

Modification of Chitosan by Zincke Reaction: Synthesis of a Novel Polycationic Chitosan-Pyridinium Derivative

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Chitosan is a biodegradable aminopolysaccharide produced by deacetylation of acetamide groups of chitin, one of the most abundant organic materials in nature. Many different types of organic reactions have been described to modify the functional groups of chitosan and produce materials with applications in a large number of areas. However, the Zincke reaction, an old method which is commonly used to convert primary amine in pyridinium salts, has not been reported to transform the chitosan amino group at C-2 position in glucosamine units. In this paper, our efforts are described to carry out this reaction, employing different conditions to covalently anchor pyridinium salts on the polymer surface. Optimized synthesis conditions using water/ethanol as solvent, triethylamine and Zincke salt excess yielded a novel polycationic chitosan-pyridinium derivative with a weight gain of 52% after 48 h of reaction. The modified biopolymer is water insoluble and exhibits a high degree of chemical modification. The ¹³C solid state nuclear magnetic resonance (SS-NMR) spectrum of chitosan-pyridinium derivative showed signals at 147.0, 142.0 and 129.0 ppm, attributed to aromatic carbons, confirming the presence of a quaternary pyridinium ring directly attached to the biopolymer. The Zincke reaction was employed for the first time to modify the chitosan backbone.

Keywords: chitosan, Zincke reaction, pyridinium salts

Introduction

Chitosan, a carbohydrate biopolymer composed of β (1 → 4) linked units of *N*-acetyl-*D*-glucosamine and *D*-glucosamine (Figure 1), is classically obtained by the controlled deacetylation of chitin, the second most abundant natural polysaccharide after cellulose.¹ For several decades, partly due to its abundance and biofunctionality, chitosan has been widely studied.² Moreover, this polymer displays interesting properties such as biodegradability, biocompatibility, low toxicity and great potential for chemical modification, providing an exciting platform for the development of different materials with various physicochemical properties.³ A great number of modulated chemical reactions may be performed on chitosan C-2 free amino and C-3 and C-6 hydroxyl groups,⁴ yielding novel polymers with applications in different fields such as biomedicine,⁵ environmental chemistry,⁶ agriculture,⁷ biotechnology industry,⁸ food products,⁹ molecular biology,¹⁰ etc. The most common chitosan chemical modifications

include *N*-phthaloylation, *N*-carboxyalkylation, *N* and/or *O*-acylation/alkylation, *O*-carboxymethylation, quaternization, Schiff base formation, phosphorylation, graft copolymerization, *O*-sulfonation, among others.¹¹

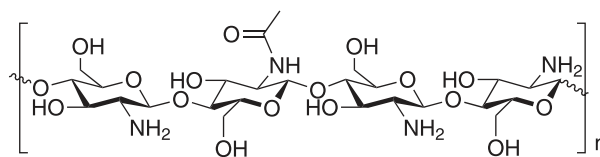


Figure 1. Chemical structure of chitosan.

Pyridinium salts represent a versatile class of heterocycles found in many natural and synthetic bioactive compounds.¹² These cationic substances have demonstrated biological uses in drug delivery and gene therapy.¹³ They may also act as antimicrobial agents¹³ and enzyme inhibitors,¹⁴ among other applications. Furthermore, these salts have been employed as phase transfer catalysts,¹⁵ ionic liquids¹⁶ and cationic polymerization initiators.¹⁷ These quaternary compounds are commonly used as important intermediates in organic synthesis for the preparation

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of highly complex nitrogenous substances, including N-substituted dihydropyridines, tetrahydropyridines and hexahydropyridines.¹⁴

