

WATER-SOLUBLE *N*-SUBSTITUTED CHITOSAN DERIVATIVES

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ABSTRACT

Many water-soluble *N*-substituted chitosan derivatives have been prepared having the typical -NH-CH₂- linkage. Both the Michael reaction and alkylation with monochloroacetic acid at pH 8 - 8.5 offer relatively simple ways for their synthesis.

Keywords: *N*-carboxymethyl chitosan; *N*-dicarboxymethyl chitosan; *N*-carboxyethyl chitosan; *N*-carboxybutyl chitosan; *N*-trimethyl chitosan; 5-methylpyrrolidinone chitosan

1. INTRODUCTION

Chitin is a natural biopolymer but its derivative - chitosan - is almost an artificial one. Both of them are insoluble in water, a metabolism medium of both animals and vegetables. *N*-acetyl-D-glucosamine (NADG), a monomer of chitin, is abundant in human body. It is a template for the glucosaminoglycans and glycoproteins and is also one half of the repeat unit of hyaluronic acid, an important substance in the human body [1, 2]. Chitosan *N*-derivatives are susceptible to cleavage by lysozyme in the human body, thereby liberating NADG.

In order to obtain polymers whose structures are as close as possible to that of NADG/chitooligomers (i.e. their degree of acetylation is as high as possible) and which are also water-soluble, biodegradable and biocompatible, the following *N*-substituted derivatives have been investigated:

- *N*-carboxymethyl chitosan (*N*-CMC) [3, 4].
- *N*-dicarboxymethyl chitosan (*N*-diCMC) [3, 6].
- *N*-carboxyethyl chitosan (*N*-CEC).
- *N*-carboxybutyl chitosan (*N*-CBC) [7, 8].

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- 5-methylpyrrolidinone chitosan (*N*-PC) [7, 8].
- *N*-trimethyl chitosan (*N*-TMC) [6].

Some potential applications are mentioned below:

- Wound healing dressings (histological and immunological tests, animal and human trials in hospital): *N*-CMC [3, 4], *N*-CBC [7], *N*-PC [5].
- Osteogenesis agents :*N*-diCMC [9].
- Drug absorption enhancer:*N*-TMC [10 - 12].
- Food preservatives *N*-CMC [13].

Their structures have been investigated carefully by NMR (1D, 2D) and some of them were applied as treatments in hospital trials.

The *N*-CMC and *N*-CEC derivatives may be easily synthesized respectively by *N*-alkylation with monochloroacetic acid at a controlled pH and by the Michael reaction between chitosan and acrylic acid [14]. This publication briefly reviews some remarkable techniques for synthesizing all chitosan *N*-derivative series, and additionally describes some new ways for obtaining *N*-CMC and *N*-CEC by reaction with ClCH_2COOH and acrylic acid respectively.

2. EXPERIMENTAL

Experimental details for the preparation of *N*-CMC, *N*-PC, *N*-CBC and *N*-TMC are given in references [3 - 8] respectively.

The samples of β chitosan were from squid pens (DA > 50%). The monochloroacetic acid and acrylic acid were from commercial pure reagent.

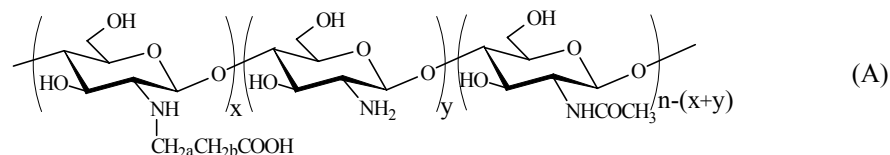
The ^1H and ^{13}C -NMR spectra were recorded on a 300 and 500 MHz Bruker Avance spectrometer. Their assignments were made using 1D-DEPT sequence and 2D ^{13}C - ^1H -NMR correlations. The samples of *N*-CMC and *N*-CEC were freeze-dried and dissolved in D_2O solution. The signal of residual H_2O is also separate from the spectrum of the polymers.

