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N-(2-Carboxyethyl)chitosans: regioselective synthesis, characterisation and protolytic equilibria

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Abstract

N-(2-Carboxyethyl)chitosans were obtained by reaction of low molecular weight chitosan with a low degree of acetylation and 3-halopropionic acids under mild alkaline media (pH 8–9, $NaHCO_3$) at 60 °C. The chemical structure of the derivatives obtained was determined by ^1H and ^{13}C NMR spectroscopies. It was found that alkylation of chitosan by 3-halopropionic acids proceeds exclusively at the amino groups. The products obtained are described in terms of their degrees of carboxyethylation and ratio of mono-, di-substitution and free amine content. The protonation constants of amino and carboxylate groups of a series of *N*-(2-carboxyethyl)chitosans were determined by pH-titration at ionic strength 0.1 M KNO_3 and 25 °C.

Keywords: *N*-(2-Carboxyethyl)chitosan; Polysaccharides, alkylation; Carboxyethylation; Polyampholyte; Protonation constants

1. Introduction

Chitosan is an enzymatically biodegradable, biocompatible and almost non-toxic polymer. Much interest has been paid to its biomedical, environmental, agricultural, food processing and other industrial applications in the past decade.[1–6] However, chitosan related applications are limited by its insolubility in water at pH higher than 6.

One of the most popular ways to provide a hydrophilic character to this polysaccharide is by carboxyalkylation. Carboxymethyl- and carboxybutylchitosan derivatives are the most known of.[7,8] However, the information about (2-carboxyethyl)chitosans (CE-chitosans) is rather poor. Lee et al.[9] have already reported the synthesis of *O*-CE-chitosan by alkaline hydrolysis of *O*-(2-cyanoethyl)chitosan. Recently a number of *N*-CE-chitosans with degree of substitution (DS) up to 1.40 have been synthesized via Michael-type 1,4-conjugate addition of ethyl acrylate[10] or methyl acrylate[11] followed by alkaline hydrolysis. Orienti et al.[12] have prepared a sample of CE-chitosan with DS 0.43 by alkylation with 3-bromopropionic acid in water–pyridine solution and have used it as supporting material for an aqueous topical gel containing vitamin B_6. In spite of these studies, the systematic investigation of chitosan alkylation by 3-halopropionic acids (3-*X*-PA, *X* = Cl, Br, I) has not been carried out, as much as we know.

Generally, the reaction of chitosan with 3-*X*-PA allows the introduction of 2-carboxyethyl group at the 3-*O*, 6-*O* and N-positions as was observed in the reactions of chitosan with chloroacetic[13] or 2-chloropropionic[14] acids under strong alkaline conditions. However, as far as we know, data about the regioselectivity alkylation of chitosan by halocarboxylic acids in neutral or mild alkaline conditions are not available.

The present work describes the preparation of CE-chitosans by the reaction of low molecular weight chi-

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tosan with 3-*X*-PA (*X* = Cl, Br, I), the characterization of their chemical structure and the protolytic equilibria in aqueous solution.

2. Experimental

2.1. Materials

The starting material was a low molecular weight chitosan from coarse ground crab (Aldrich 44,886-9). The average degree of acetylation (DA) was 0.08 as determined by ^{1}H NMR spectroscopy. 3-Chloropropionic acid (Fluka), 3-bromopropionic acid (Aldrich), 3-iodopropionic acid (Aldrich), deuterium oxide (Aldrich 15,188-2), deuterium chloride (Aldrich 54,304-7) were used without additional purification.

2.2. Synthesis

Chitosan (1.0 g, 5.4 mmol of N) was dissolved in water (70 mL) containing a prescribed amount of 3-*X*-PA during 1 h. The insoluble fraction was separated by filtration. An equivalent amount of $NaHCO_3$ was added portionwise to the dissolved chitosan, which was stirred for additional 30 min to remove the excess of CO_2 (pH ~ 6.5). After stirring at 60 °C for 6 h, $NaHCO_3$ (2 g) was added (pH ~ 8.5) and stirring was continued until the end of the synthesis (see reaction time in Table 1). The product was precipitated in EtOH to remove it from the excess of low molecular contaminants. Then the precipitate was filtered and dissolved in water (~ 0.03 g/ml). The solution was acidified to pH 1–2 with concd HCl (E. Merck, p.a.), dialysed against deionised water during 2–3 days (Spectra/Por dialysis tube membrane Mw cut-off 1 kDa, no. 132638) and freeze dried (Christ alpha 1-4 lyophiliser). The yields of all products were higher than 80%.

