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Determination of the average degree of quaternization of *N*,*N*,*N*-trimethylchitosan by solid state ^{13}C NMR

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Abstract

A novel method in which the quaternary salt of chitosan, *N*,*N*,*N*-trimethylchitosan (TMC) was synthesized using dimethylsulfate as the methylant agent is described. Although the synthesis of chitosan quaternary salts has been reported extensively, there remains some uncertainty in determining the resultant average degree of quaternization, $\overline{DQ}$, in the final products. Here we used CP-MAS ^{13}C NMR spectroscopy, that was able to calculate the $\overline{DQ}$ from the acetate salt of TMC. Chitosan in three different morphological forms (powder, wet-gel and flake) yielded TMC with $\overline{DQ} = 40.1\%$; 24.7% and 20.8%, respectively.

Keywords: *N*,*N*,*N*-trimethylchitosan; Dimethylsulfate; CP-MAS ^{13}C NMR spectroscopy

1. Introduction

The quaternary salt *N*,*N*,*N*-trimethylchitosan (TMC) is a cationic hydro-soluble polyelectrolyte. The quaternization process of the chitosan imparts permanent positive charges (Britto & Campana-Filho, 2004; Curti, Britto, & Campana-Filho, 2003; Dung, Milas, Rinaudo, & Desbrières, 1994; Sieval et al., 1998) and the derivative becomes soluble in a wide pH range (including acid, neutral and alkaline regions) whereas the parent chitosan is soluble only in pH < 6.5. Certain studies have demonstrated that the TMC has good intestinal absorption enhancing properties for hydrophilic and macromolecular drugs (Florea, Thanou, Junginger, & Borchard, 2006; Kotzé et al., 1997, 1998; Tahanou et al., 2000) and have been used to complex and deliver plasmid DNA in several gene delivery studies to treat genetic disease (Kean, Roth, & Thanou, 2005). In addition, the TMC has antibacterial activity (Jia, Shen, & Xu, 2001; Kim, Choi, Chun, & Choi, 1997), described as the resultant of ionic interaction between TMC positive charges and the negatively charged cell surface of the bacteria.

The TMC can be synthesized by either covalent addition of a substituent containing a quaternary ammonium group or by quaternization of the amino groups of the parent polymer (Britto & Campana-Filho, 2004; Curti et al., 2003). Generally quaternization methods use methyl iodide as methylant agent although recently a new and more efficient method was described by Britto and Assis (2007a, 2007b) in which dimethylsulfate is used as the methylant agent.

The main parameter that characterizes a chitosan derivative is its average degree of quaternization, $\overline{DQ}$, (relative number of positive charges available on the molecule for interactions) and, secondly, its molecular weight. The usual method of determining the TMC's $\overline{DQ}$ has been ^{1}H NMR spectroscopy (Curti et al., 2003; Sieval et al., 1998; Tahanou et al., 2000) although potentiometric and conductimetric methods have also been proposed (Curti & Campana-Filho, 2006; Curti et al., 2003; Jia et al., 2001). The ^{1}H NMR spectroscopy is particularly effective when applied qualitatively, as the signal referent to quaternary site is quite clear, but it is limited as a quantification tool once

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it is very difficult to obtain a reference signal for proportional intensity analysis. Generally, the $\overline{DQ}$ via ^{1}H NMR spectroscopy is calculated by the ratio between the signal area due to the hydrogen of the methyl groups of the amino quaternary site (3.4 ppm) and those corresponding to the hydrogen bonded to C1 of the glycoside ring (4.5–5.5 ppm). However, the C1 hydrogen signal has low intensity, low signal/noise ratio and ordinarily it appears as broad multiplets due to several substitutions at the amino moiety, affecting its intensity analysis (Britto & Campana-Filho, 2004; Curti et al., 2003; Dung et al., 1994; Sieval et al., 1998; Tahanou et al., 2000). Recently, Curti and Campana-Filho (2006) reported a new method of calculating TMC's $\overline{DQ}$ based on the intensities of all methyl hydrogen signals on the ^{1}H NMR spectrum that does not require the use of the C1 hydrogen signal as reference. It is important to note however, that in using this method, the intensity of the signal referent to dimethyl site is super estimated, once the signals of the C2 hydrogen in the glycoside ring and that of the dimethyl site are overlapped.