The ^1H -NMR spectra of *N*-CMC and *N*-CEC were recorded at 300 and 353°K. The HPLC-MALLS data were obtained using a Waters Alliance GPCV 2000 coupled with DAWN DSP-F (MALLS) and Shodex OH pak 803 + 805 columns.

1g of β -chitosan was swollen in 100 ml water for a day, 1 ml of acrylic acid (AA) was added, stirred until all the chitosan was highly swollen, (in some runs 5 ml of 5% HCl was added as a catalyst) heated at 90°C for three hours then cooled to ambient temperature and let stand overnight to give the product *N*-CEC (A).

1g of β -chitosan was swollen in 100 ml water for a day, then 1÷2 g of ClCH_2COOH was added, stirred until all the chitosan was highly swollen, then heated at 90°C for 3 hours while keeping the pH at 8-8.5 by addition of NaHCO_3 or Na_2CO_3 , to give the product *N*-CMC (B) [6, 7]. The two mixtures *N*-CEC (A) and *N*-CMC (B) [3, 4] are treated by the same way of using high speed centrifuge at 10,000 rpm to separate the residue, filtered under pressure through 0.45 μm

Sartorius membrane, precipitate them (at pH = 8 - 8.5) by 95% EtOH and lyophilised or washed it many times by 95% EtOH, then dried in vacuum oven at 50°C and 50mbar for two days.



3. RESULTS AND DISCUSSION

Decrystallised chitin/chitosan, previously shown to have many advantages over non-decrystallised chitin/chitosan for the preparation of derivatives [6], was used as starting material. Moreover, as mentioned above, in developing a simple technique for obtaining these derivatives it is considered preferable for the structures to be as similar as possible to that of chitin itself. This means that the modified molecular chain should contain the highest possible content of NADG units, which is the repeat unit in chitin, while retaining total water solubility. Decrystallized (lyophilized) β -chitin, having a DA of about 50 - 60%, was therefore used as starting material. Although it was not dissolved totally in acidic medium it was swollen strongly so that it could be readily modified during the reaction process, then step by step be dissolved totally in the aqueous reaction medium.

The synthesis of *N*-CMC, *N*-CBC and *N*-PC through reaction of chitosan with the appropriate carbonyl compound involves two reaction steps:

- First is the formation of imine (or ketimine) ($-N = CHR$; $-N = CR_1R_2$) between the primary or secondary carbonyl group of the reagent with the $-NH_2$ group of a chitosan unit.
- Secondly, hydrogenation by $NaBH_4$ to convert the imine or ketimine into saturated compounds, forming $-NH-CH_2R$ or $-NH-CHR_1R_2-$ linkages in all chitosan *N*-carboxyalkylated derivatives.

Only the mono-*N*-CMC was formed when the molar ratio of glyoxylic acid:chitosan amine groups is $\leq 2:1$. If the ratio is greater than 3:1 the product was found to contain both mono-*N*-CMC and di-*N*-CMC units [3 - 6]. If the procedure using a molar ratio $\geq 3:1$ was repeated 4 times, a compound of more than 90% di-*N*-CMC was obtained [6]. The *N*-CMC products were readily soluble in water at any such molar ratio, with no aggregation (gelation) being observed, provided that the pH is carefully controlled at 4.5 - 5 by gradual addition of a diluted HCl solution throughout the hydrogenation reaction stage.

The *N*-CBC derivative was also obtained without difficulty using fully deacetylated chitosan (DA~O) [5, 6] and a molar ratio of levulinic acid: amine group $> 5:1$; some di-*N*-CBC was also formed under these conditions. If the molar ratio was $< 1.5:1$, cyclisation took place to give 5-methylpyrrolidinone chitosan *N*-PC [12]. The structures of these compounds were demonstrated by 1D and 2D-NMR spectra [15, 16].

