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Two competing reactions of tetrabutylammonium alginate in organic solvents: Amidation versus γ-lactone synthesis

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ABSTRACT

Biocompatibility and thickening properties predetermine alginates as ingredients in food, cosmetic and pharmaceutical products. Further chemical modifications are often desired for a product optimization. The introduction of hydrophobic groups can be realized by employing organic tetrabutylammonium alginate (TBA-Alg) solutions. The synthesis of alginic acid alkyl amides from TBA-Alg with 2-chloro-1-methylpyridinium iodide (CMPI) as a coupling agent, however, has so far not resulted in a high degree of amidation. The analysis of the coupling reaction revealed the formation of mannuronic acid γ-lactone structures, which required a conformation change from $^1C_4$ to $^4C_1$. The opening of the γ-lactone required a high excess of butylamine. In the case of CMPI, triethylamine had to be added prior to the coupling agent in order to suppress the assumed alginic acid formation. The degrees of amidation achieved were up to 0.8, and for propylphosphonic anhydride as the coupling agent up to 1. The molecular weights of the alginic acid butyl amide were ≥35 kDa.

1. Introduction

Alginates are unbranched polysaccharides made of (1 → 4) linked β-D-mannuronic acid (M) and its C5 epimer α-L-guluronic acid (G) blocks. Excellent gelling properties and biocompatibility resulted in numerous applications in the food and pharmaceutical industry, such as thickening or gelling agents (Saha & Bhattacharya, 2010), wound dressing materials (Schultz et al., 2003) and drug delivery systems (Anal & Singh, 2007; Tonnesen & Karlsen, 2002). Chemically modified materials are often of special interest for further product optimization, especially for pharmaceutical applications. The chemical modification of alginates (Alg) is mostly achieved under aqueous conditions by exploiting the excellent water solubility of sodium alginate (Na-Alg) (Pawar & Edgar, 2012; Yang, Xie, & He, 2011). This approach is excellent for introducing hydrophilic groups, but has disadvantages for introducing hydrophobic ones. This obstacle can be resolved by converting Na-Alg into tetrabutylammonium alginate (TBA-Alg), which is soluble in polar aprotic organic solvents (Schleeh, Madau, & Roessner, 2014). Recently, the esterification of TBA-Alg in organic solvents with alkyl halides was reported to be efficient in terms of yield and degree of substitution (Della Valle & Aurelio, 1992; Pawar & Edgar, 2013; Pelletier, Hubert, Lapicque, Payan, & Dellacherie, 2000). In contrast, the amidation of TBA-Alg in organic solvents did not always lead to satisfying products, especially for the attachment of longer alkylamines such as dodecylamine (Barbucci, Consumi, & Magnani, 2002; Barbucci et al., 2006; Leone, Torricelli, Chiumiento, Facchini, & Barbucci, 2008; Vallée et al., 2009).

In comparison to the esterification, the amidation required the activation of TBA-Alg with the coupling agent 2-chloro-1-methylpyridinium iodide (CMPI) in a first step, followed by the amidation in the presence of the hydrogen scavenger triethylamine. The drawback of this reaction is that an intra- and intermolecular ester formation has to be taken into account, due to the reaction of CMPI activated alginate with itself (Saigo, Usui, Kikuchi, Shimada, & Mukaiyama, 1977; Vallée et al., 2009). The intramolecular esterification would result in lactone structures, which were already synthesized from sodium alginate under dehydrating conditions (Anson, Novikova, & Iozep, 2009). The intermolecular esterification would lead to a three dimensional network formed by the crosslinking reaction between carboxylic acids and hydroxyl groups of different alginate moieties. These interesting hypotheses led to the question of whether a better understanding of these side reactions could be exploited for the development of a more efficient amidation of alginates and for the evaluation of the coupling agent performance. Therefore, the reactions of TBA-Alg with CMPI and propylphosphonic anhydride (T3P) (Nagendra, Madhu, Vishwanatha, & Sureshbabu, 2012; Wissmann & Kleiner, 1980) as

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coupling agents were first studied in the absence of amines. Then, the amidation of TBA-Alg with n-butylamine and CMPI or T3P as coupling agent was investigated and optimized by considering the findings from the reactions of the TBA-Alg with the coupling agents.

2. Materials and methods

2.1. Chemicals

H-Alg from brown algae was purchased from Sigma (St. Louis, MO, USA; Na-Alg residue: 11% and M/G ratio: between 0.61/0.39 and 0.54/0.46 (Schleeh et al., 2014). The Na-Algs Manucol DM (M/G ratio: 0.61/0.39) and Protanal GP 1740 (M/G ratio: 0.37/0.63) from FMC BioPolymer were received as a gift from IMCD Benelux N.V. (Mechelen, Belgium). Potassium bromide (KBr) and T3P in DMF (c(T3P): ~0.586 mol/L) were obtained from ACROS (Geel, Belgium), ~40% tetrabutylammonium hydroxide (TBAOH) solution from Fluka (Buchs, Switzerland), CMPI, butylamine, dimethylformamide (DMF; water ≤0.005%), sodium chloride (NaCl) and triethylamine from Sigma–Aldrich, acetone, DMF (water ≤0.01%), ethanol (99%), hydrochloric acid (HCl; 1 M), ethyl acetate (water < 0.1%) and formic acid from VWR (Radnor, PA, USA), sodium hydroxide solution (0.1 N, Titrisol) from Merck (Darmstadt, Germany), ethylenediaminetetraacetic acid (EDTA) standard from the Leco Corporation (St. Joseph, MI, USA) and nitrogen (α1) from Air Liquide (Paris, France). Milli-Q water from Merck was used in general. All materials were used without any further purification.

2.2. Synthesis

In general, commercially available H-Alg was used for the investigation of the reaction of TBA-Alg and the coupling agent. Changing the M/G ratio required the synthesis of TBA-Alg from specific Na-Algs (Manucol and Protanol), which was done according to the recently reported procedure (Schleeh et al., 2014). The amidation of TBA-Alg was studied on highly DMF soluble TBA-Alg, which was also prepared according to the same procedure.

