise, *in situ*, to the quaternary ammonium reagents. This procedure avoids the difficult and expensive synthesis of quaternary ammonium reagents and allows the obtaining of a large number of polysaccharide derivatives with different properties and various applications, simply by changing the tertiary amine used in reaction. The method can be applied to the quaternization of any neutral polysaccharide, in linear or crosslinked form, and lead to the formation of well controlled chemical structures, with pendant quaternary ammonium groups statistically distributed along the polysaccharide chain.

The procedure allowed the preparation of polysaccharides with different hydrophilic–lipophilic balance by simply changing the length of one substituent at the tertiary amine used as a reagent. These new cationic polysaccharides have self-assembling properties and potential application as hipolipemic drugs, flocculants, drug delivery systems or antibacterial agents.

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