N-CEC may be produced more simply than *N*-CMC or *N*-CBC through the Michael addition reaction between acrylic acid (AA) and chitosan. This involves α,β -unsaturated carbonyl reagents such as acrylic acid reacting with the reactive hydrogen in the $-\text{NH}_2$ groups of chitosan. Using 5% HCl as catalyst gives a higher yield of water soluble *N*-CEC, so that very little residue separates out after high speed centrifuging. One possible explanation is that hydrolysis of the chitosan chains occurs in the acidic medium thereby increasing the solubility. The Michael reaction product was precipitated with EtOH, and washed extensively with 95% EtOH at pH > 8.5 to remove nearly all residues of AA and AA homopolymer. The typical signal in ^1H -NMR of the $-\text{CH}_2-$ of the *N*-CEC product was readily identified, unlike a previous report [14] in which only the $-\text{CH}_2-$ signal was assigned (this can sometimes be easily confused with the $-\text{CH}_2-$ group in AA or its polymer). The AA residue signals are readily assigned because their chemical shifts are located separately to those of *N*-CEC. Moreover, the value of the $(-\text{CH}_{2b}-)$ peak area versus those of $(\text{H}_{1a} + \text{H}_{1b})$ (3.357/1 + 1.18) demonstrated that the signal of the AA homopolymer is overlapped simultaneously. The assignments (ppm) of the *N*-CEC spectra by ^{13}C -NMR DEPT at 300°K (Fig. 1) are as follows:

$-\text{CH}_3$:	23.73
$-\text{C}3$:	71.82 - 73.09
$-\text{CH}_{2b}$:	34.08
$-\text{C}5$:	76.01 - 76.40
$-\text{DMSO}$:	39.17 - 39.84
$-\text{C}4$:	77.73 - 78.49
$-\text{NH}-\text{CH}_{2a}$:	46.24
$-\text{Cl}$	99.41 - 102.91
$-\text{C}2$:	57.69
$-\text{COOH}$:	181
$-\text{C}6$:	61.64

AA residue : 128 – 135.

The ^1H -NMR spectra of chitosan and *N*-CEC are recorded at 300 and 353°K (the first one is not shown). The latter one is better for assigning the *N*-CEC spectrum: (Fig. 3) δ (ppm, peak area in brackets).

$-\text{COCH}_3$:	2.57 (2.088)
$-\text{H}_{1c}$ of GINAc :	5.14 (0.23)
$-\text{CH}_{2b}$:	3.075 (3.357)
$-\text{H}_{1b}$ of GINH_2 :	5.26 (1.181)
$-\text{H}_2$ of <i>N</i> -CEC :	3.54 (2.737)

- H1a of N-CEC : 5.44 (1)
- H2 of GINH₂ : 3.64 (1.712)
- AA residue : 6.46-6.89
- NH-CH_{2a}⁻ : 3.90 (2.194)
- H3, 4, 5, 6 : 4.115 - 4.474.

It will be getting better if using the H1 (a, b, c) signals as reference since their presence in all units of chitosan (GINAc, GINH₂, N-CEC). The degree of substitution is estimated from the peak area at $\delta = 3.90$ ppm of the (-NH-CH_{2a}⁻) proton (2.194) compared with the sum of those of H1_b and H1_a at (ppm) 5.26 (1.18) and 5.47 (1) is 49%.