Ignoring these drawbacks of the ^{1}H NMR spectroscopy, the use of a suitable deutered solvent is an additional necessity. Besides, concentrated chitosan solutions results in a very viscous solutions or are even insoluble for some derivatives, requiring measurements at 80 °C in order to obtain narrow spectral line widths.

An alternative method is to use the solid state CP-MAS ^{13}C NMR technique to characterizes and calculates the TMC's $\overline{DQ}$. The ^{13}C solid state NMR technique had been used successfully to calculate the degree of acetylation of chitosan (Cervera et al., 2004; Duate, Ferreira, Marvão, & Rocha, 2001; Holappa et al., 2004; Nunthanid et al., 2004; Velde & Kienkens, 2004) and it is a technique with potential to be explored. In this sense, the purpose of this study was to discuss the $\overline{DQ}$ calculation of three samples of TMC synthesized from chitosan in three different initial morphological forms: raw, powder and wet, by means of the ^{13}C solid state NMR spectroscopy.

2. Experimental

Chitosan of medium molecular weight was purchased from Aldrich Chemical Company Inc. (USA), having Brookfield viscosity 200,000 cps, accordingly to the manufacturer. Dimethylsulfate was obtained from Vetec (R. Janeiro, Brazil) and the other chemicals were obtained from Synth (S. Paulo, Brazil).

The basic quaternization reaction sequence (Britto & Assis, 2007a) comprised a suspension of 1 g of chitosan (0.005 mol) in 16 cm^3 of dimethylsulfate and 4 cm^3 of deionized water. 1.2 g of NaOH (0.015 mol) and 0.88 g of NaCl (0.015 mol) were added. The reaction was conducted under magnetic stirring at room temperature for 6 h. After this period, the mixture was divided in two parts: one part underwent dialysis against 0.1 N NaCl aqueous solution and another underwent dialysis against 0.1 N $NaCH_3COO$ aqueous solution for three days, changing the solution at every 12 h. Cellophane membrane with cut-off 12,000–14,000 g/mol, from Aldrich Chemical Co. Inc. (USA) was used for dialysis. After the dialysis, the pH of the TMC solution was corrected to 7.0 with 0.1 N NaOH aqueous solution. The final products were obtained by precipitation with acetone. After exhaustive rinsing, the derivatives were filtered and vacuum dried.

The three initial chitosan morphological forms were prepared as follows:

- Wet chitosan: one gram chitosan was dissolved in 300 cm^3 of 1% aqueous acetic acid solution and precipitated out by the addition of a concentrated NH_4OH solution. The precipitated was washed with distilled water to remove excess of NH_4OH and filtered until the weight of chitosan + water remains constant at 5 g. This wet chitosan was submitted to quaternization reaction as described above, with the exception of the addition of 4 cm^3 of water. The final product was named TMC_{wet}.
- Powder chitosan: one gram of chitosan was dissolved in 300 cm^3 of 1% aqueous acetic acid solution and precipitated out by the addition of a concentrated NH_4OH solution. The precipitated was washed with distilled water to remove excess of NH_4OH, followed by rinsing with ethanol and acetone and dried at 100 °C. The chitosan sample was then pulverized until able to pass through a 125 μm nominal sieve opening. This chitosan was quaternized and named TMC_{powder}.
- Raw chitosan (flake): for this, Aldrich commercial chitosan was directly submitted to quaternization reaction without any pre-treatment stage. The morphology of this chitosan was like small flakes whose dimensions were in order of 0.5 mm. The final sample was named TMC_{raw}.

FTIR spectra were obtained from cut films samples of $10 \times 10 \times 0.01$ mm. A typical resolution of 2 cm^{-1} and 16 scans were employed in a Perkin Elmer spectrometer (model Paragon 1000) to take the spectra in transmittance mode. Films were prepared by dissolution of the sample in deionized water and casting in Petri dishes.

The ^{1}H NMR spectra of chitosan and TMC were acquired at 353 K by using a 200 MHz spectrometer (Bruker AC200). Samples of chitosan and TMC were dissolved in D_2O/HCl (100/1 v/v) and in D_2O, respectively, at a concentration of 10 mg cm^{-3}. The parameters for the acquisition of the ^{1}H NMR spectra were as follows: a pulse of 90°, corresponding to a pulse width of 8.2 μm; LB = 0.3 Hz and 16 transients were acquired. The chemical shifts were internally referenced by setting the hydrogen resonance of residual H_2O to 4.1 ppm (Sieval et al., 1998).