2.3. General methods

^{1}H and ^{13}C NMR spectra were measured on an Avance 400 spectrometer at 70 °C for improving the signal resolution. The samples were dissolved in D_2O/DCl (concn 10 mg/mL). 3-(Trimethylsilyl)-1-propanesulfonic acid was used as an external standard.

The elemental analyses were performed on a Carlo Erba EA 1108 analyzer.

Potentiometric titrations (PT) were carried out with a PC-controlled system assembled with a Crison MicropH 2002 pH meter, a Crison MicroBU 2030 microburette, an Orion 90-02-00 (double junction AgCl/Ag) reference electrode and a Russel SWL/S7 glass electrode. Titration of polymer solutions, concentration range $(1-5) \times 10^{-3}$ monomole·L^{-1}, as performed by addition of standard carbonate-free 0.1 M KOH solution under an atmosphere of CO_2-free N_2 at 25.0 ± 0.1 °C and ionic strength 0.1 M KNO_3. For complete protonation, a stoichiometric excess of 0.1000 M HNO_3 (9964 Titrisol, E. Merck) was added to the solution before titration. The glass electrode was calibrated in terms of hydrogen ion concentration by using buffers in the pH range 4–9 with ionic strengths adjusted to 0.1 with KNO_3.[15]

3. Results and discussion

The reaction of chitosan with a calculated amount of 3-*X*-PA per glucosamine residue as carried out at 60 °C and pH 8–9 ($NaHCO_3$). The experimental conditions followed are summarized in Table 1.

The chemical structure of the CE-chitosans was determined by ^{1}H and ^{13}C NMR spectroscopies. Figs. 1 and 2 shows typical ^{1}H and ^{13}C NMR spectra of the reaction product. It was found that the spectra do not depend on the reagent used for alkylation.

The ^{1}H NMR spectrum (Fig. 1) shows the formation of two species during the reaction of NH_2 with 3-*X*-PA, namely the mono-carboxyethylated and the di-carboxyethylated amine. These two forms are distinguishable on the spectrum because of two different chemical shifts of *H-1* (4.94 and 5.13 ppm) and C***H***$_2$COOH (2.79 and 2.93 ppm). The δ(*H-1*) and δ(C***H***$_2$COOH) integral ratios for GlcNHR and $GlcNR_2$ residues are 1:2 and 1:4, respectively. The suggested structure was confirmed by ^{13}C NMR spectroscopy. No methyl resonance of acetylated unit expected at 25 ppm and chemical shift corresponding to NH***C***OCH_3 (about 170 ppm) was observed because the *DA* was quite low. The obtained spectra are comparable with those obtained for *N*-substituted derivatives of CE-chitosan: *N*-CE-chitin Na salt[10], *N*-CE-chitosan methyl ester[11] or *N*-CE-chitin ethyl ester[10].

Therefore, it was concluded that alkylation of chitosan by 3-*X*-PA under the reaction conditions used in this work proceeds exclusively at the amino groups of the D-glucosamine residue (Scheme 1). In our opinion, the regioselectivity of the alkylation (more precisely a chemoselectivity) arises from the mild alkaline reaction conditions used that give the possibility to differentiate the reactivity of –OH and $–NH_2$ groups.