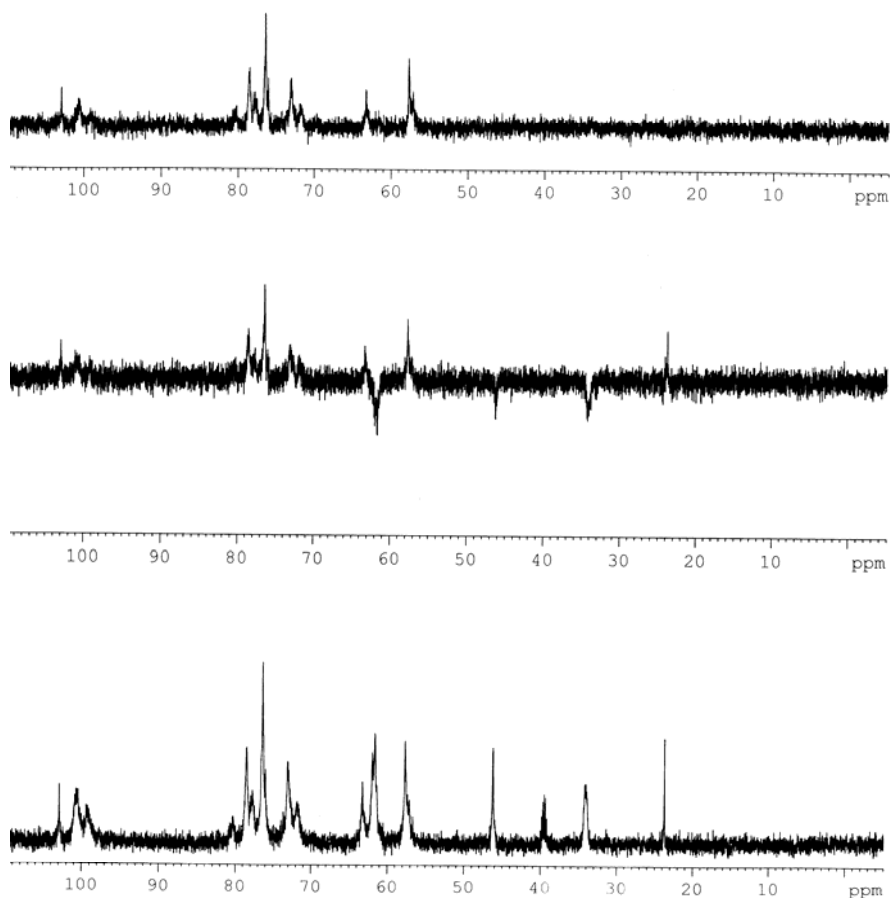


Fig. 1: ¹³C NMR.DEPT spectrum of N-CEC at 300 °K, 500 MHz

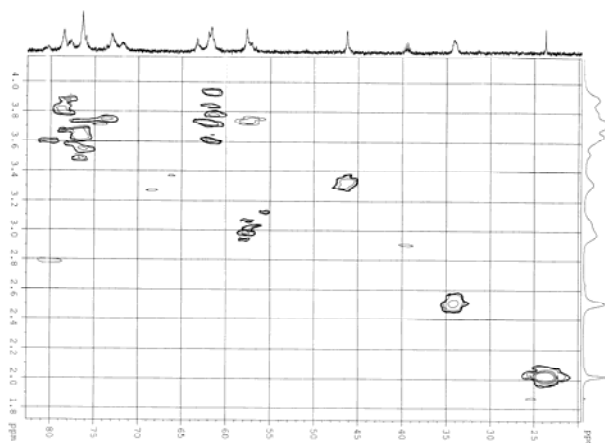


Fig. 2: ^{13}C - ^1H - NMR cosy spectrums of *N*-CEC at 300 °K, 500 MHz

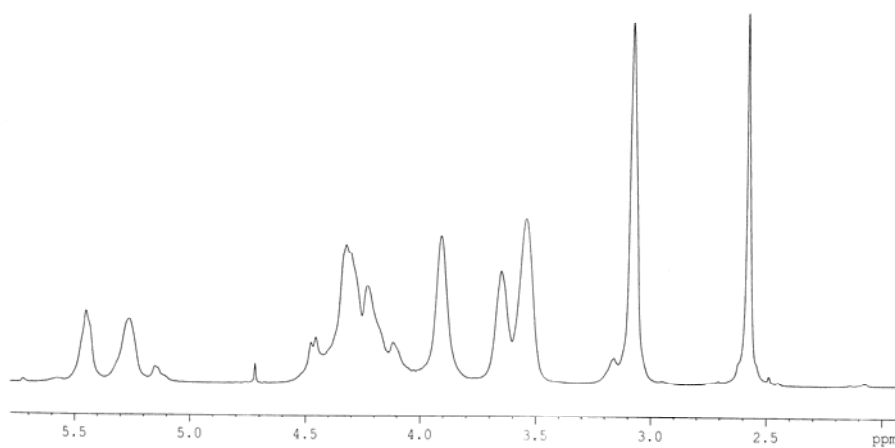


Fig. 3: ^1H - NMR spectrum of *N*-CEC at 353 °K, 500 MHz

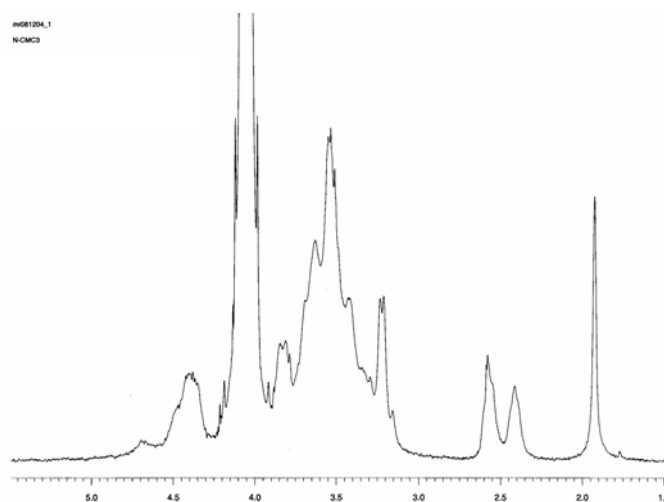


Fig. 4: ^1H -NMR of *N*-CMC (300MHz, 353°K)

All the *N*-carboxyalkylated chitosan derivatives of this series were investigated carefully by 1D and 2D NMR spectra.

It should be noticed that the ^1H -NMR signal of the $(-\text{CH}_{2b}-)$ group in the *N*-CEC compound sometimes overlaps with that of $(-\text{CH}_2-)$ in AA homopolymer (it will not be the case if it is washed well) so the $(-\text{NH}-\text{CH}_{2a}-)$ linkage of *N*-CEC is better for characterising both *N*-CEC and the other *N*-carboxyalkylated chitosan derivatives.

The *N*-TMC derivative was formed by reacting methyl iodine (CH_3I) with decrystallized (lyophilised) chitosan in suitable condition as presented previously [6]. It must be emphasised that only a one-stage reaction was required to reach a degree of substitution of *N*-TMC of 59%, and not 4 stages as is reported in [10 - 12]. It was completely soluble over a large pH range.

In the alkylation of chitin/chitosan by ClCH_2COOH using 25 % NaOH at 60°C , the products obtained had more than 70% of the $\text{C6}(\text{OH})$ and 47% of the $\text{C3}(\text{OH})$ groups substituted, but only 20% of the $\text{C2}(\text{NH}_2)$ groups. This means that although *N,O*-carboxymethylation of chitosan has occurred, almost all substitution has been at C6 and C3. It should be noted that this alkylation reaction will run smoothly in basic medium if the chitosan was dispersed well, but not if it is present as a separated gel in the modification process.

To obtain water-soluble *N*-CMC, in which chemical modification has taken place only at the $\text{C2}(\text{NH}_2)$ position, the reaction was carried out at pH 8 - 8,5 catalysed by NaHCO_3 or Na_2CO_3 . The products were characterised using 300 MHz ^1H and ^{13}C DEPT nmr and compared with the previous data [3, 4]. This simple preparation route gave *N*-CMC without any *O*-carboxymethylation. The mono-*N*-CMC shows a peak at 58.6 ppm (^{13}C - nmr) and the di-*N*-CMC at 3.27 ppm (^1H -nmr) and 53.7 ppm (^{13}C -nmr), (Fig. 4, 5). The degree of substitution is 0.53.