The most common method to prepare pyridinium salts is the alkylation of the pyridine nitrogen atom with primary alkylating agents by an S_N2 reaction.¹⁸ However, in special cases (for example in the synthesis of N-arylpyridinium or pyridinium salts containing a chiral group which presents a significant risk of racemization during alkylation),¹⁴ the best choice is the reaction between primary amines with highly electrophilic species, such as pyrylium¹⁹ or Zincke salts (N-2,4-dinitrophenyl-pyridinium salt).²⁰ The latter salt, which may be prepared via S_NAr from pyridine and 2,4-dichloronitrobenzene (Scheme 1),²¹ is an important starting material for the Zincke reaction. In this reaction, the salt suffers an attack at the pyridinium C-2 atom by the primary amines and generates a new pyridinium derivative through a transannulation reaction. In sum, the nitrogen atom of the primary amine is incorporated in the pyridine cycle without C–N amine bond cleavage.¹⁴

Despite the great number of chitosan derivatives synthesized up to date and the variety of primary amines employed as starting materials for the Zincke reaction, no reports have been made so far in the literature describing the synthesis and characterization of covalently anchored pyridinium salt into chitosan backbone using the electrophilic Zincke salt. Considering the experience of our group in the Zincke chemistry¹² and the interest on the obtention of a novel polycationic chitosan-based material with potential biological applications, this paper describes our efforts to evaluate the viability of this reaction to chemically modify chitosan.

Experimental

Materials

Medium molecular weight chitosan (75-85% deacetylated, Sigma-Aldrich, Brazil), pyridine (Vetec, Brazil), 1-chloro-2,4-dinitrobenzene (Sigma-Aldrich,

Brazil), triethylamine (Et₃N) (Dinâmica, Brazil), acetone (CRQ, Brazil), ethanol (95%, Labsynth, Brazil), NaOH and HCl were used in this study.

Synthesis of a chitosan-pyridinium derivative (C1)

The Zincke salt was prepared according to Vianna *et al.*¹² Chitosan (0.5 g, 2.5 mmol of NH₂), Zincke salt (1.125 g, 4 mmol, 1.6 equiv.) and 20.0 mL of ethanol/distilled water (1:1) were added to a 250 mL round-bottom flask, then 4 mmol (1.6 equiv.) of triethylamine was added. The reaction mixture was refluxed at 100 °C with magnetic stirring for 48 h. At the end of the reaction, the suspension was separated by vacuum filtration using a Büchner funnel, and the modified chitosan (C1) was washed with ethanol, dichloromethane, distilled water and exhaustively with acetone, then C1 was dried in an oven at 70 °C for 1 h.

Percent weight gain (pwg)

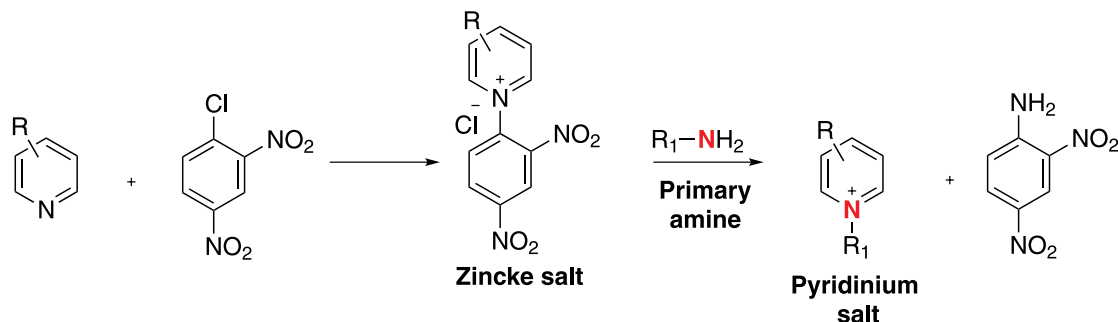
The pwg after modification of chitosan with Zincke salt was calculated using equation 1.

$$\text{pwg}(\%) = \left(\frac{w_{c1} - w_c}{w_c} \right) \times 100 \quad (1)$$

where w_c (g) and w_{c1} (g) are the weights of the chitosan and C1, respectively.

