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Optimized synthesis of O-carboxymethyl-N,N,N-trimethylamino chitosan

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Abstract:

We present here the synthesis of a highly O-carboxymethylated chitosan derivative. First, an improved protocol for the two-step synthesis of N-trimethyl chitosan (TMC) from chitosan was developed, yielding a maximum degree of quaternization (DQ) of up to 46.6 %. Successively, the chitosan derivative O-carboxymethyl-N,N,N-trimethyl chitosan (CMTMC) was synthesized from the TMC obtained by applying an optimized synthesis pathway. In contrast to the previous reports, the optimized protocol was shown to yield very high rates (>85%) of O-carboxymethylation of CMTMC, as shown by ^{1}H NMR and heteronuclear single quantum correlation (^{1}H- ^{13}C HSQC). Finally, *in vitro* cytocompatibility (viability >80 %) of the polymer was demonstrated using human dermal fibroblasts.

Keywords: chitosan, chitosan derivatives, drug/gene delivery, methylation, quaternization

Abbreviations: N,N,N-trimethyl chitosan (TMC); O-carboxymethyl-N,N,N-trimethyl chitosan (CMTMC); degree of quaternization (DQ).

1. Introduction

Chitosan, a biopolymer used in numerous medical applications, is obtained through partial deacetylation of chitin (Sieval et al., 1998). Chitosan has been widely used in medical applications, wastewater treatment, textile industry, cosmetics and agriculture, because of its unique properties. Indeed, chitosan is a biodegradable, biocompatible, haemostatic, and non-toxic polymer, with antibacterial and antimicrobial activities (Jayakumar et al., 2010; Lee et al., 2014; Muzzarelli, 2009; Rinaudo, 2008), and is also an efficient biosorbent useful for environmental purposes (Patrulea et al., 2013). However, the application of chitosan is limited due to its poor solubility at physiological *p*H, which in turn decreases efficiency of drug delivery below *p*H 6 (Sieval et al., 1998). In this view, several methods were investigated to improve chitosan solubility. The preferred approach to increase chitosan solubility at neutral *p*H is to introduce permanent charges into the chitosan backbone. In order to obtain a water-soluble chitosan derivative, Domard et al. (1986) prepared quaternized chitosan through methylation (Domard et al., 1986), while Chen and Park (2003) developed carboxymethylated chitosan, and Holme and Perkin (1997) proposed sulfonated chitosan.

N,N,N-Trimethyl chitosan chloride (TMC) is a positively charged chitosan derivative soluble at neutral *p*H. It has been shown that TMC has an enhanced solubility, promotes intestinal absorption of peptides and drugs, and has mucoadhesive properties (Amidi et al., 2006; Polnok et al., 2004). Nanoparticles based on TMC are used as delivery systems for drugs, DNA or RNA therapeutics. Domard et al. and later Sieval et al. prepared TMC by treating chitosan with N-methyl-2-pyrrolidone followed by reaction with methyl iodide in the presence of sodium hydroxide and sodium iodide (Domard et al., 1986; Sieval et al., 1998). The reaction was carried out in several steps in order to obtain a higher degree of substitution (up to 64%), but drawbacks were reported, such as a reduced molecular weight attributed to chain scission, decreased solubility, and uncontrolled degree of trimethylation leading to O-methylation (Verheul et al., 2008). Generally, during quaternization, O-methylation may occur, leading to merely loosely controlled polymer characteristics. In order to avoid O-methylation, Muzzarelli et al.

reported on a method for N-dimethyl-chitosan (DMC) formation followed by synthesis of TMC with formaldehyde and borohydride (Muzzarelli & Tanfani, 1985). The same principle was used by Verheul et al. (2008) and Xu et al. (2010) by switching from borohydride to using formic acid.