2.2.1. H-Alg synthesis in brief

Na-Alg (4 g, 20 mmol) was dispersed in aqueous formic acid (200 mL, water content 30% (v)) at 4 °C. The dispersion was stirred at 4 °C for 1 h before the ice bath was removed. The reaction mixture was then allowed to warm up by stirring for 6 h or overnight. The product was filtrated, washed with aqueous ethanol (3 × 100 mL, ethanol/water: 70% (v)/30% (v)) and then with acetone (100 mL), dried under vacuum and kept in a desiccator over a drying agent until further use. FTIR (KBr pellet): υ(OH) 3900–3064 cm^{-1}, υ(CH) 3064–2787 cm^{-1}, (sat COOH) 1744 cm^{-1}, $\upsilon_{as}(COO^-)$ 1636 cm^{-1}, $\upsilon_s(COO^-)$ 1412 cm^{-1}, (C—O) 1323 cm^{-1}, (C—O—C) 1180 cm^{-1} and 1101 cm^{-1}, (C—OH) 1032 cm^{-1}. Elemental Analysis: C: 41.00%; H: 4.92%. Degree of acidification (DS_H): 0.94 (Titration with 0.1 N NaOH). Yield: 2.91 g (93.8%).

2.2.2. TBA-Alg synthesis

H-Alg (1.12 g, 6.4 mmol) was dispersed in 100 mL Milli-Q water, followed by a pH adjustment to between 7 and 9 with a TBAOH solution (approximately 0.5 M). The solution was then immediately frozen to avoid any carbon dioxide absorption, lyophilized and kept in a desiccator over a drying agent until further use. FTIR (casted film): υ(OH) 3685–3041 cm^{-1} (with maxima at: 3501 cm^{-1}, 3383 cm^{-1} and 3200 cm^{-1}), υ(CH) 2961 cm^{-1}, 2937 cm^{-1} and 2879 cm^{-1}, $\upsilon_{as}(COO^-)$ 1611 cm^{-1}, $\delta(CH_2$ and $CH_3)$ 1485 cm^{-1} and 1468 cm^{-1}, $\delta_s(CH_3)$ 1385, (C—O) 1317 cm^{-1} and 1285 cm^{-1}, (C—O—C) 1173 cm^{-1}, 1148 cm^{-1} and 1101 cm^{-1} and (C—OH) 1036 cm^{-1}. Elemental analysis: C 61.92%, H 10.35%, N 3.27%. Degree of substitution with TBA (DS_{TBA}) 0.98. Yield: 2.39 g (91.3%).

2.2.3. Ester synthesis (side product)

The studied parameters of this reaction were the coupling agent (none, CMPI and T3P), the TBA-Alg concentration (0.5 wt.-% to 10 wt.-% for CMPI, to 9 wt.-% for T3P), the ratio of TBA-Alg:coupling agent (1:0.25 to 1:2 for CMPI and 1:0.25 to 1:10 for T3P) and the reaction duration (0.5 h to 24 h, ambient temperature). For the reaction with CMPI, TBA-Alg (6.4 mmol) and coupling agent (6.4 mmol) were separately dissolved in DMF (TBA-Alg: 220 mL; coupling agent: 50 mL) at ambient temperature. The TBA-Alg solution was cooled down to 0 °C before the coupling agent solution was added drop-by-drop under stirring. The reaction mixture was kept at 0 °C for 1 h before the ice bath was removed. Then, the reaction mixture was allowed to warm up to ambient temperature under continuous agitation overnight. The products were isolated either by hydrolysis of the remaining activated alginate moieties and replacing the remaining TBA^+ by Na^+ ions (procedure (b)) or without (procedure (a)). *Procedure (a)*: The reaction mixture was precipitated directly in heavily stirred absolute ethyl acetate and then allowed to settle for 30 min. The product was then filtrated, purified in a soxhlet with acetone and vacuum dried. All samples were kept in a desiccator until further use. *Procedure (b)*: A 2.5 M NaCl solution (80 mL) was added to the reaction mixture drop-by-drop. Then, the product was precipitated in heavily stirred aqueous ethanol (1100 mL, ethanol/water (7/1)), followed by a period without agitation of 30 min, in which the polymer was allowed to settle down. The product was filtrated, purified by a soxhlet extraction with acetone and finally vacuum dried. A few selected samples were additionally purified to remove the coupling agent and solvent traces. This was done in a stirred cell equipped with a membrane (molecular cut-off: 10 kDa) with Milli-Q water (at least 10 times the stirred cell volume). These samples were finally freeze-dried and kept in the desiccator, as with all other samples.

In case where the coupling agent was absent (negative control), TBA-Alg was directly dissolved in the complete amount of DMF (270 mL). Working with T3P as a coupling agent occasionally required the solvent volume for dissolving TBA-Alg to be adjusted to keep the desired TBA-Alg concentration, because T3P was delivered as a DMF solution.

2.2.3.1. Negative control. FTIR (KBr pellet)): υ(OH) 3717–3079 cm^{-1}, υ(CH) 2978–2819 cm^{-1}, (sat COOH) 1738 cm^{-1}, $\upsilon_{as}(COO^-)$ 1614 cm^{-1}, $\upsilon_s(COO^-)$ 1416 cm^{-1}, (C—O) 1258 cm^{-1}, (C—O—C) 1094 cm^{-1}, (C—OH) 1026 cm^{-1}. Elemental Analysis: C 32.02%, H 4.43%. Yield: 1.4 g.

2.2.3.2. Ester synthesis with procedure (b) and CMPI as a coupling agent. FTIR (KBr pellet): υ(OH) 3726–3070 cm^{-1}, υ(CH) 2991–2828 cm^{-1}, (γ-lactone) 1780 cm^{-1}, (sat COOH) 1742 cm^{-1}, $\upsilon_{as}(COO^-)$ 1618 cm^{-1}, $\upsilon_s(COO^-)$ 1418 cm^{-1}, (C—O) 1248 and 1207 cm^{-1}, (C—O—C) 1096 cm^{-1}, (C—OH) 1036 cm^{-1}. Elemental Analysis: This could not be meaningfully performed due to limited purity. Yield: 1.07 g.

2.2.3.3. T3P as a coupling agent. FTIR (KBr pellet): υ(OH) 3717–3070 cm^{-1}, υ(CH) 2988–2840 cm^{-1}, (γ-lactone) 1782 cm^{-1}, (sat COOH and sat. COOR) 1742 cm^{-1}, $\upsilon_{as}(COO^-)$ 1622 cm^{-1}, $\upsilon_s(COO^-)$ 1416 cm^{-1}, (C—O) 1246 and 1205 cm^{-1}, (C—O—C) 1092 cm^{-1}, (C—OH) 1030 cm^{-1}. Elemental Analysis: This could not be meaningfully performed due to limited purity. Yield: 0.97 g.