N-CE-chitosans were quantitatively characterized by their ^{1}H NMR spectra in terms of $DA = n/(n+m) = n/(n+x+y+z)$ and $DS = (y+2z)/(x+y+z)$. Integrals of the signals were compared with the δ(H-1) integral considered as internal standard. The DA values for the starting material and all products were determined from the δ(CH_3) integral[16] and there was no modification of the DA = 0.08 during the reaction. The DSs on the amino group of the D-glucosamine residue were estimated by the integrals of δ(CH_2COOH) or

Table 1
Reaction conditions and some properties of *N*-(2-carboxyethyl)chitosans [a]

Product	*X*	Reagent equivalents [b]	Time, day	Elemental analysis (EA), % [c]			DS [d]			x:y:z [e]	$\log K_1^{H}$ [f]	$\log K_2^{H}$ [f]
				C	H	N	EA	PT	NMR			
Chitosan				42.22	7.88	7.58					6.42 ± 0.05	
I	Cl	5	5	42.09	6.25	5.08	1.15	1.12	1.08	0.10:0.74:0.16	7.31 ± 0.02	3.15 ± 0.02
II	Br	5	5	40.71	6.31	5.21	0.95	0.97	0.95	0.15:0.75:0.10	7.30 ± 0.03	3.36 ± 0.02
III	I	5	5	42.54	6.55	4.95	1.28	1.17	1.12	0.09:0.70:0.21	7.35 ± 0.02	3.25 ± 0.02
IVa	Br	2	3	38.50	6.67	5.91	0.40	[g]	0.43	0.57:0.43:0.0	6.72 ± 0.02	3.24 ± 0.05
IVb	Br	2 equiv × 2 [h]	3 d × 2	40.85	6.72	5.12	1.02	[g]	0.90	0.21:0.68:0.11	7.19 ± 0.04	3.21 ± 0.08
IVc	Br	2 equiv × 3 [h]	3 d × 3	40.85	6.57	4.88	1.18	1.19	1.14	0.07:0.72:0.21	7.33 ± 0.02	3.15 ± 0.01
Va	Br	5	3	41.63	6.62	5.33	0.95	[g]	0.92	0.18:0.72:0.10	7.20 ± 0.02	3.20 ± 0.03
Vb	Br	5 equiv × 2 [h]	3 d × 2	42.43	6.74	4.71	1.46	1.45	1.41	0.0:0.59:0.41	7.66 ± 0.04	3.35 ± 0.03
Vc	Br	5 equiv × 3 [h]	3 d × 3	42.35	6.65	4.44	1.68	1.67	1.56	0.0:0.44:0.56	7.75 ± 0.07	3.44 ± 0.03

[a] T 60 °C, pH 8–9 ($NaHCO_3$).
[b] 3-*X*-PA:D-glucosamine residue ratio.
[c] Each value represents the mean of two independent determinations.
[d] Degree of substitution was calculated per D-glucosamine residue of chitosan.
[e] See the reaction Scheme 1.
[f] T 25.0 °C, 0.1 M KNO_3.
[g] Sample contains HCl that is not removed by dialysis.
[h] As indicated in [b] × 2 or 3 times.

δ(H-1) and x:y:z ratio by δ(H-1) integrals. The results are shown in Table 1.

From elemental analysis data, the DS was calculated as: $DS = \Delta_{C/N}/[3M_{C/N}(1 - DA)]$, being $\Delta_{C/N}$ the C/N percentage ratio difference of this elements in the derivative and in the original chitosan. $M_{C/N}$ is the ratio of C to N molar masses and DA has the meaning already mentioned. A good agreement were obtained with NMR results, despite the former value had be calculated from 5 experimental values (with their inherent associated errors) namely %C and %N, both from the derivative and chitosan, and DA, obtained by NMR.