The solid state ^{13}C NMR measurements were performed on a NMR spectrometer Varian INOVA 400 Unity with a magnetic field of 9.4 T using the cross polarization sample technique, CP-MAS. The parameters for the acquisition were: pulse width of $\pi/2$, contact time of 1 ms, recycle time

of 4 s, 60 kHz of decoupling band width and spectral window of 18 kHz. The samples were packed into zirconia rotors and spun at 5 kHz. All spectra were filtered by an exponential decay function (LB = 10). The chemical shifts were externally referenced by setting the methyl resonance of hexamethylbenzene (HMB) at 17.3 ppm. A Lorentzian fitting was applied to the CP-MAS ^{13}C NMR spectra in the range 15–35 ppm.

3. Results and discussion

It is feasible to isolate cationic polyelectrolyte-like TMC in several counter-ion forms, though the chloride form is preferred due to its stability in the solid state, easiness to isolate and non-toxicity. By altering the type of counter-ion, changes in physical chemistry properties like solubility, hydrophilic/hydrophobic character, density, morphology etc., can be observed. Chitosan in different salty forms, such as, acetate, citrate and lactate, have been studied, showing different properties while the derivative TMC have been studied exclusively in the chloride form. Chitosan in several counter-ion form, mainly acetate, have shown different mechanical properties (Bégin & Calsteren, 1999); different capacity to drug release, swelling, erosion and mucoadhesiveness of matrices (Cafaggi et al., 2005; Orienti et al., 2002) and important feature in drug-polymer and excipient-polymer interaction (Nunthanid et al., 2004). Unfortunately, the TMC in different salty form have been unexplored and represent a potential research area. In the present work the TMC in acetate form was chosen for analysis since it was possible to detect this counter-ion by solid state ^{13}C NMR.

The FTIR spectra of films of the TMC, in the chloride (TMCCl) and acetate (TMCAc) forms clearly show difference, mainly in the 2000–1200 cm^{-1} range (Fig. 1). In the TMCAc spectrum, the bands at 1570 and 1402 cm^{-1} are more intense being assigned, respectively, to carbonyl stretching (C═O) and C—O asymmetric stretching, present in the acetate cation. The FTIR spectra also show a band at 1480 cm^{-1}, corresponding to an asymmetrical stretching of C—H in the methyl groups, that is characteristic of a highly methylated chitosan quaternary salt (Britto & Assis, 2007a, 2007b; Britto & Campana-Filho, 2004; Kim et al., 1997). The type of counter-ions Cl^- or Ac^- does not influence the position or the intensity of this band.

As previously mentioned the ^1H NMR spectroscopy is suitable for good qualitative analysis but is poor for quantitative data of the TMC. This fact can be depicted from Fig. 2 which shows the TMCAc ^1H NMR spectrum. Note that the signals referent to hydrogen bonded to C1 (4.5–5.5 ppm) (which is frequently taken as internal standard to calculate the $\overline{DQ}$) have low intensity. However, the peak referent to hydrogen in trimethylated site (at 3.27 ppm, labeled as "T") is very intense. Other important signals attributions in the spectrum of Fig. 2 are: *N*,*N*-dimethylated sites (2.76 ppm, labeled as "D"); *O*-methylated sites (3.37–3.56 ppm, labeled as "O"); hydrogen of the methyl group of the acetamide moiety (2.01 ppm, labeled as "α") and hydrogen of the methyl group of the acetate counter-ion (1.85 ppm, labeled as "γ") (Britto & Assis, 2007a).

After the quaternization process, one very good signal used to calculate the degree of acetylation of chitosan has shifted or is overlapped by other signals. This signal relates to hydrogen bonded to C2, as observed at 3.18 ppm in the chitosan spectrum (Fig. 3). In this way, the localization of that peak after the quaternization remains uncertain (Britto & Assis, 2007a; Britto & Campana-Filho, 2004; Curti & Campana-Filho, 2006; Curti et al., 2003; Dung et al., 1994; Sieval et al. 1998), otherwise it could be used to calculate the $\overline{DQ}$ as well. From chitosan spectrum (Fig. 3), the average degree of acetylation (Britto & Assis, 2007a) was $\overline{DA} = 18\%$.