2.2.4. Amidation of TBA-Alg in organic polar aprotic solvents

A three-neck flask was purged with nitrogen for 15 min. TBA-Alg (7.2 mmol) was then placed in this flask, dissolved in DMF

(45 mL for the reaction with CMPI or 55 mL for the reaction with T3P) and cooled down to 0° C. The dissolved coupling agent (CMPI: 7.2 mmol in 11 mL DMF or T3P: 14.4 mmol in 4.8 mL DMF) was added drop-by-drop under agitation and left to react by stirring gently at this temperature for 1 h. Butylamine (CMPI: 14.4 mmol or T3P: 72 mmol) and triethylamine (7.2 mmol) were added with a micropipette immediately one after another. The reaction mixture was stirred again gently at 0 °C for 1 h, before the ice bath was removed. The reaction mixture was agitated continuously for 20 h to warm up to ambient temperature. Then next day, a sodium ion rich phosphate buffer solution (NaCl, Na_2HPO_4, NaH_2PO_4, $c(Na^+) \geq 2.5$ M, $c(PO_4^{2-}) = 0.5$ M, pH: 7.5; 29 mL) was slowly added. The polymer was precipitated in heavily stirred cold aqueous ethanol (280 mL, 70% ethanol), followed by a centrifugation step (g-force: 3488 ms^{-2}, 5 min, 5 °C). The solvent fractions were collected and unified and the solvent was removed. The remainder was transferred into acetone (500 mL) to precipitate the polymer, centrifuged, washed with acetone four times and dried under vacuum. The dry sample was dispersed in a sodium phosphate buffer (Na_2HPO_4, NaH_2PO_4, $c(PO_4^{3-}) = 0.1$ M, pH: 7–8) and let to dissolve overnight. The solution was then transferred to a stirred cell equipped with a membrane with a 10 kDa molecular cut-off and washed with Milli-Q water (at least 10 times the stirred cell volume). Finally, the product was lyophilized and kept in a desiccator until further use.

2.2.4.1. Alginic acid butyl amide (AlgNC4 synthesized with CMPI as coupling agent. ^{1}H NMR (D_2O): δ 5.66–4.34 ppm (H1-5), δ 3.84 ppm (H7), δ 1.96 ppm (H8), δ 1.93 ppm (H9), δ 1.51 ppm (H10), Degree of substitution with amide groups (DS_{amide}): 0.43. ^{13}C NMR (D_2O): δ 175.56 ppm (carboxylate C6), δ 170.38 ppm (amide C6), δ 100.7 ppm (C1), δ 80.71–66.97 ppm (C2–C5), δ 39.91 ppm (C7), δ 31.16 ppm (C8), δ 20.13 (C9), δ 13.60 ppm (C10). FTIR (KBr pellet): υ(OH) 3710–3008 cm^{-1}, υ(—CO—NH—) shoulder at 3110 cm^{-1} (2nd derivative 3095 cm^{-1}), υ(CH (of CH_2 and CH_3)) 2961 cm^{-1} and 2876 cm^{-1}, υ(CH (of Alg)) 2930 cm^{-1}, (—CO—NH—) 1657 cm^{-1} and 1558 cm^{-1}, $\upsilon_{as}(COO^-)$ 1616 cm^{-1}, δ(—CH_2—) 2nd derivative at 1466 cm^{-1}, δ_s(—CH_3—) 1377 cm^{-1}, (C—O—C) 1092 cm^{-1}. Elemental Analysis: C 40.98%, H 5.52%, N 2.56%, DS_{amide}: 0.41. Yield: 1.38 g (88.6%).

2.2.4.2. AlgNC4 synthesized with T3P as coupling agent: ^{1}*H NMR (DMSO-d6).* δ 4.86–3.35 ppm (H1-5), δ 3.11 ppm (NH and H7), δ 2.5 ppm (OH), δ 1.45 ppm (H8), δ 1.31 ppm (H9), δ 0.90 ppm (H10), DS_{amide}: 0.98. ^{13}C NMR (DMSO-d6): δ 168.12 ppm and 167.39 ppm (C6), δ 99.86 ppm (C1), δ 77.62–66.11 ppm (C2-C5), δ 37.95 ppm (C7), δ 30.56 ppm (C8), δ 19.09 ppm (C9), δ 13.02 ppm (C10). FTIR (KBr pellet): υ(OH) 3700–3017 cm^{-1}, υ(—CO—NH—) 3109 cm^{-1} (2nd derivative 3090 cm^{-1}), υ(CH (of CH_2 and CH_3)) 2961 cm^{-1} and 2874 cm^{-1}, υ(CH (of Alg)) 2936 cm^{-1}, —CO—NH—) 1665 cm^{-1} and 1551 cm^{-1}, δ(—CH_2—) 1466 cm^{-1}, δ_s(—CH_3—) 1379 cm^{-1}, (C—O—C) 1097 cm^{-1}. Elemental analysis: C 49.01%, H 7.31%, N 5.58%. DS_{amide}: 0.96. Yield: 1.28 g (77.3%).

2.3. Elemental analyses

The samples were vacuum dried and the analyses were performed on a True Spec instrument from the Leco Corporation. The furnace and afterburner temperatures were set at 950 °C and at 850 °C, respectively and the instrument was calibrated with EDTA. The degree of substitution (DS) of nitrogen containing alginates (TBA-Alg, CMPI activated Alg and Alg amide) was calculated based on the *N*/*C* ratio according to the formula (1) (Hu et al., 2013).

$$DS = \frac{(\alpha * 6M_C)}{(M_N - \alpha * I * M_C)} \quad (1)$$

with $\alpha = N/C$ (determined by elemental analysis) and I = number of carbon atoms of the substituent (TBA: $I = 16$; CMPI: $I = 6$; butylamine: $I = 4$).

2.4. FTIR

256 spectra were recorded for each sample with a Vertex 70 FTIR from Bruker (Billerica, MA, USA) in the wavenumber range between 4000 and 400 cm^{-1} with a resolution of 4 cm^{-1} in transmission mode, using a sample holder with an aperture diameter of 5 mm. Humidity and carbon dioxide compensation, rubber band base line correction and a normalization of the spectra were carried out before signal evaluation. Some signals were identified on the basis of the analysis of the second derivative of the FTIR spectrum, because they were not fully resolved or were hidden in the spectrum. The signal assignment of FTIR averaged spectra was carried out according to Hesse, Meier and Zeh (Hesse, Meier, & Zeh, 2008). The γ-lactone content was estimated from the lactone absorbance (A) at 1782 cm^{-1} by assuming that the sum of the absorbance signals of the lactone at 1782 cm^{-1}, the carboxylic acid at 1744 cm^{-1} and carboxylate groups at 1611 cm^{-1} (formula (2)) were representing all carbonyl carbons (equivalent to all alginate moieties) of the polymer.

$$A(\text{carbonylcarbons}) = A(\gamma\text{-lactone}) + A(\text{carboxylicacid}) + A(\text{carboxylate}) = 1 \quad (2)$$

2.5. NMR spectroscopy

2.5.1. ^{13}C solid-state

The spectra were acquired with an Avance I, 400 MHz spectrometer from Bruker, equipped with a magic angle spinning (MAS) probe. The instrument was operated at a resonance frequency of 100.62 MHz (B0 = 9.4 T) and with 4 mm rotors at a rotation speed of 12.5 kHz. The spectra were acquired via cross polarization (^{1}H–^{13}C) and total decoupling of ^{1}H at 85 kHz. The contact time was 1 ms and the relaxation time between the pulses 5 s. The spectrum of the material received from the reaction with CMPI was averaged from 2048 scans and that of the material received from the reaction with T3P from 1024 scans.