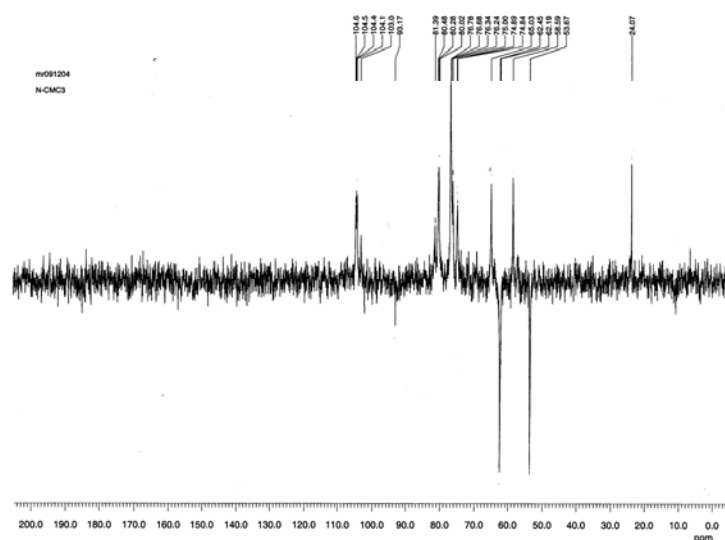
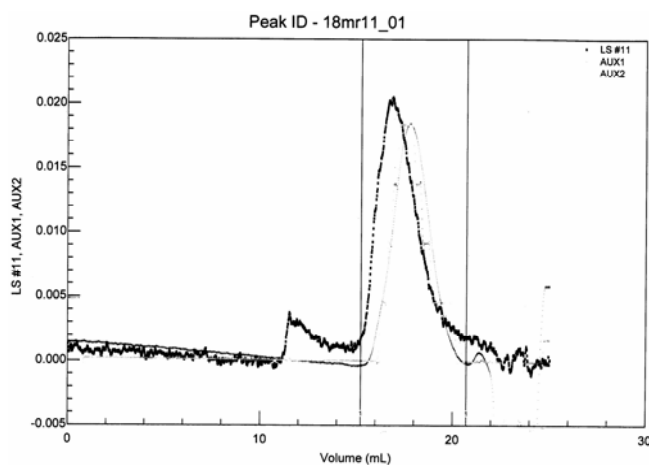


Fig. 5: ^{13}C DEPT-NMR of *N*-CMC (300MHz, 353°K)

**Fig. 6:**

The ^1H - NMR is recorded at 353°K Bruker 300 MHz as followed (ppm) [6 - 8]:

- NAc:	1.94
- H2 of <i>N</i> -diCMC:	2.40
- H2 of <i>N</i> -mono CMC: and chitin unit	2.60
- N-(CH) ₂ -:	3.15 ÷ 3.30
- N-CH ₂ :	3.25 ÷ 3.4
- H3,4,5,6:	3.45 ÷ 3.95
- H1 of <i>N</i> -diCMC:	4.45
- H1 of <i>N</i> -mono CMC:	4.70

and chitin unit.

The molecular weight and radius of gyration values were determined by Waters Alliance GPCV 2000 coupled with DAWN DSP-F (MALLS) and are shown in Table 1 and Fig. 6.

Table 1: Molecular weight and gyration radius

Samples (g/mol)		N - CMC	N - CEC
Mn		$1.353.10^5$	$4.335.10^4$
Mw		$1.485.10^5$	$6.714.10^5$
Mz		$1.621.10^5$	$9.334.10^6$
polydispersity	Mw/Mn	1.098	1.549
	Mz/Mn	1.198	2.153
Radius moments (nm)	Rn	59.1	42.5
	Rw	56.3	45.6
	Rz	53.7	48.3

3. CONCLUSIONS

Three types of reaction are available for preparing water-soluble chitosan *N*-carboxylated derivatives: a) alkylation; b) imine-hydrogenation; c) Michael addition. Alkylation by ClCH_2COOH at $\text{pH} = 8 - 8.5$ is a new and simple way of forming a water-soluble *N*-carboxylated chitosan derivative. To preserve as much of the chitin structure as possible, a starting chitosan of 50 - 60% DA was used.

The $(-\text{NH}-\text{CH}_2-)$ linkage is typical for all compounds of this series. located separately in NMR spectra so that it could be estimated easily.

In the Michael addition reaction homopolymerisation of acrylic acid was sometimes observed, depending on the reaction conditions (temperature, reagent molar ratios). Only the *N*-CH_{2a} signal could be assigned for this kind of derivative, but not $-\text{CH}_2\text{b}-$ one.

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