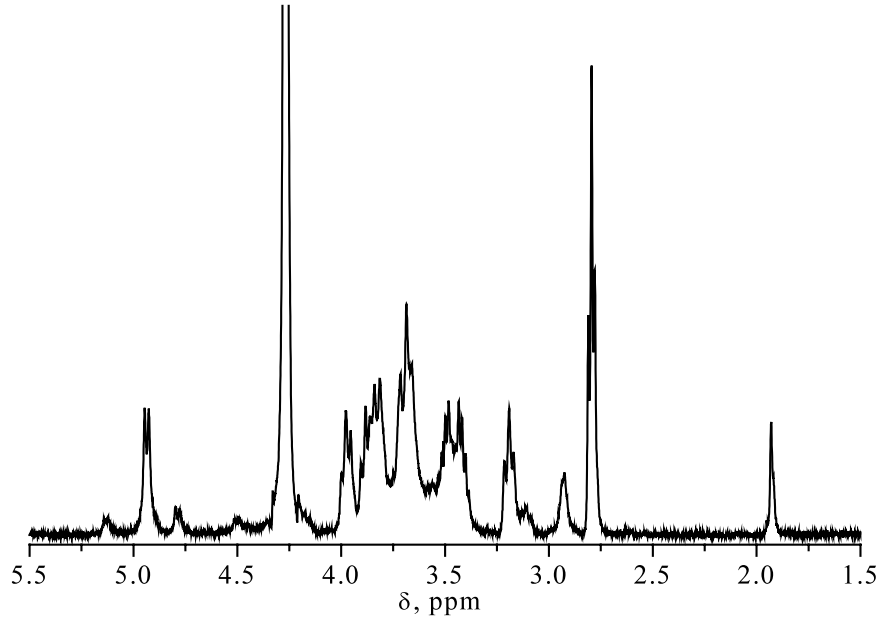


Fig. 1. 400 MHz ^{1}H NMR spectrum (δ, ppm) of *N*-CE-chitosan solution (sample II) in D_2O/DCl at 70 °C: 1.93 (s, 0.27 H, CH_3), 2.79 (t, 1.53 H, CH_2COOH of GlcNHR), 2.93 (m, 0.36 H, CH_2COOH of $GlcNR_2$), 3.11 (t, ~0.14 H, *H-2* of $GlcNH_2$), 3.19 (t, ~0.76 H, *H-2* of GlcNHR), 3.39–4.00 (m, 7.58 H, *H-3,4,5,6*, *H-2* of $GlcNR_2$ and GlcNHAc, NCH_2), 4.50 (d, ~0.09 H, *H-1* of GlcNHAc), 4.79 (d, 0.15 H, *H-1* of $GlcNH_2$), 4.94 (d, 0.75 H, *H-1* of GlcNHR), 5.13 (d, 0.10 H, *H-1* of $GlcNR_2$).

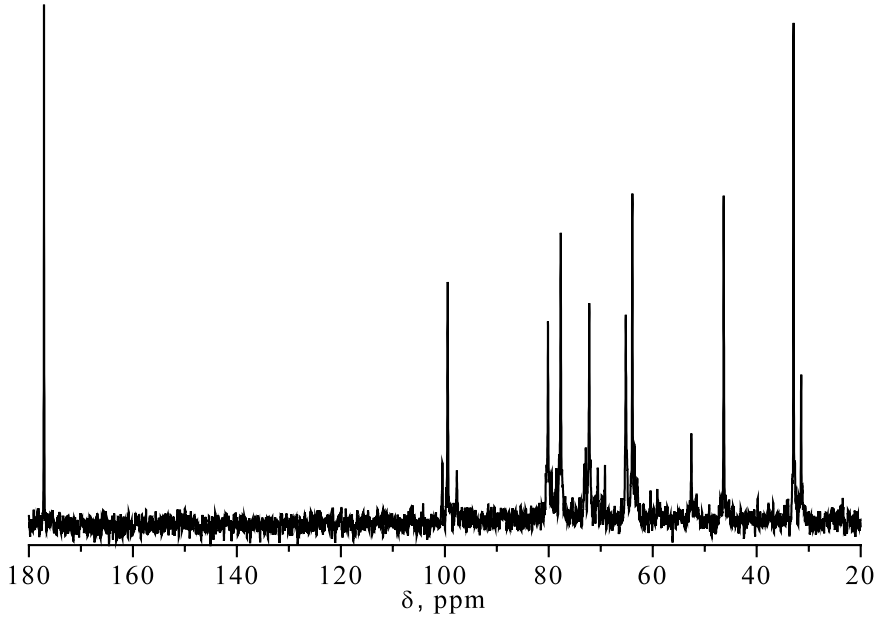
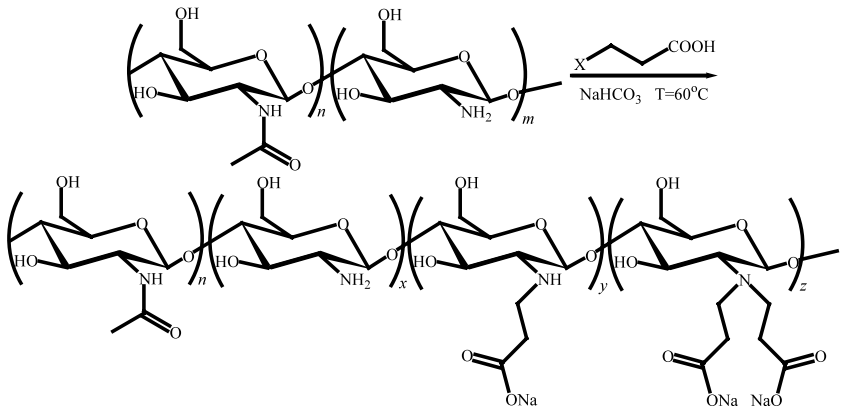


