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Short communication

Efficient activation of carboxyl polysaccharides for the preparation of conjugates

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Abstract

We demonstrated here a highly efficient activation of carboxyl groups applicable to any polysaccharide. It was done by using the alternative activation agent, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride. More specifically, we prepared glucans bearing linkers of different lengths that possessed masked carbonyl groups.

Keywords: Conjugation; Glucan; DMTMM

Polysaccharides are of great interest as immunogenic effectors and biocompatible carriers of small bioactive molecules. However, their chemical modifications are limited by low reactivity of their hydroxyl groups. Carboxylated polysaccharides that occur naturally, or are easily prepared, are more suitable for chemical modification. The effective activation of the carboxyl groups here is a critical step. The methods used rely, almost solely, on the activation with water-soluble carbodiimide (e.g., 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, EDCI) forming intermediates that are able to link to the nucleophil groups of ligands. This strategy is widely used mainly for preparation of conjugate vaccines based on capsular polysaccharides (Armitt, 2001). The problem, which draws the attention, is that the considerable amount of undesired side groups is formed. For the explanation, the part of active *O*-acylisourea intermediate groups quickly undergoes structural rearrangement to form relatively stable *N*-acylurea groups (Bystrický, Alföldi, Machová, Steiner, & Šoltés, 2001). These unwanted side groups can change the nature and function of the polysaccharide, especially when it is used for the preparation of a glycoconjugate vaccine. Therefore an alternative synthetic method is desired so we came up with the use of alkoxychloro 1,3,5-triazine as the carboxyl group activator. It was originally used in peptide syntheses in anhydrous solvents (Garret, Jiang, Prasad, & Repič, 2002). *In situ* preparation of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) reagent precluded primarily the irritant properties of 2-chloro-4,6-dimethoxy-1,3,5-triazine CDMT component (Kamiński, 2000). Since DMTMM became recently commercially available (Kunishima et al., 1999), we started to study the possibility of this reagent as more effective as well as water stable condensing agent for the activation of the polysaccharide carboxyl groups. The ammonium salt of DMTMM reacts by S_NAr mechanism forming reactive triazinyl ester capable of reacting with a nucleophile (e.g., amine group) (Lee, Wong, Lee, Yue, & Lee, 1989).

We present here the outcome from this chemistry highlighting potential effectiveness of this technique compared to the carbodiimide procedure.

For our purposes, we chose two types of model polysaccharides, namely carboxymethyl glucan (CMG) (Lindberg, 1999) and oxidized glucan (OXG) (Machová, Kogan, Alföldy, Šoltés, & Šandula, 1995). The degree of substitution (i.e., carboxylation) was 0.87 and 0.45, respectively.

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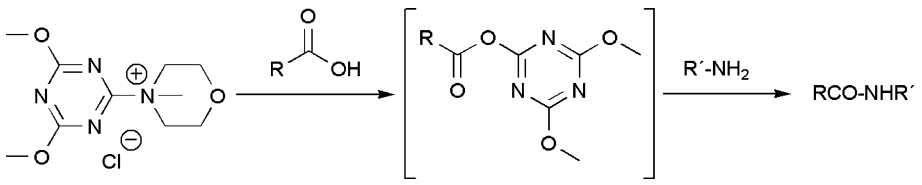


Fig. 1. Activation of carboxyl groups with DMTMM and amide bond.

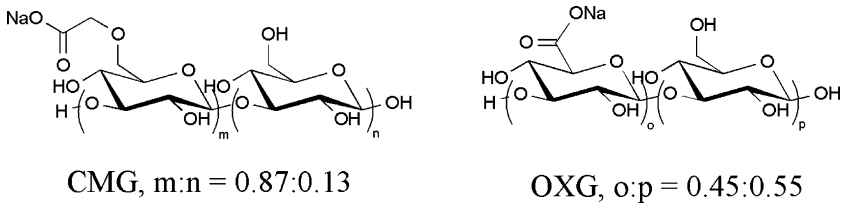
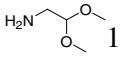
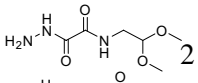
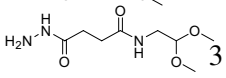
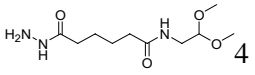


Fig. 2. Structure of glucans.