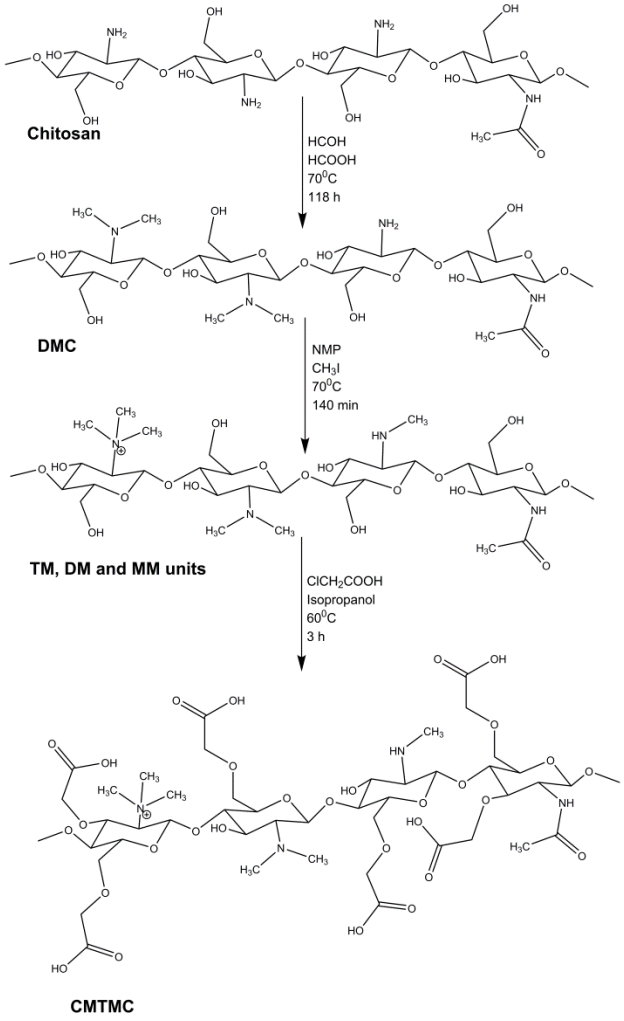

Figure 1. **Optimized synthesis of O-carboxymethyl-N,N,N-trimethyl chitosan (CMTMC).**

N,O-Carboxymethyl-chitosan (CMC) has drawn much attention due to its improved solubility in water. CMC is a polyelectrolyte derivative, which contains carboxymethyl substituents at carboxyl and amino sites. CMC is a water-soluble polymer, with its solubility being related to the degree of substitution (George & Abraham, 2006; Muzzarelli, 1988). Some authors reported that CMC is biocompatible

and mucoadhesive polymer, which does not induce immune responses (Jayakumar et al., 2010; Yin et al., 2007) and is of low toxicity (Tokura et al., 1996). Such characteristics turn CMC into a unique platform for grafting peptides, proteins or drugs for effective delivery (Wang et al., 2010; Wang et al., 2013), cell delivery vehicle for *in situ* bone tissue engineering (Mishra et al., 2011) and safe parenterally applied system (Fu et al., 2011). In view of functionalizing chitosan with high amount of peptide for increased bioactivity, chitosan bearing a high number of O-carboxymethyl alkyl groups is a highly desirable product.

The aim of this study was therefore to develop a water-soluble O-carboxymethyl-N,N,N-trimethyl chitosan (CMTMC) with a degree of substitution higher than previously reported, based on TMC without O-methylation, as shown in Figure 1. To this end, optimization of each synthesis step – N-trimethylation and O-carboxymethylation - was carried out.

2. Materials and methods

2.1. Materials

Chitosan (79% degree of deacetylation, ChitoClear Cg10, 7–15 mPa*s) was bought from Primex (Siglufjordur, Iceland). Chloroacetic acid, methyl iodide (CH_3I) (99%), N-methyl-2-pyrrolidone (NMP) (99%), deuterium oxide (D_2O), deuterium chloride (DCl), deuterium oxide supplemented with 0.05% 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid and sodium salt (TSP) were purchased from Sigma–Aldrich (Buchs, Switzerland). Sodium hydroxide (98.5%) and diethylether (99.5%) were obtained from Acros Organics (Geel, Belgium). Formic acid (85%) was bought from Reactolab (Servion, Switzerland) and formaldehyde (37%) was ordered from Merck (Darmstadt, Germany). Ethanol (99.9%) and isopropanol (99.9%) were obtained from Fischer (Wallingford, UK). Dulbecco`s modified Eagle medium (DMEM) and all other reagents were purchased from Sigma–Aldrich (Buchs, Switzerland).

2.2. Methods

2.2.1. O-Carboxymethyl-N,N,N-trimethyl chitosan (CMTMC) synthesis

CMTMC was obtained by O-carboxymethylation of N-trimethyl chitosan (TMC). TMC was synthesized in two ways, either by a “conventional” 1-step method ("TMC 1"), or an “optimized” 2-step method ("TMC 2"). CMTMC was further obtained by carboxymethylation of TMC 2 based either on a “conventional” protocol previously used in our group (Hansson et al., 2012a) yielding a product designated "CMTMC 1", or using a new method to increase the substitution degree and potentially enhance drug, peptide or protein grafting ("CMTMC 2").