Determination of degree of deacetylation (DD) and amount of free amine groups (n_{NH_2}) of chitosan

The DD (in percentage) and n_{NH_2} (mmol g⁻¹) of chitosan were determined by potentiometric titration. A sample of 25.0 mg of chitosan was dissolved in 25.0 mL of standardized aqueous HCl solution (0.1 mol L⁻¹), after complete dissolution that solution was titrated with standardized aqueous NaOH solution (0.1 mol L⁻¹). A pH meter (PHS-3BW, Aprolab, Brazil) was used for pH measurements, three replicates were performed. The degree of deacetylation was calculated



Scheme 1. The Zincke reaction.

by equation 2^{22,23} and the amount of free amine groups was calculated using equation 3.²⁴

$$DD(\%) = \frac{V_{\text{NaOH}} \times C_{\text{NaOH}} \times 0.016}{0.0994 \times w_c} \quad (2)$$

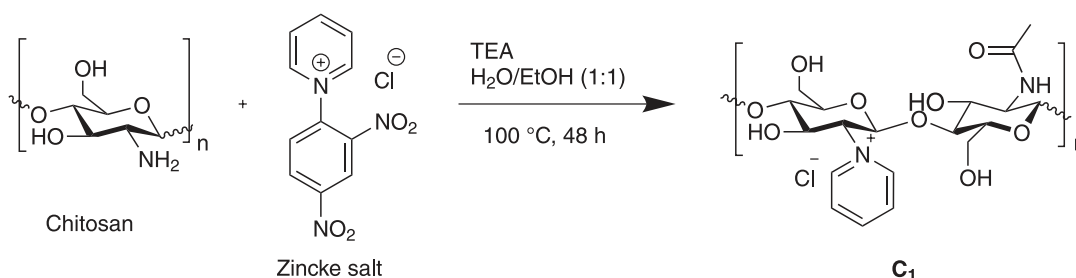
$$n_{\text{NH}_2}(\text{mmol L}^{-1}) = \frac{C_{\text{NaOH}} \times V_{\text{NaOH}}}{w_c} \quad (3)$$

where V_{NaOH} (mL) is the volume of aqueous NaOH solution required to neutralize the ammonium groups, C_{NaOH} (mol L⁻¹) is the concentration of aqueous NaOH solution, 0.016 is the molar mass weight of NH₂ (kg mol⁻¹) and 0.0994 is the theoretical NH₂ percentage in chitosan.²⁵

Fourier transform infrared spectroscopy (FTIR) analysis

Chitosan and **C1** were analyzed by FTIR spectroscopy. Samples were dried in an oven at 70 °C for 1 h, then 1.0 mg of each material was mixed with 100.0 mg of spectroscopy grade KBr. The FTIR spectra were recorded on a Shimadzu IR-408 spectrometer (ICEX-DQ, UFMG, Brazil), with the detector at a resolution of 4 cm⁻¹, from 400 to 4000 cm⁻¹ and 32 scans *per* sample.

Table 1. Conditions tested for the synthesis of **C1** via Zincke reaction



| Reaction | Solvent | time / h | Temperature / °C | Et ₃ N / equiv. | Zincke salt / equiv. | Heating | pwg / % |
|----------|--|----------|------------------|----------------------------|----------------------|---------------------------|---------|
| 1 | <i>n</i> -butanol | 15 | 115 | – | 1.6 | conventional | 0 |
| 2 | <i>n</i> -butanol | 36 | 115 | – | 1.6 | conventional | 2.3 |
| 3 | <i>n</i> -butanol | 48 | 115 | 1.6 | 1.6 | conventional | 0 |
| 4 | H ₂ O | 48 | 100 | 1.6 | 1.6 | conventional | 35.5 |
| 5 | H ₂ O | 15 min | 100 | – | 1.6 | microwave | 0 |
| 6 | H ₂ O/EtOH (20:80) | 1 | 100 | 1.6 | 3 | ultrasound | 2 |
| 7 | H ₂ O/EtOH (20:80) | 48 | 100 | 1.6 | 1.6 | conventional | 18.4 |
| 8 | H ₂ O/EtOH (20:80) | 48 | 100 | 1.6 | 3 | conventional | 30 |
| 9 | H ₂ O/EtOH (50:50) | 48 | 100 | 1.6 | 3 | microwave | 17 |
| 10 | H ₂ O/EtOH (50:50) | 48 | 100 | 1.6 | 1.6 | conventional | 52 |
| 11 | H ₂ O/EtOH (50:50) 1% AcOH | 48 | 100 | 1.6 | 1.6 | conventional ^a | 25 |
| 12 | AcOH-AcO/EtOH (50:50) (buffer pH 4.5) | 48 | 100 | – | 1.6 | conventional ^a | 28 |
| 13 | AcOH-AcO/EtOH (50:50) (buffer pH 4.5) | 48 | 100 | 1.6 | 1.6 | conventional ^a | 34 |