2.5.2. ^{1}H and ^{13}C NMR

Deuterium oxide and DMSO-d6 were chosen as solvents in dependence of the solubility for preparing 3–4% (w/v) solutions. The NMR spectra were acquired with a DRX-500 NMR spectrometer from Bruker at 500 MHz (^{1}H) and at 125 MHz (^{13}C) at ambient temperature. The spectrum of the AlgNC4 synthesized with CMPI as coupling agent was averaged from 16 (^{1}H) and 7770 scans (^{13}C) and that of the reaction with T3P as coupling agent from 16 (^{1}H) 20,480 scans (^{13}C). The DS was calculated on the basis of ^{1}H NMR spectra by evaluating the ratio of the signal integrals of a representative group (I_S) for the substituent and for the complete polymer (I_R) according to formula (3).

$$DS_{amide} = \frac{x * I_S}{(z * I_R - y * I_S)}. \quad (3)$$

The signal of methyl group of the substituent aliphatic chain (D_2O at 1.51 ppm or DMSO-d6 at 0.90 ppm) representing $3 \times DS_{amide}$ protons ($z = 3$) was integrated for the determination of I_S. Where D_2O was a solvent, the spectral range of the alginate backbone (~6.0 and 4.1 ppm) was integrated for I_R. It represented 5 protons ($x = 5$, $y = 0$), since the polymer was dissolved in D_2O and a complete exchange of the hydroxyl and amide protons by deuterium was assumed. In the case of DMSO-d6, the selected

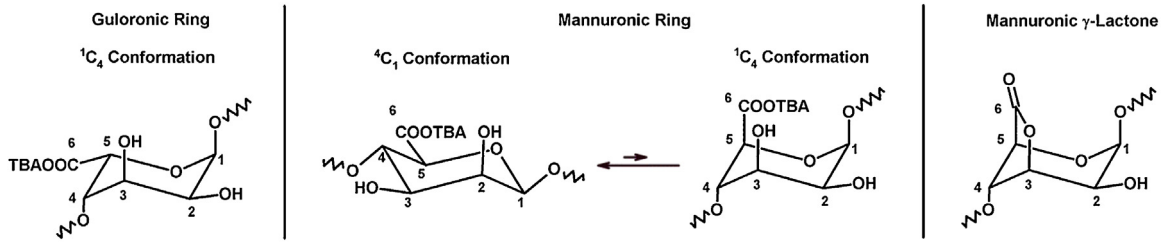

Fig. 1. On the left side the $^1C_4$ chair conformation of the guluronic ring is drawn with carboxylate and hydroxyl groups on opposite sides of the ring plane. In the middle the conformations of the mannuronic acid ring with carboxylic and two hydroxyl groups are on the same side of the ring plane. The $^4C_1$ chair conformation (left) is the preferred one, with an equatorial positioning of the carboxylate group (C6). The carboxylate group and the hydroxyl group are in an axial position in the $^1C_4$ chair conformation (right), which enables the formation of a γ-lactone, as displayed on the right hand side.

spectral range (~5.4 to 2.7 ppm) for I_R comprised the integrals of the alginate backbone, the amide proton and the C7 methylene group (3.11 ppm) due to signal overlaps, but not the protons of the hydroxyl groups attached to the backbone (2.5 ppm). Five protons contributed to the alginate backbone signals ($x = 5$) and $3 \times DS_{amide}$ protons to the substituent signals ($y = 3$).

2.6. Size exclusion chromatography – multi angle light scattering (SEC-MALS)

The chromatographic system consisted of a pump (Agilent G1311B, Santa Clara, CA, USA) with an integrated online degasser, and a temperature controlled autosampler (Agilent G1329B) kept at 22 °C. One 7.8 mm OH pack SEC column (Shodex SB-806 M, SHOWA DENKO EUROPE GmbH, Munich, Germany) was employed. A DAWN HELEOS II multi-angle light scattering detector (Wyatt Technology Corporation, Santa Barbara, CA, USA) and an Optilab T-rEX (Wyatt Technology Corporation) differential refractive index detector were connected sequentially to the SEC column. The aqueous mobile phase consisted of 100 mM NaCl with 0.02% sodium azide, at a flow rate of 0.5 mL/min, using the isocratic mode at room temperature. All reagents for SEC were of HPLC grade and the mobile phase was filtered through Durapore VVPP 0.1 μm membrane filters (Millipore). The injection volume was 20 μL. Data collection and processing were performed using the ASTRA software, Version 6.1.1.17 (Wyatt Technology Corporation). Zimm formalism with first order regression was used for the processing of light scattering data.

3. Results and discussions

3.1. Ring conformations

The formation of a lactone requires the positioning of the carboxylic group and a hydroxyl group in axial position on the same side of the ring plane (Fig. 1). Therefore, the formation of a guluronic lactone is impossible due to the positioning of these groups on opposite sides of the ring plane. In the case of the mannuronic acid ring the groups are located on the same side of the ring plane, but the carboxylic group is in an equatorial position for the assumed thermodynamic preferred conformation $^4C_1$. Bringing the mannuronic acid ring into the $^1C_4$ conformation moves the carboxylic (C6) and the C3 hydroxyl groups into axial positions, which enables the formation of the γ-lactone.

3.2. Negative control

TBA-Alg did not react in the absence of coupling agents under the given conditions, as the expected lack of a lactone signal in the FTIR spectra (Fig. 2) approved. The small signal at 1739 cm^{-1} was assigned to carboxylic acid structures, which resulted from the treatment with aqueous solutions at the end of the reaction. This signal could also be interpreted as resulting from ester groups, but this reaction was considered too unlikely under these conditions.