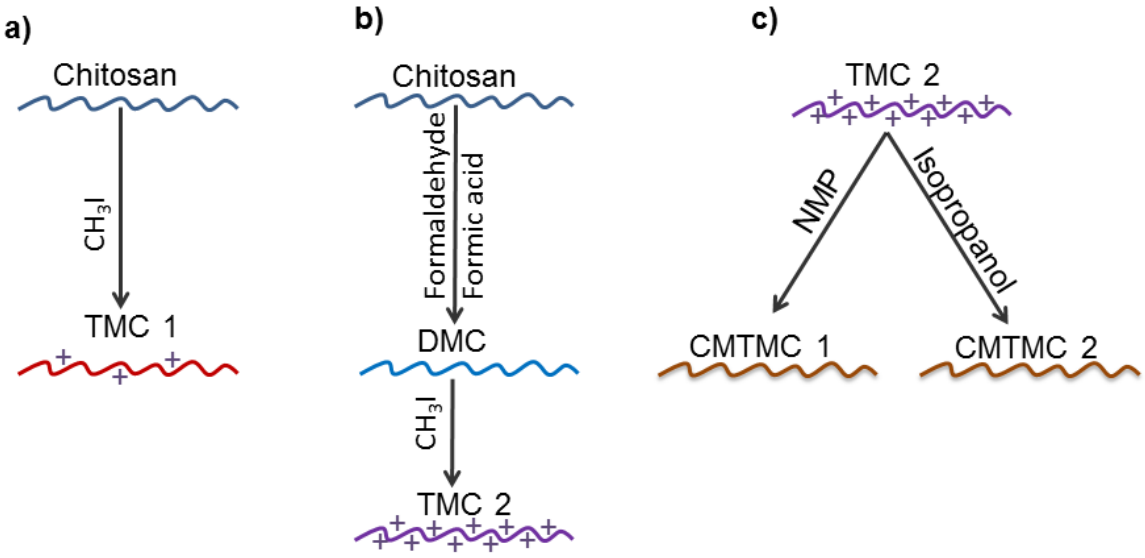

Figure 2. Scheme of the experiments performed for (a) TMC 1 synthesis by conventional 1-step method, (b) TMC 2 synthesis by optimized 2-step method and (c) O-carboxymethylation of TMC 2.

2.2.2. Conventional synthesis method – TMC 1

For the conventional synthesis, TMC was synthesized through nucleophilic substitution with CH_3I as a reagent, sodium iodide as catalyst and sodium hydroxide

(NaOH) as base with NMP as solvent, modifying the protocol described by Hansson et al. (Hansson et al., 2012b). We evaluated the effects of reaction time and solvent used for precipitation on quaternization.

Briefly, 4.0 g of chitosan and 9.6 g of NaI (molar ratio 4:1) were added to 160 mL pre-warmed NMP at 60 °C. After 30 min of reaction, the *p*H of the solution was adjusted to a value of 10 using 15% (w/v) NaOH and left to react for 15 min. Later, 24 mL of CH_3I (chitosan:CH_3I molar ratio of 1:2) were added and left to react for the desired time (varying, see below). The solution was filtered through a P3 glass filter under vacuum in order to remove any insoluble residues. Then, the solution was precipitated by adding 5 volumes of ethanol and diethylether at different ratios (varying, see below). Subsequently, TMC was separated from the supernatant by centrifugation (Beckmann Avanti TM 30, UK) at 3220 x *g* for 15 min. The precipitate was dissolved in 10% NaCl and then stirred overnight at room temperature. Furthermore, dialysis (Spectra/Por 4, cut-off 12-14 kDa, Spectra, USA) was performed for 3 days with water changes twice daily. Samples were lyophilized (Christ Alpha 2-4 LD Plus, Switzerland), which was followed by analysis.

In order to study the effect of reaction time, we left reactions for 20, 40, 60, 100, 140 min or overnight. After filtration, TMC was precipitated in a 1:1 mixture of ethanol and diethylether. We also investigated the effect of ethanol:diethylether ratio (1:0; 1:1; 2:1; 3:1; 4:1; 5:1; 1:2; 1:3; 1:4 or 1:5) used for precipitation, for a given reaction time of 140 min.

2.2.3. Optimized method – TMC 2

The optimized synthesis of TMC was carried out in two steps: first by the Eschweiler-Clarke reaction, treating chitosan with formaldehyde in the presence of formic acid using a protocol modified from Verheul et al. (2008) and second by treating the obtained N-dimethyl-chitosan (DMC) with CH_3I in order to obtain TMC 2, using the best reaction time and precipitation solvent ratio determined for TMC 1.

Briefly, 2 g of chitosan were added to 6 mL of formic acid, 8 mL of formaldehyde and 36 mL of distilled water. This reaction was carried out at 70 °C under reflux (Liebig condenser) for 118 h. Afterwards, the volume was reduced with a Rotavapor and the *p*H was subsequently adjusted to 12 using 1M NaOH. The precipitate was isolated by paper filtration, followed by washing with water in order to remove any impurities and un-reacted formaldehyde. Filtrated N-dimethyl-chitosan (DMC) was suspended in water at a concentration of 2% (w/v) and the *p*H adjusted to 4 using 1 M HCl and further stirred until complete DMC dissolution. The solution was filtered and dialyzed for 3 days, with water changes twice daily. Then the product was lyophilized for 2 days and analyzed.