^aHomogeneous medium. pwg: percent weight gain.

Solid state ¹³C nuclear magnetic resonance (NMR) analysis

The solid-state ¹³C NMR spectrum of **C1** was obtained on a Varian-Agilent 400 MHz spectrometer (NCQP, UFES, Brazil) at the frequency of 100 MHz, acquisition time of 20.48 ms, with an accumulation of 1232 scans.

Results and Discussion

The Zincke salt (*N*-2,4-dinitrophenyl-pyridinium salt) was easily prepared by the reaction between pyridine and 1-chloro-2,4-dinitrobenzene under acetone reflux for 15 h, following an S_NAr mechanism.¹² The next stage was the treatment of chitosan with 1 equivalent of the Zincke salt in refluxing 1-butanol for 15 h, an ideal condition established by Marazano and co-workers.²⁰ In these heterogeneous conditions, no change from the original color of the suspension was observed, even prolonging the reaction time or adding Et₃N to enhance the nucleophilic properties of the amino groups (Table 1, entries 1-3). Thus, it was decided to study the reaction between *D*-glucosamine (the monomeric unit of chitosan) and the Zincke salt since a thorough search of the literature revealed that no carbohydrate-based pyridinium salts were

efficiently prepared by the Zincke reaction. However, all conditions used to promote this reaction did not provide the desired pyridinium salt, suggesting that the amino group in glucosamine is a poor nucleophile to pyridinium salts, probably by steric hindrance. Rivard and co-workers¹⁸ described the use of water as a solvent to promote the reaction of poorly nucleophilic amines with Zincke salts. Thus, in our hands, the use of water (Table 1, entry 4) resulted in an immediate formation of a deep red color in the reaction (Figure 2), indicating a facile and rapid formation of dianil salts, important intermediates in the Zincke reaction. After 48 h, the red color disappeared, and the reaction medium turned brown.