3.3. Activated intermediates

3.3.1. Reaction and isolation under non-hydrolytic conditions

The analysis of the activated alginate intermediate required isolation and purification in the absence of water to avoid any hydrolysis, which was achieved by the following purification procedure (a). The ^{13}C solid state NMR spectra of both intermediates (Fig. 3) contained the expected signals of coupling agents, but did not provide any information about potential side product.

The corresponding FTIR spectra confirmed the findings of the ^{13}C solid state NMR spectra with signals from the aromatic ring in the case of CMPI and signals from the propyl groups in the case of T3P. The spectra further supported the hypothesis of a side reaction, as strong signals of γ-lactone structures were found at 1782 cm^{-1} in the case of the CMPI intermediate and at 1792 cm^{-1} for the T3P intermediate. Deriving any quantitative information about the efficiency of activation and lactone formation was impossible, as the FTIR spectra quality was poor due to the limited purification in procedure (a).

3.3.2. Isolation under hydrolytic conditions

Samples isolated under hydrolytic conditions according to procedure (b) resulted in better FTIR spectra. Samples made by using

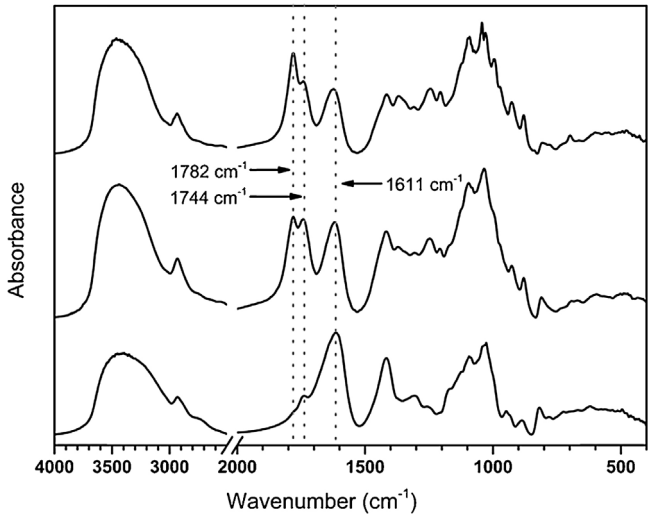

Fig. 2. FTIR spectra of the products, which were obtained from the negative control (lower) and from the reaction in the presence of the coupling agents 2-chloro-1-methylpyridinium iodide (middle) or propylphosphonic anhydride (upper). All samples were isolated according to method (a). Wavenumbers correspond at 1782 cm^{-1} to γ-lactones, at 1744 cm^{-1} to carboxylic acids and/or esters and at 1611 cm^{-1} to carboxylate ions.

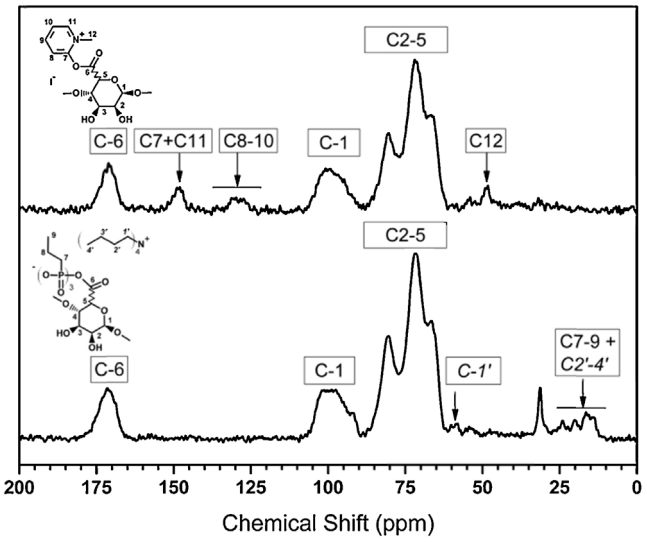

Fig. 3. ^{13}C solid state NMR Spectra of 2-chloro-1-methylpyridinium iodide (upper spectrum) and propylphosphonic anhydride activated alginates (lower spectrum) isolated according to method (b). Carbon atoms of the tetrabutylammonium ion were marked as *C-1′* to *C-4′* in the lower spectrum.

one or the other coupling agent were giving very similar spectra (Fig. 3). Signals of γ-lactone (1782 cm^{-1}), carboxylic acid or ester (both at 1744 cm^{-1}) and carboxylate ions moieties (1611 cm^{-1}) were found for all materials. The presence of a clear γ-lactone signal and the absence of coupling agent signals in these spectra were correlated with an unexpected resistance of the γ-lactone to hydrolysis and to a fast and efficient scission of the coupling agent complex. Furthermore, the reproducibility of the normalized FTIR absorbance for the γ-lactone, carboxylic acid or ester and carboxylate ion was also good from batch to batch. This allowed a comparison of the different materials synthesized by applying the method described above (formula (2)). A variation of the TBA-Alg source resulted in a change of the normalized absorbance values (Table 1) due to the different M/G block structures and different M/G contents in these samples. The synthesis with TBA-Alg made from an Alg with a low content of mannuronic acid moieties resulted in a lower γ-lactone absorbance than expected.

Optimizing the γ-lactone synthesis revealed highest yields for CMPI with a TBA-Alg concentration of 5% (w/v), a TBA-Alg:CMPI ratio between 1:0.5 and 1:1, a reaction duration of at least 30 min (test conditions and FTIR results are given in Table S1 of the supplementary data). These conditions led only to a marginal improvement of the γ-lactone content of about 0.377 (61.8% yield) in comparison to 0.349 (57.1% yield) for the standard conditions.

Best conditions for the synthesis of the γ-lactone with T3P as coupling agent were a TBA-Alg concentration of 1 or 2% (w/v), a TBA-Alg:T3P ratio of 1:2 with a reaction duration of at least 1 h. The lactone content increased from 41.6% (±1.2%) under standard conditions to 50.7% (84% of mannuronic acid moieties).

At a first glance, the signal at 1744 cm^{-1} in the received FTIR spectra seemed to support the proposed formation of an intermolecular alginate–alginate ester (Vallée et al., 2009), because the

Table 1
Normalized FTIR absorbance values of the γ-lactone (1782 cm^{-1}), carboxylic acid respectively ester (1744 cm^{-1}) and carboxylate (1611 cm^{-1}) groups and the corresponding γ-lactone yields of mannuronic γ-lactones obtained from the reaction between 2-chloro-1-methylpyridinium iodide (CMPI) or propylphosphonic anhydride (T3P) as coupling agents and tetrabutylammonium alginates[a] (TBA-Alg) of different origins. Coupling agents were removed by a short treatment with a sodium chloride solution.