1 g of dried DMC was solubilized in 160 mL of water under stirring at room temperature. The *p*H was adjusted to 11 by drop-wise addition of 1M NaOH until gel formation occurred. This step was followed by washing twice with water and three times with acetone through a filter. Then, dried precipitate was suspended in 50 mL NMP and the dispersion was stirred at 70 °C under reflux conditions until the complete dissolution of the chitosan derivative (DMC). Next, CH_3I was added at a ratio of 1:25 to the reaction medium and left for 140 min to complete the reaction. Subsequently, precipitation with ethanol:diethylether (4:1) and centrifugation at 3220 x *g* for 15 min were performed. Dialysis was done for 3 days. In order to obtain a better ion exchange, the first day of dialysis was done against a 1% NaCl solution in water and the last 2 days against water, changing dialysis media twice per day. The obtained TMC was lyophilized and analyzed as described below.

2.2.3. CMTMC synthesis: TMC carboxymethylation

2.2.3.1. CMTMC obtained with conventional carboxymethylation protocol – CMTMC 1

A quantity of 0.2 g of dried TMC 2 was suspended in 20 mL NMP and left to stir overnight at ambient temperature. The *p*H was adjusted to 10 with 15% NaOH, and kept constant by NaOH addition for 1 h. Then, 20 equivalents of mono-chloroacetate were added to the reaction and the *p*H was kept constant during 3 h of the reaction. After 3 h, reaction product was precipitated in a mixture of ethanol:diethylether (4:1). After

centrifugation at 3220 x *g* for 15 min, 10 mL of Milli-Q water were added to dissolve the precipitate and the *p*H was adjusted to 5 with 1M HCl. The solution was dialyzed for 3 days, changing water twice per day, and lyophilized samples were analyzed.

2.2.3.2. CMTMC obtained with new carboxymethylation protocol – CMTMC 2

An amount of 0.1 g of dried TMC was suspended in 10 mL of NMP or isopropanol as solvent and left to stir overnight at 45 °C. During 20 min, under continuous stirring, 0.5 mL of 50% NaOH was slowly added to the reaction mixture that was stirred for another 45 min. Subsequently, 0.12 g of mono-chloroacetate (molar ratio 1:3 to TMC) was added during 25 min and reaction was left for 3 h at 60 °C under a nitrogen atmosphere in order to avoid TMC degradation. Afterwards, *p*H was adjusted to neutral with 1M HCl followed by filtration, washing with 70% ethanol and subsequently with anhydrous ethanol. CMTMC precipitate was dried under vacuum, dissolved in distilled water and dialyzed for 3 days against water, changing dialysis medium twice per day. The dialyzed CMTMC 2 was lyophilized and later analyzed.

2.3. Characterization of polymers

2.3.1. Determination of the degree of substitution

^{1}H-NMR and ^{13}C-NMR spectra were recorded with Varian Gemini 300 MHz or 500 MHz (Agilent Technologies, Santa Clara, CA, USA). Chemical shifts are reported as part per million (ppm) at room temperature. The samples were dissolved in 1% DCl/D_2O with 0.05% trimethylsilyl propionic acid (TSP) as internal standard for δ=0.00 ppm. The protons in the chitosan derivatives were attributed according to literature (Hansson et al., 2012b; Heuking et al., 2009; Polnok et al., 2004) as follows: δ=2.0 ppm (COCH3); δ=2.8 ($NHCH_3$); δ=3.0 ($N(CH_3)_2$); δ=3.3 ($N^+(CH_3)_3$); δ=4.15-4.5 (OCH_2COOH) and δ=5-5.7 (H-1).

The degrees of monomethylation (Heuking et al.), dimethylation (Luca et al.), trimethylation (Howling et al.) and carboxymethylation (CM) were calculated with the methods previously described, using Equation (1-4) as shown below (Polnok et al., 2004):

$$MM(\%) = \left[\frac{[CH_3]}{[H]} \times \frac{1}{3}\right] \times 100 \quad (1)$$

$$DM\ (\%) = \left[\frac{[(CH_3)_2]}{[H]} \times \frac{1}{6}\right] \times 100 \quad (2)$$

$$TM\ (\%) = \left[\frac{[(CH_3)_3]}{[H]} \times \frac{1}{9}\right] \times 100 \quad (3)$$

$$CM\ (\%) = \left[\frac{[(OCH_2 - COOH)]}{[H]} \times \frac{1}{2}\right] \times 100 \quad (4)$$

$[CH_3]$ is the value of the integral for the MM functions at 2.8 ppm; $[(CH_3)_2]$ is the integral of dimethyl amino DM at 3.0 ppm, $[(CH_3)_3]$ is the integral for trimethyl amino functions at 3.3 ppm, $[(OCH_2—COOH)]$ represents the integral for carboxymethyl groups at 4.15-4.5 ppm and [H] is the value for the integral attributed to H-1 peaks between 5 and 5.7 ppm.