Table 1
Reaction conditions and yields of various linkers with glucans

Entry	Linker	Glucan	Linker	Agent	Yield (%)	Amidation (%)
1	 1	CMG	1	1.5	74	74.3
2		OXG	1	1.5	75	38.6
3		OXG	0.3	1.5	88	12.4
4	 2	CMG	0.5	1.5	77	42.8
5		OXG	0.5	1.5	66	27.4
6	 3	CMG	1	3	84	73.5
7			1	3[a]	58	22.3 (47.7)[b]
8			0.5	1.5	88	48.1
9		OXG	0.5	1.5	81	38.1
10	 4	CMG	0.5	1.5	82	46.3
11		OXG	0.5	1.5	84	38.5
12	No linker	CMG	0	1	100	0[c]

[a] EDCI.
[b] Brackets: both linker and urea derivatives.
[c] 0% of nitrogen in the product.

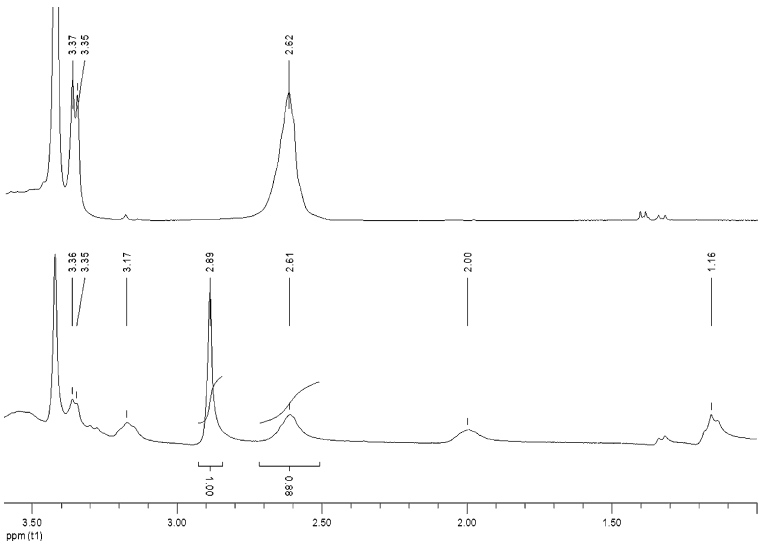


Fig. 3. ^{1}H NMR spectrum in D_2O of CMG derivatives, entries 6 (upper spectrum) and 7 (lower spectrum).