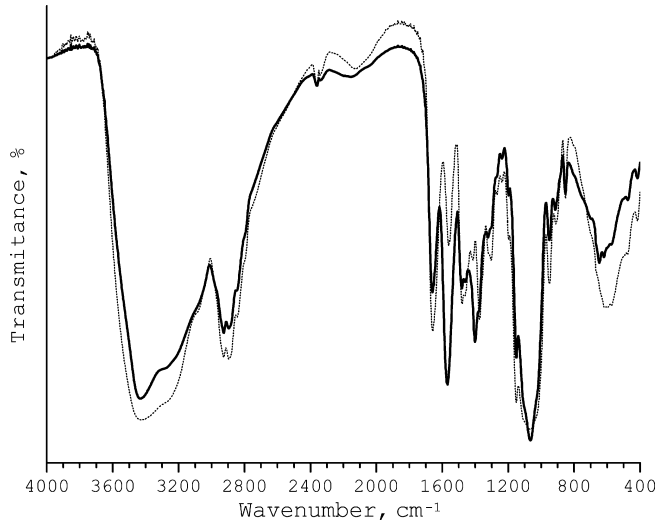

Fig. 1. FTIR spectra of the TMC_{raw} in the forms: TMCAc (solid line) and TMCCl (dotted line).

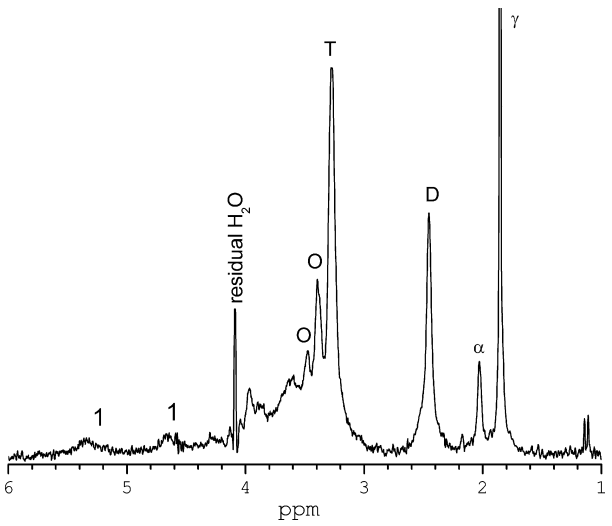

Fig. 2. ^1H NMR spectrum of the TMC_{raw} derivative dissolved in D_2O. For further information and legend, see the chemical structure in Fig. 4.

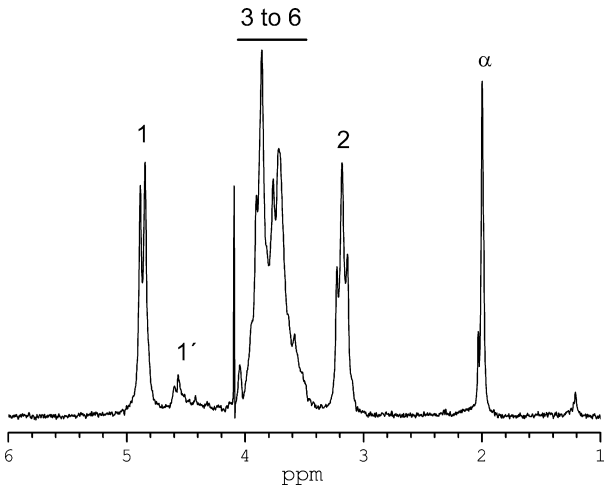

Fig. 3. ^{1}H NMR spectrum of the chitosan dissolved in D_2O/HCl (100:1 v/v).