2.3.2. Cytotoxicity test WST-1

In vitro toxicity of CMTMC was evaluated using the cell proliferation reagent WST-1 (Roche, Switzerland) on human dermal fibroblasts (provided by Centre Hospitalier Universitaire Vaudois under Ethics Committee Protocol #62/07, Lausanne, Switzerland). Human dermal fibroblasts were seeded in a 96-well plate at an initial seeding density of 5×10^3 cells per well and incubated for 48 h at 37 °C and 5% CO_2. Afterwards, the culture medium was replaced by a polymer solution in DMEM with concentrations of either: 1 mg mL^{-1}, 0.5 mg mL^{-1} or 0.1 mg mL^{-1}. Cells were incubated for 0, 2, 4, 7 and 10 days. Positive (DMEM) and negative controls (sodium dodecyl sulfate (SDS) 1%) were used. Polymer suspension was replaced every 3 days. Subsequently, polymer suspensions were aspirated and replaced by 100 µL of WST-1 (diluted 1:10 in DMEM) and incubated

for 0.5 h to 4 h. Absorbance (BioTek Microplate Reader, GmbH, Luzern, Switzerland) was measured at wavelengths 450 and 690 nm according to (Fisichella et al., 2009).

3. Results and discussions

3.1. TMC characterization

TMC 1 synthesis through direct trimethylation of chitosan with iodomethane led to O-methylation, as shown by NMR peak at δ=3.4 ppm (Figure. 3A), which can be attributed to O-CH_3. This is in agreement with uncontrolled O-methylation at C-3 and C-6 as previously reported (Verheul et al., 2008). A similar process was followed by Li et al. (2010), which is likely to result in non-selective O- and N-trimethylation. Hence, the degree of quaternization (DQ) and in turn the degree of carboxymethylation would be difficult to control.

Table 1. Increase of the degree of trimethylation during the reaction (n=3).

Reaction time (min)	Degree of quaternization (%)
100	24.5±1.2
140	36.8±9.3
overnight	22.7±3.3

Varying reaction time (from 40, 60, 100, 140 and overnight) influences DQ (Table 1). For 40 and 60 min (data not shown), the DQ achieved was about 20%. While increasing reaction time to 100 and 140 min, DQ increased linearly reaching a plateau after 140 min. These results are in good agreement with Kean et al., who showed that the DQ of TMC increased linearly until 120 min of reaction reaching a plateau (Kean et al., 2005). In addition, we observed that changing the solvent ratio influenced the DQ. The best DQ (41±9.8%) was obtained with a 4:1 solvent ratio. Higher and lower ratios yielded DQ in the range of 17 to 24%, by changing solvent ratio ethanol: diethylether

(1:0; 1:1; 2:1; 3:1; 5:1; 1:2; 1:3; 1:4; 1:5).

For TMC 2 optimized synthesis, we used the reaction time (140 min) and the solvent ratio (4:1) that lead to highest degrees of TMC 1 quaternization. We obtained a higher degree of substitution (46.6%) in the absence of O-methylation at C-3 and C-6, as confirmed by NMR (Figure. 3). Moreover, chain scission was prevented (size exclusion chromatography data, not shown). This method is expected to control the DQ without altering the structural properties of the glucosamine units (Verheul et al., 2008).

In Figure. 3 are presented chemical shifts for TMC 1 and TMC 2. The signal intensity present in TMC 1 at 3.4 ppm (peak 8) demonstrates that O-methylation occurred during the reaction. In contrast, spectra (B) of TMC 2 obtained through the optimized method shows O-methylation-free TMC. As a result, DQ increased from 29.4% for TMC 1 to 46.6% for TMC 2. This increased quaternization was also related to an improvement of solubility and is considered to be advantageous for complexation with anionic polymers in order to form nanoparticles, or to carry drug or nucleic acids for pharmaceutical applications.