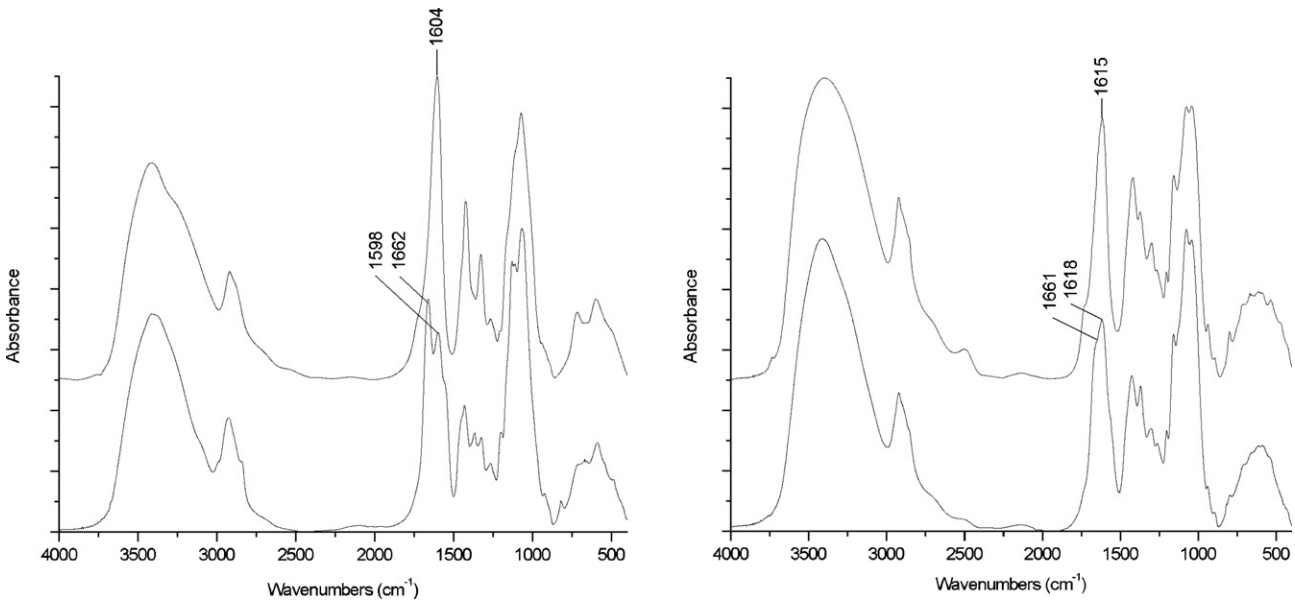


Fig. 4. FT-IR spectra of amine-glucan derivatives. The original carboxymethylglucan (upper spectrum) and its aminoderivate (entry 1, lower spectrum) is on the left. Oxidized glucan (upper spectrum) with its aminoderivate (entry 2, lower spectrum) is on the right.

The structures of both glucans used in this experiment are in Fig. 2. In the case of CMG, the carboxymethyl groups are on C-2 and C-6 positions, randomly distributed over the polymer. In the case of OXG C-6, only the primary hydroxyl group is oxidized.

The function of activated carboxyl groups was tested by binding of heterobifunctional linkers. These linkers, as the ligands, carry an amino group at one end and a protected aldehyde group on the other (Painter, Cesaro, Delben, & Paloeti, 1985). Both glucans were dissolved in water, typically 30 mg in 1 mL without any buffer, and then linkers mentioned above were added as well as the DMTMM activator. The mixtures were stirred for 12 h at room temperature. Then ether extraction (3×3 mL) followed by dialysis against distilled water and lyophilization was performed which gave products in good yields (Table 1). The reaction was monitored by TLC (H_2O/isopropanol, 1:1), where movement of the polysaccharide band was observed.

Various equivalents of the linkers and activator were used in the reactions (Table 1, columns 4 and 5). In the table, the amount of bound linker is expressed as a percentage of amidated carboxyl groups in glucans (column 7, Table 1). Equity of molar ratio of nitrogen and carbon, $(N/C)_{EXP}$ obtained from elemental analysis and ratio of sum of nitrogens to sum of carbons in derivative, Σ_C/Σ_N was used as the basis for calculation of percentage of amidation.

The amount of linkers introduced to glucans is evidently lower in the case of OXG compared to CMG (Table 1). CMG in comparison to OXG, does have carboxyl groups shifted from the ring by $–CH_2–O–CH_2$-moiety and therefore may be more flexible and more accessible to activation reagents. Slight excess of DMTMM activator (1.5 equiv) is optimal and sufficient for reaching ~75% amidation. For comparison, the reaction was also performed using carbodiimide activator (EDCI) documented as Entry 7 in Table 1. As can be seen, the binding capacity of the ligand, under the same conditions as that used with DMTMM, is markedly lower. In the case of carbodiimide activator, the amidation was only ~22% compared to ~75% using DMTMM under the same reaction conditions, even at high excess of EDCI (3 equiv). Additionally, in this case there was ~25% of *N*-acylurea groups. The differences in the structure of products are evident from ^{1}H NMR spectra shown (Fig. 3).

^{1}H NMR spectra of both derivatives contain signals of bound linker 3. Singlet at $\delta \approx 3.45$ ppm ($–OH_3$); doublet at $\delta \approx 3.35$ ppm, $J = 5.1$ Hz ($NH–CH_2–CH=$) and broad singlet at δ 2.62 ppm ($–CH_2–CH_2–$). However, in the case of entry 7 (Fig. 1, lower spectrum) there were additional signals of *N*-acylurea at δ 2.9 ppm ($–N(CH_3)_2$), δ 2.0 ppm ($–CH_2–CH_2–CH_2–$) and δ 1.16 ppm ($–N–CH_2–CH_3$). The ratio of the amount of undesired *N*-acylurea groups to the amount of bound linker can be roughly estimated from NMR signal integration as 1:0.88 (Fig. 4).

The two main advantages of use of DMTMM over conventional carbodiimides are certainly the structural purity of products as well as higher reaction yields. Moreover, DMTMM is more stable in water solution, whereas EDCI is prone to decomposition at lower pH.

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