Fig. 2. 100 MHz ^{13}C NMR (δ, ppm) spectrum of a solution of *N*-CE-chitosan (sample II) in D_2O/DCl at 70 °C: 31.45 (CH_2COOH of $GlcNR_2$), 32.91 (CH_2COOH of GlcNHR), 46.35 (NCH_2 of GlcNHR), 52.58 (NCH_2 of $GlcNR_2$), 62.99–63.93 (C-6, C-2 of $GlcNH_2$), 65.18 (C-2 of GlcNHR), 69.19 (C-2 of $GlcNR_2$), 70.57–73.20 (C-3), 77.29–78.55 (C-5), 79.46–80.57 (C-4), 97.68–100.47 (C-1), 177.10 (COOH).



Scheme 1. Carboxyethylation of chitosan with 3-halopropionic acids. Different residues are distributed statistically.

The degree of carboxyethylation of chitosan was measured vs the reaction time. The results are shown in Fig. 3 and in Table 1 (compounds I–III). Two parts with a boundary at approximately 40 h can be distinguished in the slopes. In the first part, the DS strongly increases with reaction time following the expected order of the reactivity of the alkylating agent 3-*X*-PA: Cl < Br < I. In the second part, a pronounced stabilization of the DS was observed, in addition to an inversion in the 3-*Cl*-PA and 3-*Br*-PA order.

The increasing of the DS in the case of 3-*Cl*-PA could be explained by the reaction rate of the concurrent reactions of substitution and elimination. In the case of the former reaction, a DS value is expected in the following order for 3-*X*-PA: Cl < Br < I. However, the rate of the elimination reaction slightly depends on the nature of the halogen atom in a reverse order I < Br < Cl following the hydratation ability. Otherwise, the acrylic acid accumulated during the elimination reaction can react with the amino group of chitosan increasing the DS.

The effect of repetition of the reaction procedure on the DS value was examined additionally in order to increase the DS. As shown in Table 1 (samples IVa–c, Va–c), DS of the products increased with an increase in the repeating times.

N-CE-chitosans are weak amphoteric polymers and, in aqueous solution the following equilibria have to be considered:

$$-NR_2 + H_3O^+ \rightleftharpoons -NHR_2^+ + H_2O \qquad K_1^H$$

$$-COO^- + H_3O^+ \rightleftharpoons -COOH + H_2O \qquad K_2^H$$

Because of intramolecular electrostatic attraction between $-COO^-$ and $-NHR_2^+$ groups, *N*-CE-chitosan showed typical ampholytic polyelectrolyte behavior in aqueous solution during the potentiometric titration, precipitating in the pH range near the isoelectric point, which depends on DS. *N*-CE-chitosans with low DS (about 0.5) was completely soluble in all pH range. Some pH-titration curves are presented in Fig. 4.