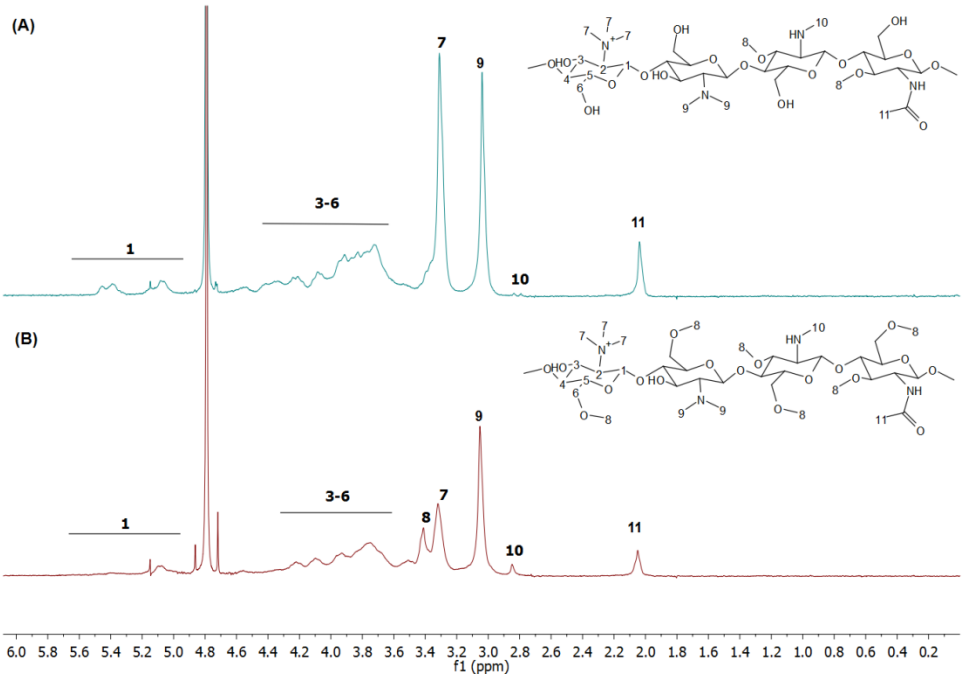

***Figure 3*. ^{1}H-NMR spectra for TMC 1 (A) and for TMC 2 (B) in D_2O.**

3.2. CMTMC characterization

Concerning carboxymethylation, Hansson et al. used chloroacetic acid in NMP under basic conditions (*p*H 10). By varying reaction time, they substituted up to 27% carboxymethyl groups (Hansson et al., 2012b). This relatively low value may be due to previous O-methylation at C-3 and C-6, resulting in a low availability of OH groups for carboxymethylation (Figure. 4A). Using NMP, we also observed a very low degree of substitution (data not shown). Switching reaction medium to isopropanol led to better results, achieving degrees of O-carboxymethylation in extent of 85% (Figure. 4B) at an overall yield of 74%. This might be related to a better conformation of TMC in isopropanol offering higher accessibility to the reaction sites.