The CP-MAS ^{13}C NMR spectrum of the chitosan (Fig. 4) was discussed previously (Britto & Assis, 2007a) and the following main signals can be identified: (a) $\delta = 24.3$ ppm attributed to the carbon atom of the methyl moieties of the acetamide groups; (b) $\delta = 60$ ppm two overlapped signals are observed and assigned to carbon C6 and C2; (c) $\delta = 76.4$ ppm due the carbon C5 and C3; (d) $\delta = 84$ ppm corresponding to carbon atom C4; (e) $\delta = 106.2$ ppm corresponding to carbon atom C1 and finally (f) $\delta = 174.5$ ppm that corresponds to carbon of carbonyl group. From the following equation (Ottøy, Vårum, & Smidsrød, 1996):

$$\overline{DA} = \frac{I_{CH_3}}{(I_{C1} + I_{C2} + I_{C3} + I_{C4} + I_{C5} + I_{C6})/6} \quad (1)$$

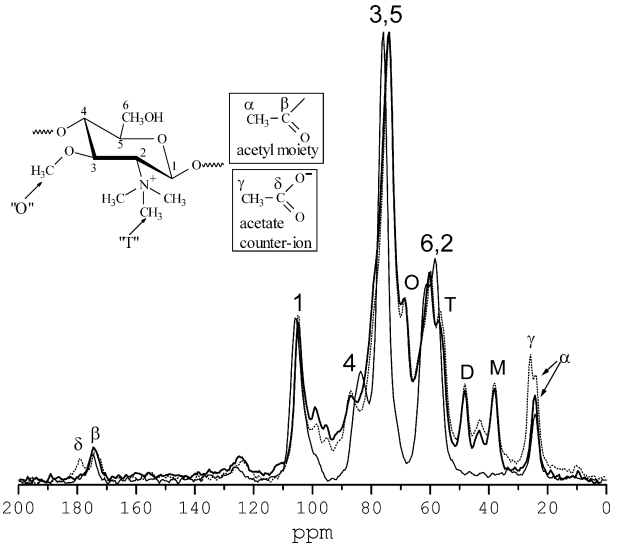

Fig. 4. Solid state CP-MAS ^{13}C NMR spectra of the chitosan (thin line) and TMC_{raw} in the forms: TMCCl (thick line) and TMCAc (dotted line). The labels O, T, D and M stand for *O*-methylated; *N*,*N*,*N*-trimethylated; *N*,*N*-dimethylated and *N*-monomethylated signals, respectively.

where, I_x is the intensity of the signal, the average degree of acetylation of chitosan was calculated as $\overline{DA} = 26\%$. Comparing the $\overline{DA}$ calculated by CP-MAS ^{13}C NMR and that calculated by ^{1}H NMR, there is a difference of about 7%, which according to Ottøy et al. (1996) is acceptable.

The CP-MAS ^{13}C NMR spectra of the TMCCl and TMCAc (Fig. 4) show new signals that can be attributed as follow: (a) $\delta = 37.9$ ppm from carbon of the methyl groups in a *N*-monomethylated site; (b) $\delta = 48.1$ ppm from carbon of the methyl groups in a *N*,*N*-dimethylated site; (c) $\delta = 56.4$ ppm from carbon of the methyl groups in a *N*,*N*,*N*-trimethylated site and (d) $\delta = 68.5$ ppm from carbon of the methyl groups in a *O*-methylated site (Britto & Assis, 2007a).

An important feature of the spectra in the Fig. 4 is that the quaternization process does not induce a deacetylation of chitosan once the intensity of the carbonyl and methyl acetamide signals did not change from chitosan to the derivative spectrum.

Fig. 5 is the expansion of the TMCAc spectrum in the 15–35 ppm region, showing that the deconvolution of the methyl signals allows quantifying the contribution of each signal and identifying different intensities according to each chitosan initial morphologic form. From the ratio A_γ/A_α, in which A_γ and A_α represent, respectively, the deconvoluted areas of the "γ" (acetate counter-ion) and "α" (acetamide moiety) signals, and considering $A_\alpha = 26\%$ (as calculated before for $\overline{DA}$), enables a satisfactory calculation of the $\overline{DQ}$ for the quaternized derivative of chitosan (Table 1).

From the data of the Table 1, the derivative TMC_{raw} (obtained from chitosan without any purification processes and used as received) attained the highest $\overline{DQ}$. This is a very relevant result, especially when considering large scale production. The result provides evidence that it is unnecessary to submit the chitosan to long and expensive processes before the reaction. The TMC_{wet} did not show a high $\overline{DQ}$ value due to the low dispersion in the DMS medium during the reaction as well as the low affinity between the water and DMS. Finally the TMC_{powder} showed an intermediary value probably due to, despite finely powdered, particles which are very compact and dense. Indeed, after the purification and drying processes, the chitosan becomes a very hard material that demands some mechanical force to powder it.