The intrinsic protonation constants were calculated using the Katchalsky equation[17]: $pH = \log K^H + n \cdot \log[a/(1-a)]$, where a is the degree of neutralization and n is an empirical parameter. The $\log K^H$ and n values were determined from the intercept and the slope

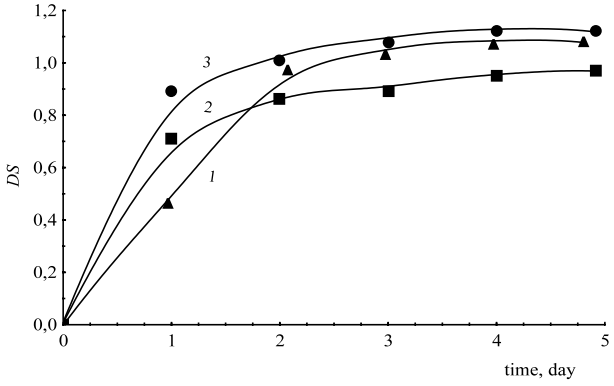


Fig. 3. DS values of 2-carboxyethyl groups as a function of reaction time of the chitosan with 3-*Cl*-PA (*1*), 3-*Br*-PA (*2*) and 3-*I*-PA (*3*). The DSs were determined by ^{1}H NMR spectra. The reaction conditions are shown in Table 1.

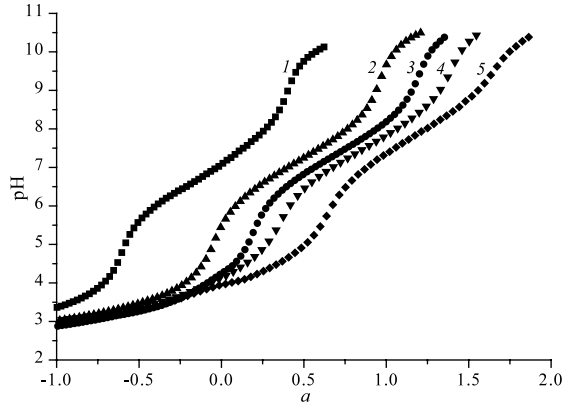


Fig. 4. pH-Titration curves (a is the degree of neutralization) for *N*-CE-chitosans IVa (*1*), II (*2*), IVc (*3*), Vb (*4*) and Vc (*5*) with equimolar amount of HNO_3 at 25.0 °C and 0.1 M KNO_3.

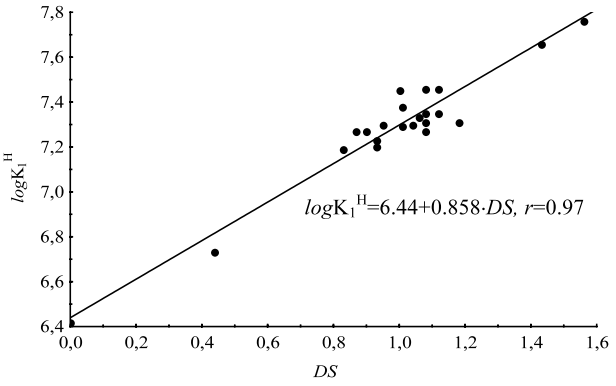


Fig. 5. Relationship between the DSs of *N*-CE-chitosans and log K_1^H (25.0 °C, 0.1 M KNO_3).

of the plot of pH vs $log[a/(1-a)]$. The results are illustrated in Table 1 together with the DSs calculated from potentiometric data. The protonation constant of mother chitosan sample was also determined at the same conditions, being $\log K^H = 6.42 \pm 0.05$. This value is in good agreement with the literature 6.4 (0.1 M KCl).[18]

The value of $\log K_1^H$, which corresponds to the protonation of the amino group of *N*-CE-chitosan, increases with the DS (Table 1, Fig. 5), while $\log K_2^H$ is independent on DS (Table 1). Generally, the basicity of the amino groups depends on contribution of the inductive effect of the substituents, electrostatic effect, steric hindrance, hydrogen bonding, solvation etc. The slight negative inductive effect carried out by 2-carboxyethyl groups leads us to expect a decrease in the basicity of the amino group. Therefore, the increase observed can be attributed to steric hindrance of the 2-carboxyethyl groups and hydrogen bonding rearrangements.