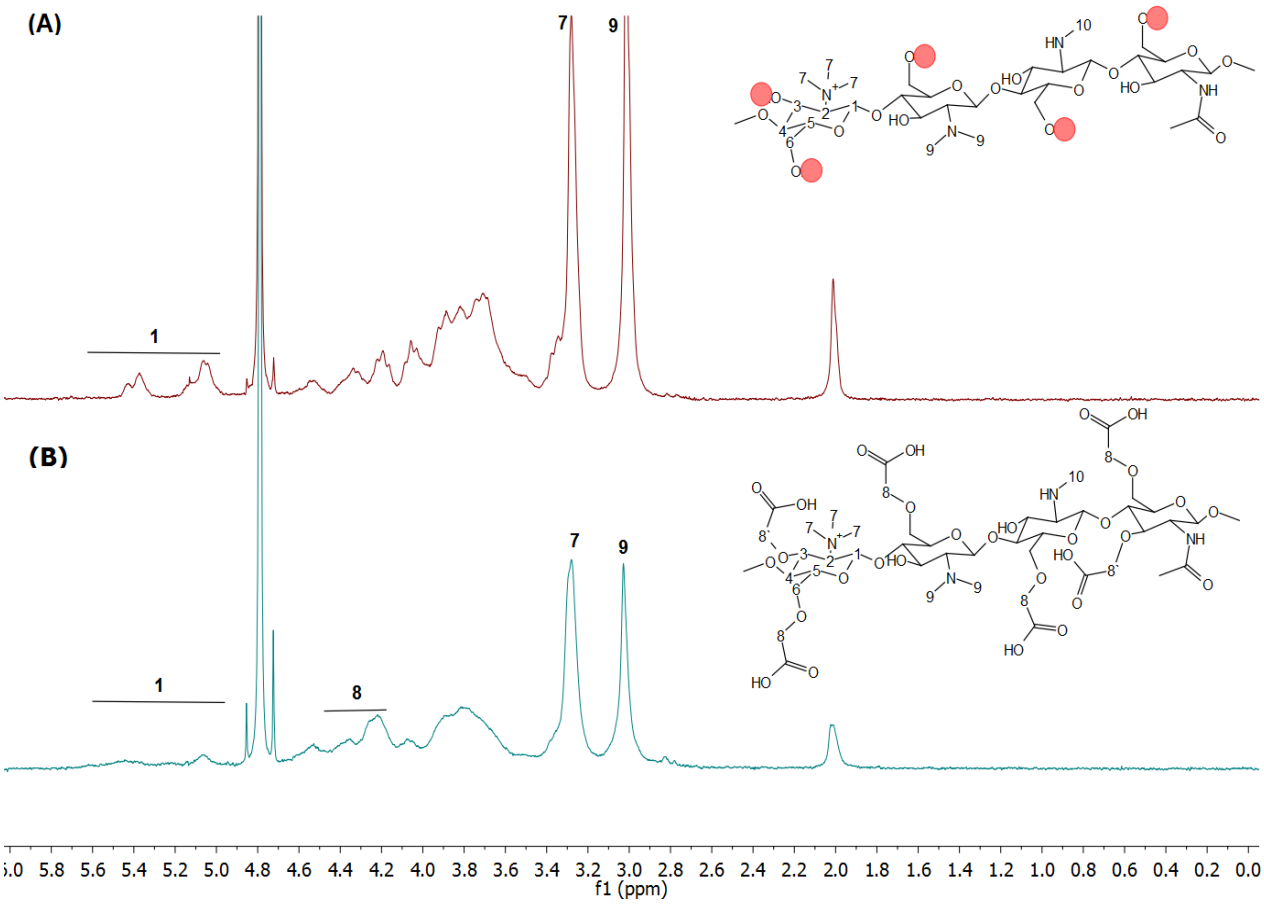

Figure. 4. ^{1}H-NMR spectra for CMTMC 1 (A) and CMTMC 2 (B). Red bullets correspond to O-methylated C-3 and C-6.

Successful O-carboxymethylation of TMC is further demonstrated by heteronuclear single quantum correlation (^{1}H-^{13}C HSQC) map (Figure. 5). The ^{13}C-NMR signals that belong to the –COOH group substituted on OH-3 and OH-6 are present at f_1 =71.1 ppm and 71.5 ppm, respectively, and also appear on ^{1}H-NMR spectra (Figure. 4B) at 4.15-4.5 ppm (peak 8). Their positions on HSQC map indicate that there are two possible sites for O-carboxymethylation on the TMC backbone, but the major active site is on C-6 as shown by the presence of a peak at 4.15 ppm (Chen et al., 2003; Chen & Park, 2003).

The high degree of O-carboxymethylation was obtained by using a strong basic medium, pointing out the importance of alkaline conditions for O-carboxymethylation. A sufficiently strong base is needed to allow chloroacetate penetration on the whole TMC chain, avoiding side reactions between NaOH and chloroacetate (Barros et al., 2013).