TBA-Alg		Intramolecular reaction				
Source	M/G ratio[b]	Coupling agent	FTIR: Normalized absorbance values			Mannuronic γ-lactone yield[c]
			γ-Lactone	Carboxylic acid or ester	Carboxylate	
Commercially available alginic acid	0.61/0.39	CMPI	0.349	0.328	0.323	57.1%
		T3P	0.416	0.325	0.259	68.2%
Protanal GP 1740	0.37/0.63	CMPI	0.284	0.356	0.360	76.9%
		T3P	0.344	0.449	0.208	92.9%
Manucol DM	0.61/0.39	CMPI	0.384	0.308	0.309	62.9%
		T3P	0.510	0.295	0.196	83.5%

[a] c(TBA-Alg) = 1% (w/v) in DMF.
[b] M: content of mannuronic acid moieties, G: content of guluronic moieties.
[c] γ-lactone yield = (normalized γ-lactone absorbance) × 100/M.

Table 2
Degree of amidation and molecular weight analysis for alginic acid butyl amide obtained from reactions with 2-chloro-1-methylpyridinium iodide (CMPI) or propylphosphonic anhydride (T3P) as a coupling agent under various reaction conditions.

Coupling agent	TBA-Alg in DMF	Ratio: TBA-Alg to coupling agent	Ratio: TBA-Alg to n-butylamine	DS_{amide}[a]	Molecular weight analysis		
	(% (w/v))				Mn (kDa)	Mw (kDa)	Polydispersity
CMPI	0.5	1:1	1:1	0.275	–	–	–
CMPI	0.5	1:1	1:2	0.259	–	–	–
CMPI	5	1:1	1:2	0.409	–	–	–
CMPI	5	1:1	1:10	0.403	20.4 ± 1.1	35.5 ± 0.1	1.7 ± 0.1
CMPI[b]	5	1:1	1:1	0.68	–	–	–
T3P	0.5	1:2	1:1	0.307	–	–	–
T3P	0.5	1:2	1:2	0.472	–	–	–
T3P	5	1:0.5	1:10	0.391	25.1 ± 2.1	44.1 ± 1.1	1.8 ± 0.1
T3P	5	1:1	1:2	0.587	–	–	–
T3P	5	1:2	1:2	0.528	–	–	–
T3P	5	1:2	1:10	0.961	46.4 ± 3.5[c] 40.4[d]	109.3 ± 9.8[c] 54.7[d]	2.4 ± 0.3[c] 1.4[d]

[a] DS_{amide} calculation based on data from the elemental analysis.
[b] Proton scavenger was added twice, before adding CMPI and before adding butylamine.
[c] Obtained results for the assumed micelle structure.
[d] Estimated result for the single alginic acid butyl amide chain.

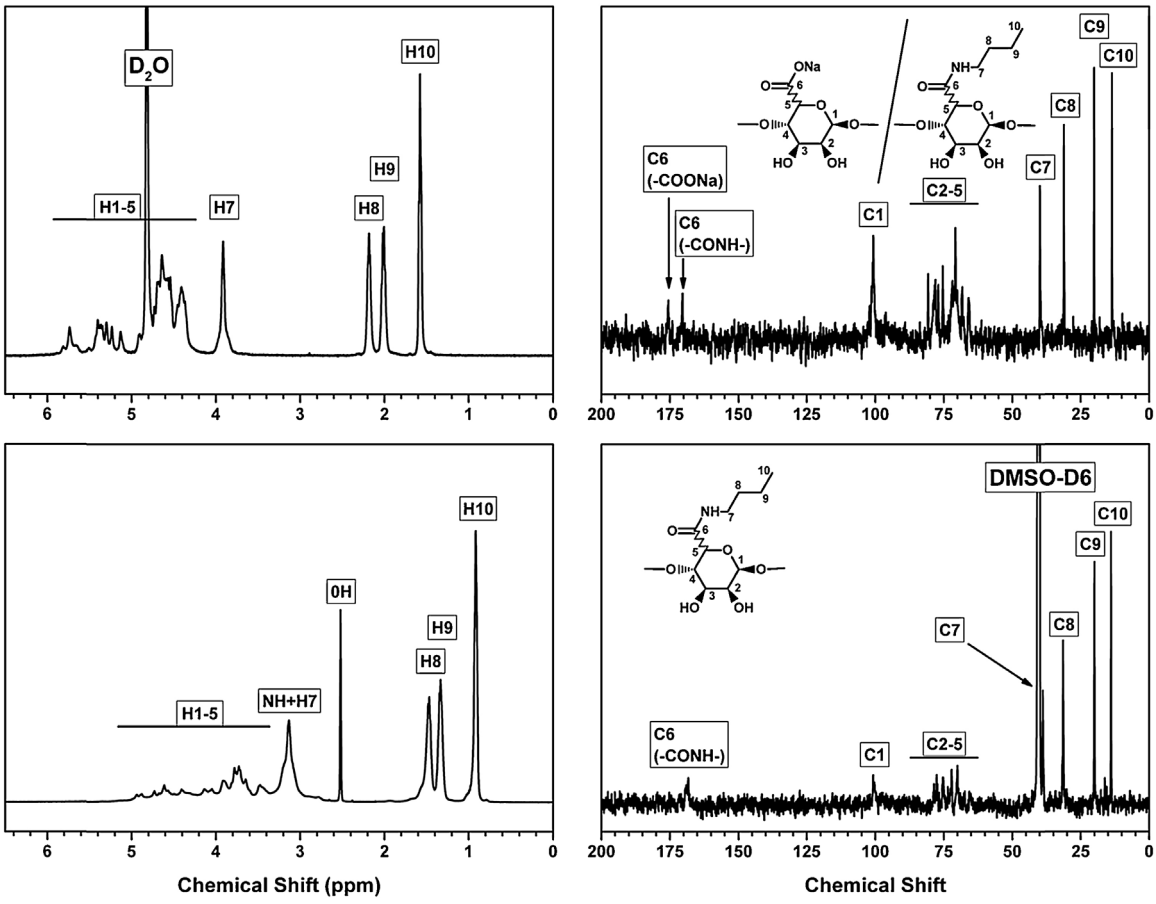

Fig. 4. ^{1}H NMR (left) and ^{13}C NMR (right) spectra of alginic acid butyl amide obtained from the reactions of tetrabutylammonium alginate with the coupling agent 2-chloro-1-methylpyridinium iodide (DS_{amide} = 0.41; upper spectra; solvent: D_2O) and the coupling agent propylphosphonic anhydride (DS_{amide} = 0.96; lower spectra; solvent: DMSO-D6).

signal correlates with the carboxylic ester group. The activated alginate esters moieties did not contribute, because they were no longer present, due to the hydrolysis with a salt solution. However, the latter reaction led to the formation of carboxylic acid moieties, which gave a signal in the same spectral range as esters. This was also confirmed by putting a sample purified with procedure (a) into water, resulting in an acidic dispersion. Therefore, it was not possible at this point to confirm or disprove the presence of alginate–alginate ester structures.