4. Conclusion

The synthesis of the chitosan quaternary salts in others salty forms like acetate is imperative to know others properties and applications of this important polyelectrolyte. The study of TMC in the acetate form allowed the determination of the degree of quaternization by means of CP-MAS ^{13}C NMR technique, that is a powerful technique and have been used for chitosan derivative characterization. Other relevant feature of this study is that the highest $\overline{DQ}$ of the TMC was achieved for the derivative obtained from chitosan without any pre-treatment (morphological

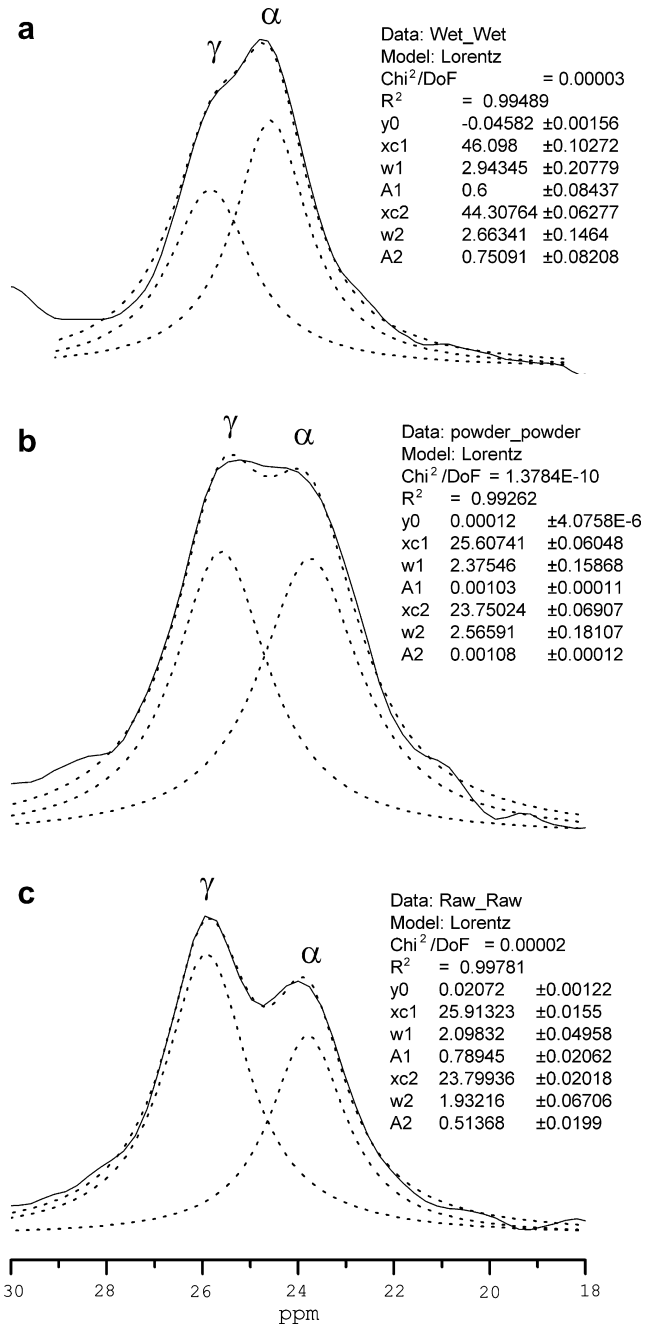

Fig. 5. Expansion of the solid state CP-MAS ^{13}C NMR spectra in the range 15–35 ppm for (a) TMC_{wet}; (b) TMC_{powder} and (c) TMC_{raw} in the acetate form. The solid line is referent to experimental spectrum and the dotted lines are referent to the calculated Lorentzian fitting.

Table 1
Degree of substitution, $\overline{DQ}$, calculated from the ratio of the deconvoluted areas of the signals "α" and "γ"

Sample	Ratio A_γ/A_α	$\overline{DQ}$ (%)
TMC_{raw}	1.54	40.04
TMC_{wet}	0.80	20.80
TMC_{powder}	0.95	24.70

form like flake) which can signify cost and time reduction in a large scale production.

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