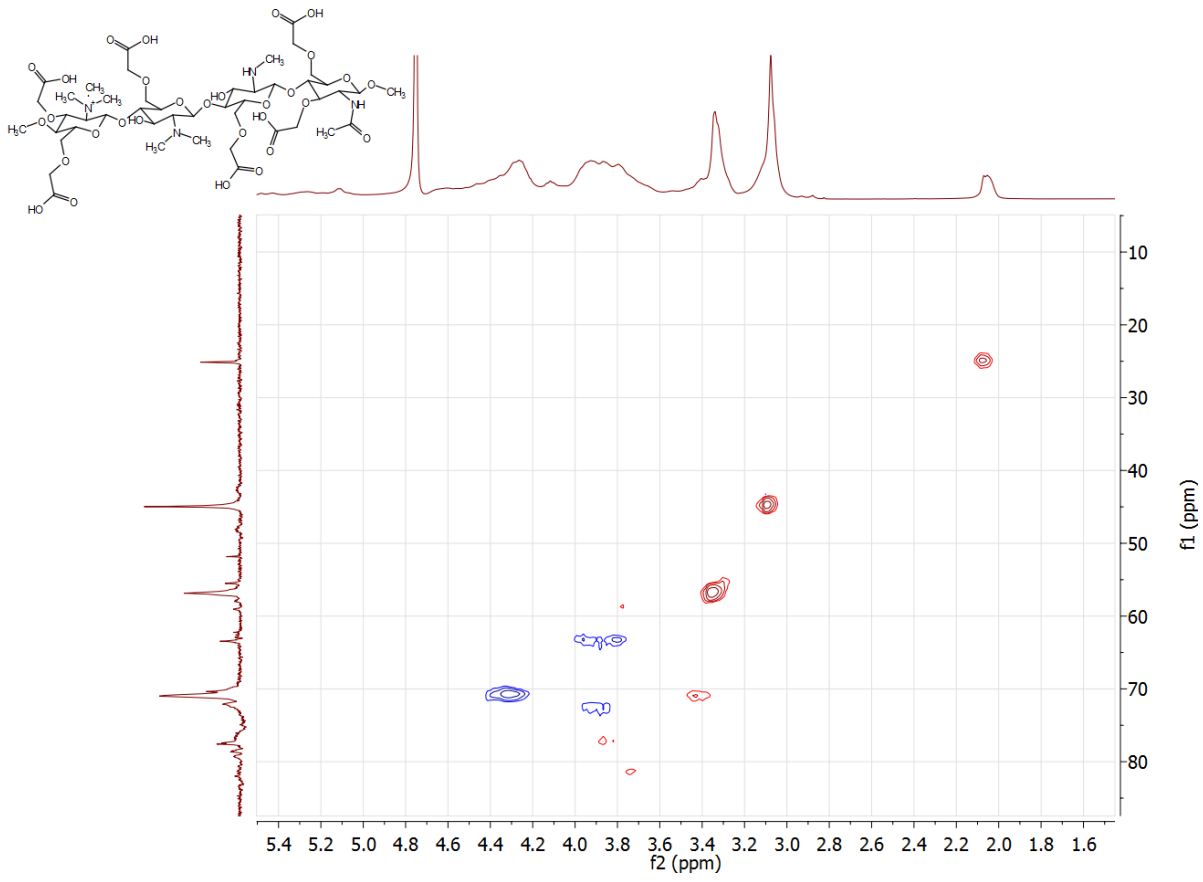

Figure 5. ^{1}H-^{13}C HSQC spectrum for CMTMC 2 with TSP as an internal standard.

3.3. Cytotoxicity

After 10 days of CMTMC suspension exposure to human dermal fibroblasts, the results showed no significant toxicity (viability > 80% in all cases) (Figure. 6). The lowest CMTMC concentration of 0.1 mg/mL resulted in highest cell viability (> 100%), while 1 and 0.5 mg/mL CMTMC showed a minor decrease in viability (≈82%), as compared to the negative control (SDS 1%, 7 – 11% viability). This absence of CMTMC cytotoxicity is consistent with literature reports (Yin et al., 2007).

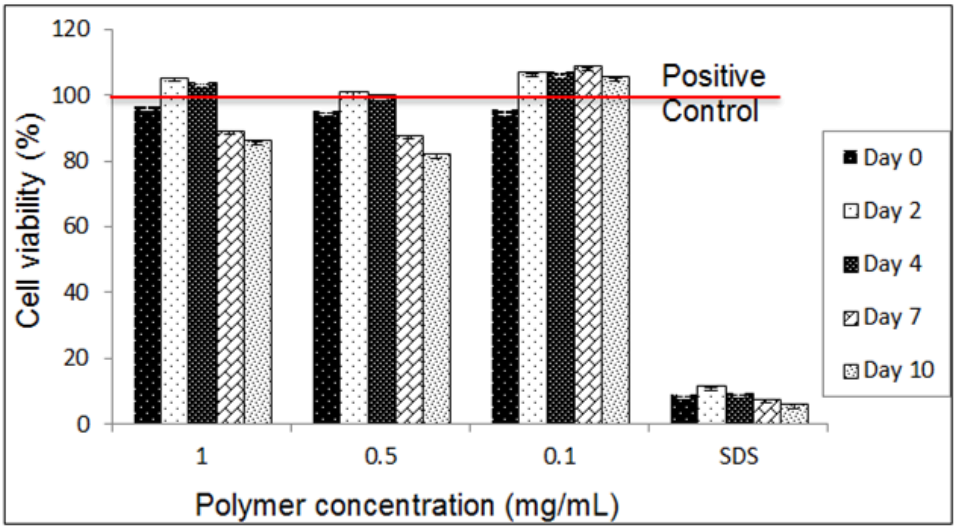

Figure. 6. Human dermal fibroblast viability upon exposure to CMTMC polymer solutions for 0 to 10 days. Results are given as mean ± SD (n=3).

4. Conclusions

The present work demonstrates that O-carboxymethyl chitosan can be obtained with unprecedented degree of carboxymethylation, higher than 85% based on O-methylation free TMC. As for TMC synthesis, we determined optimal reaction time and solvent ratio for precipitation. O-methylation-free TMC was obtained using the Eschweiler-Clarke reaction step. O-Carboxymethylation was carried out using sufficiently strong base in

isopropanol. Further in vitro results indicated that CMTMC was devoid of toxicity on human dermal fibroblasts with no negative influence on cell growth and morphology.

Taken together, these results provide a chitosan derivative platform for drug delivery. The highly O-carboxymethylated chitosan provides a material for subsequent derivatization with peptide, e.g., through carbodiimide coupling chemistry, proteins or targeting moieties. The presence of charges enables formulation of nanoparticles, gels, or dried sponges as carriers, allowing multiple delivery modes for various biomedical applications.

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