3.4. Ester hydrolysis

The intensive purification of dispersed esterified Alg with Milli-Q water (pH ~ 6) via ultra-filtration in a stirred cell led to a clear solution, which was assumed to correspond to the complete hydrolysis of all ester structures. Furthermore, it was deduced that the γ-lactone structures were sensitive to a nucleophilic attack of the hydroxyl groups of water under gentle pH conditions as long as sufficient quantities and time were available. The final clear solution indicates also that the predicted formation of intermolecular esters was very unlikely, because the resulting three dimensional networks were expected to withstand aqueous treatments under slightly acidic conditions for a longer period of time. The FTIR spectra of these materials and the corresponding second derivatives showed only the typical bands of Na-Alg (1612 cm^{-1}) and H-Alg (1735 cm^{-1}) moieties and no longer those of γ-lactone structures (1782 cm^{-1}).

3.5. Amidation

The optimized reaction conditions for the synthesis of the mannuronic acid γ-lactone were assumed to also be the optimum for

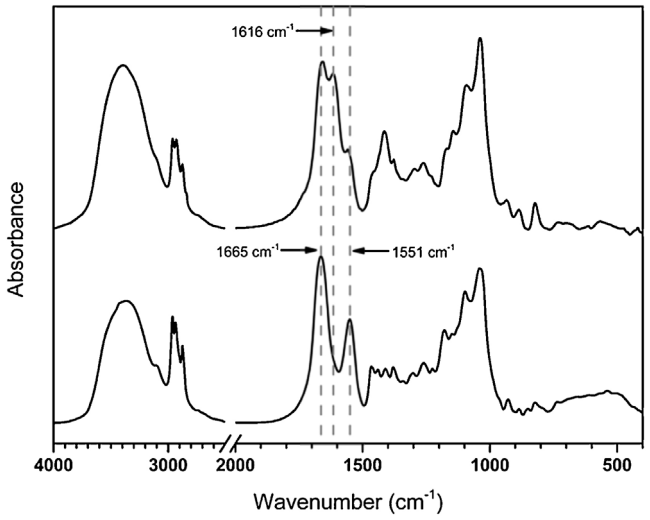

Fig. 5. FTIR spectra of alginic acid butyl amide obtained from the reactions of tetrabutylammonium alginate with the coupling agent 2-chloro-1-methylpyridinium iodide (DS_{amide} = 0.41; upper spectra) and the coupling agent propylphosphonic anhydride (DS_{amide} = 0.96; lower spectra). Wavenumbers correspond at 1616 cm^{-1} to carboxylate ions and at 1665 cm^{-1} and at 1551 cm^{-1} to amide groups (—CO—NH—).

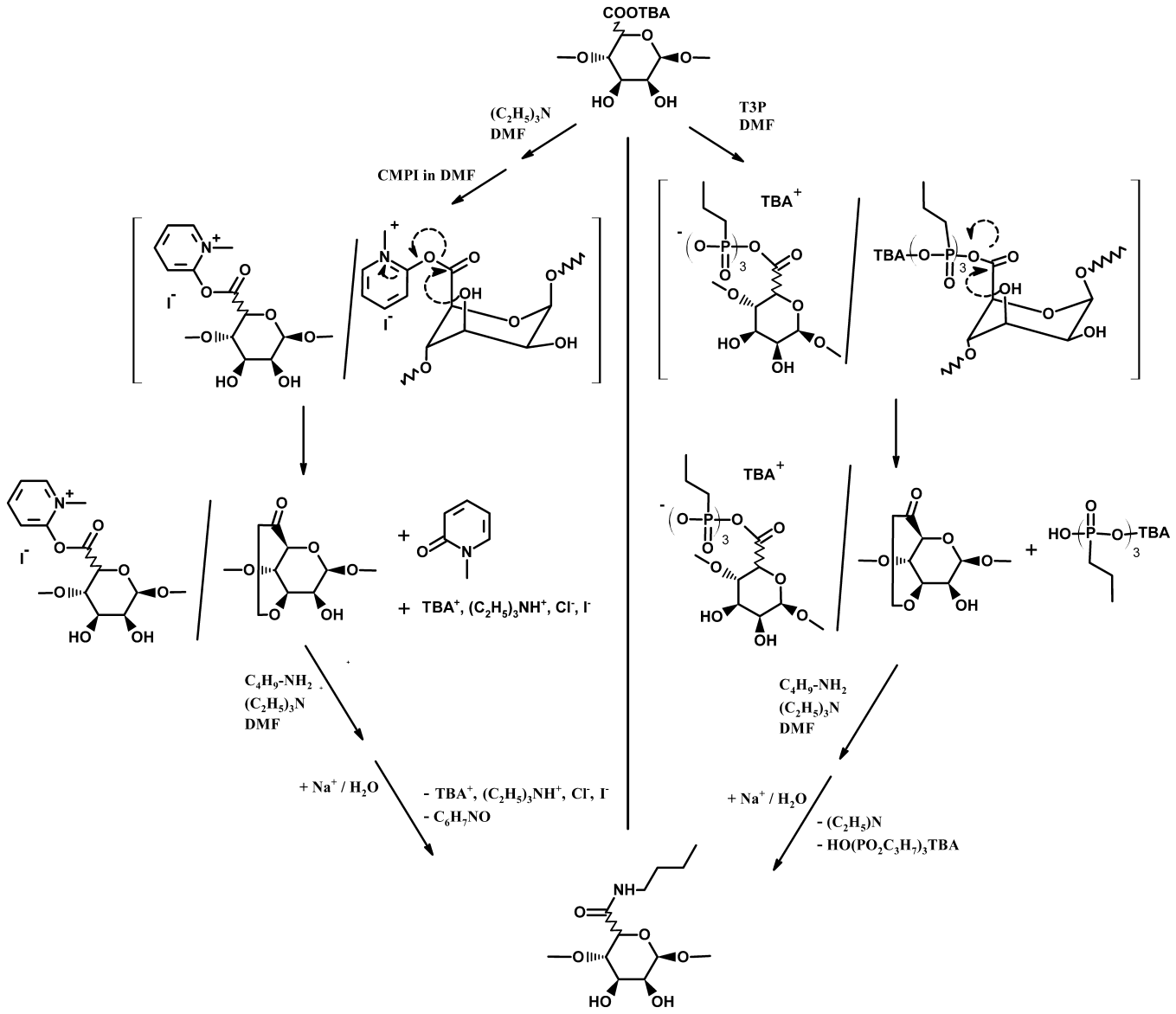

Fig. 6. Reaction schemes for the amidation of tetrabutylammonium alginate in polar aprotic organic solvents with 2-chloro-1-methylpyridinium iodide (left) and propylphosphonic anhydride (right) as coupling agent, including the mannuronic acid γ-lactone formation.