Comparing the $\log K^H$ values obtained in this work for derivatives with DS ~ 1 (Table 1) with those found in the literature for *N*-(carboxymethyl)chitosan with the same DS[7] ($\log K_1^H = 6.6$, $log\ K_2^H = 2.3$), a decrease in the acidity of the carboxylic group and an increase in the basicity of the amino group is observed, due to the screening role of an additional CH_2 group that reduces the mutual influence of these groups.

4. Conclusion

A number of *N*-CE-chitosans were successfully produced by alkylation of mother chitosan sample by 3-*X*-PA in mild alkaline conditions. The chemical identity of the *N*-CE-chitosans was assessed by ^1H and ^{13}C NMR spectrometry.

The proposed method has the following advantages:
(i) the regioselectivity of N-carboxyethylation of chitosan is about 100%;
(ii) DS can be regulated by the reaction period and repeating procedure;
(iii) no toxic reagent was used.

It appears that 3-*Cl*-PA is the most effective alkylating reagent in terms of DS yield and cost.

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References

1. Kumar, M. N. V. R. *React. Funct. Polym.* **2000**, *46*, 1–27.
2. Singh, D. K.; Ray, A. R. J. *Macromol. Sci. Rev. Macromol. Chem. Phys.* **2000**, *C40*, 69–83.
3. Borchard, G. *Adv. Drug Delivery Rev.* **2001**, *52*, 145–150.
4. Paul, W.; Sharma, C. P. *STP Pharma Sci.* **2000**, *10*, 5–22.
5. Shahidi, F.; Arachchi, J. K. V.; Jeon, Y. J. *Trends Food Sci. Tech.* **1999**, *10*, 37–51.
6. Dodane, V.; Vilivalam, V. D. *Pharm. Sci. Tech. Today* **1998**, *1*, 246–253.
7. Muzzarelli, R. A. A.; Tanfani, F.; Emanuelli, M.; Mariotti, S. *Carbohydr. Res.* **1982**, *107*, 199–214.
8. Muzzarelli, R.; Weckx, M.; Filippini, O.; Lough, C. *Carbohydr. Polym.* **1989**, *11*, 307–320.
9. Lee, Y. M.; Shin, E. M.; Noh, S. T. *Ang. Macromol. Chem.* **1991**, *192*, 169–181.
10. Aoi, K.; Seki, T.; Masahiko, O.; Sato, H.; Mizutani, S.; Ohtani, H.; Tsuge, S.; Shiogai, Y. *Macromol. Chem. Phys.* **2000**, *201*, 1701–1708.
11. Sashiwa, H.; Shigemasa, Y.; Roy, R. *Chem. Lett.* **2000**, 862–863.
12. Orienti, I.; Luppi, B.; Zecchi, V. *J. Cosmet. Sci.* **1999**, *50*, 307–313.
13. Hayes E.R. U.S. Pat. 4,619,995, 1986; Chem. Abstr. 1987, 106, 104137y
14. Shigemasa, Y.; Ishida, A.; Sashiwa, H.; Saimoto, H.; Okamoto, Y. *Chem. Lett.* **1995**, 623–624.
15. Vasconcelos, M. T. S. D.; Machado, A. A. S. C. *Rev. Port. Quim.* **1986**, *28*, 120–141.
16. Vårum, K. M.; Anthonsen, M. W.; Grasdalen, H.; Smidsrød, O. *Carbohydr. Res.* **1991**, *211*, 17–23.
17. Katchalsky, A. J. *Polym. Sci.* **1954**, *12*, 159–184.
18. Park, J. W.; Choi, K.-H.; Park, K. K. *Bull. Korean Chem. Soc.* **1983**, *4*, 68–72.