the synthesis of AlgNC4. Furthermore, it was deduced from the γ-lactone hydrolysis during ultrafiltration that an excess of amine was required. Therefore, the amide synthesis was performed accordingly for both coupling agents with a twofold excess of amine per alginate moiety. This approach resulted in a DS_{amide} value of 0.47 (Table 2) for the butyl amidation of TBA-Alg with T3P as a coupling agent. The FTIR spectra of this partially amidic alginate, taken before the ultrafiltration step, again displayed the presence of mannuronic acid γ-lactone structures in the material. The optimization of the reaction resulted in an elevation of the TBA-Alg concentration to 5% (w/v) and in an amine excess of 10 times the alginate moiety. The best DS_{amide} was determined to be 0.96 on the basis of the elemental analysis, and to be 0.98 according to the evaluation of the ^{1}H NMR spectrum (Fig. 4). The ^{13}C NMR (Fig. 4) and the FTIR spectra (Fig. 5) of this highly amidic alginate were well in line. Two amide signals were found at 168.12 (C6) and 167.39 (C6) ppm in the ^{13}C NMR spectrum and at 1665 and 1551 cm^{-1} in the FTIR spectrum. The typical carboxylate signal for partially amidic alginates was neither present in the 13C spectrum nor in the FTIR spectrum. Furthermore, DS_{amide} values of about one and the presence of the mannuronic acid γ-lactone did not, in our opinion, promote the hypothesis of the intermolecular alginate esters. We even believe that these results rather disprove this hypothesis.

The amidation of TBA-Alg with CMPI as the coupling agent according to the optimized conditions of the γ-lactone synthesis with a stoichiometric ratio of the coupling agent and a twofold excess of amine per alginate moiety led to a DS_{amide} value of 0.41 on the basis of elemental analysis and to 0.43 on the basis of 1H NMR analysis (Fig. 4). The partial amidation of Alg was also proven by the presence of two C6 carbons, carboxylate and amide carbons, in the ^{13}C NMR spectrum (Fig. 4), and by the signals of the amide and carboxylate groups in the FTIR spectrum (Fig. 5). The ^{13}C NMR signals of the alginate were in line with earlier reported findings (Grasdalen, Larsen, & Smisrod, 1981) and the butyl group signals appeared in the expected ranges. Modifications of the synthesis conditions including the usage of an extreme excess of amine (10 units/TBA-Alg moiety) resulted at best in a similar DS_{amide} in our test series so far.

An interesting fact was that the optimized reaction conditions for both coupling agents were found to be identical, but they

resulted in very different DS_{amide} values. T3P led to an almost completely amidated alginate, whereby the amidation with CMPI remained below a DS_{amide} of 0.5. This efficiency difference between the two coupling agents was unexpected. Both of them were selected for their capacity to change the alginate carboxylate moieties into activated esters, and therefore, should have a similar effect on the following amidation. This, however, did not take into account the impact of the lactone formation. Original esterification with CMPI (Bald, Saigo, & Mukaiyama, 1975) required proton scavengers for the released hydroxyl proton. This additional proton from the γ-lactone formation was not considered so far. The presence of a free proton was believed to result in the formation of alginic acid, which was then no longer available for the amidation.

Therefore, adding a proton scavenger before adding CMPI was expected to reduce or to suppress the alginic acid formation. Performing the reaction accordingly with triethylamine as a proton scavenger (Fig. 6) led to a DS of up to 0.80 for the material isolated from the solvent fraction (see Section 2.2.4). The average DS of the complete batches were found to be 0.68. These high DS_{amide} proved in our opinion the amidation of some mannuronic acid γ-lactone units, because the mannuronic acid moiety content was 61%, which was lower than the average DS_{amide}. Therefore, the hypothesis of alginic acid formation as a result of the γ-lactone formation was most probably correct. The amidation with CMPI as coupling agent, however, was still not as complete as the one with T3P as coupling agent. Further improvements of the amidation with CMPI as coupling agent were assumed to be achievable by varying the proton scavenger.

As the amidation with T3P as coupling agent resulted, under optimal conditions, in almost complete amidation of TBA-Alg, it was believed that the released phosphonic acid was binding TBA^+ and H^+, resulting from the lactone formation (Fig. 6).

The analysis of the molecular weights (Table 2) revealed the still polymeric character of the samples. In case of partial amidation, they were determined to decrease from Mw ~ 77 kDa (Schleeh et al., 2014) of TBA-Alg to 35.6 kDa where CMPI was the coupling agent and to 44.1 where T3P was the coupling agent. The SEC MALS measurement of the almost quantitative amidated alginate showed a bimodal distribution curve. The evaluation of the first signal gave a far too high molecular weight Mw. This was interpreted as the result of micelle formation in the aqueous solution. The evaluation of the second peak which was not baseline, subtracted from the first peak of the micelles, resulted in a reasonable Mw of about 55 kDa.

4. Summary

The confirmation of the hypothesis of lactone formation and the understanding of the resulting impact on the reaction were the key factors for the successful synthesis of AlgNC4 with a high DS_{amide} from TBA-Alg activated with CMPI ($DS_{amide} \leq 0.8$) or T3P ($DS_{amide} \leq 1$) as a coupling agent. Only the mannuronic acid moieties were confirmed to be able to form γ-lactone structures, which required a change from the preferred $^4C_1$ to $^1C_4$ conformation for the mannuronic acid ring. It was further deduced that the mannuronic acid γ-lactone was rather stable, because it withstood short-term exposure to mild hydrolytic conditions. The mannuronic acid γ-lactone also expressed its stability in the necessary high excess of a nucleophile required for the ring opening reaction to result in either the mannuronic acid or a derivative such as an amide. The proton released from the lactone formation had to be bound by a proton scavenger for the following amidation. This was done by the resulting phosphonic acid derivative in the case of T3P and the specifically added triethylamine in the case of CMPI. The molecular weight analysis confirmed the polymeric nature of the resulting AlgNC4 samples despite some polymer degradation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2015.11.070.

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