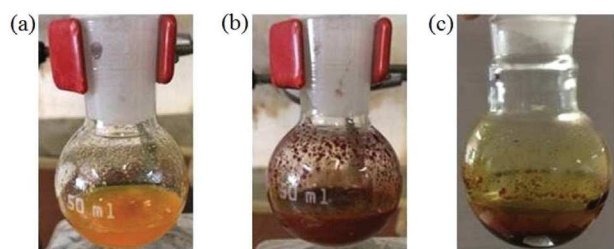
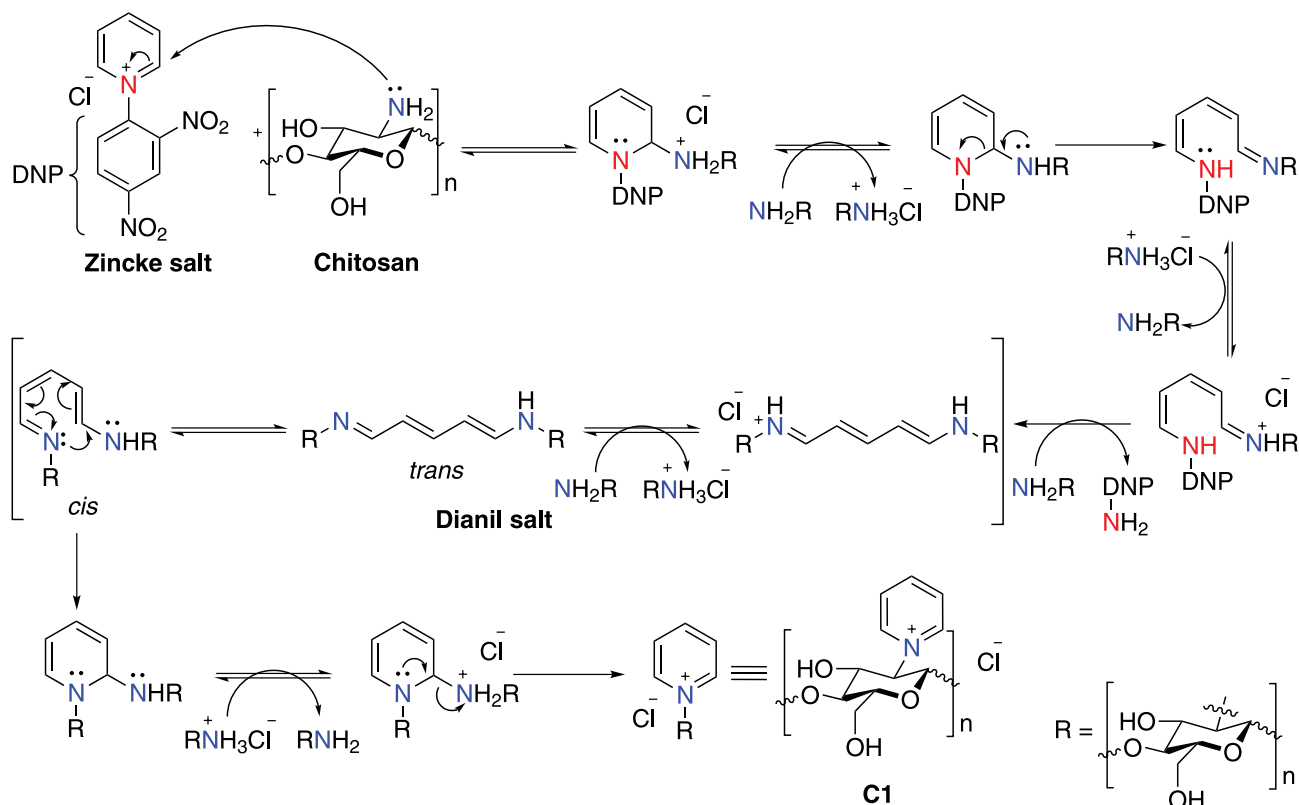


Figure 2. Reaction between chitosan and Zincke salt: (a) initial time; (b) 30 min; (c) 48 h.

After filtration and exhaustive washing with different solvents to remove the unreacted Zincke salt and the

2,4-dinitroaniline formed in the reaction, the obtained solid was dried under vacuum and characterized by usual spectral techniques. The percent weight gain of this material was 35.5%, indicating an important chemical modification degree. Other conditions were evaluated to increase the percent weight gain of the chitosan material, including the use of microwave (Table 1, entry 5), ultrasound (Table 1, entry 6), a binary solvent system (entries 6-11) and homogeneous reaction conditions (entries 11-13). In the case of microwave assisted reaction, it was observed the rapid formation of red dianil salts, but the suspension color indicated that the ring closure step was too slow to be observed. The system employing the binary solvent water/ethanol, amine and Zincke salt excess for 48 h at 100 °C provided the highest percent mass gain. The polycationic obtained material **C1** was insoluble in organic solvents and in aqueous media in pH ranges 1-12, being soluble only in concentrated nitric acid at room temperature.

The Zincke reaction follows an S_N (ANRORC) mechanism with three key steps: nucleophilic addition, ring opening, and ring closing with a key *cis-trans* interconversion (Scheme 2).²⁶ In detail: the nucleophilic attack of the primary amine leads to the ring opening of the pyridinium ring; a second equivalent of the primary amine carries out a new nucleophilic attack causing the displacement of 2,4-dinitroaniline and formation of the



Scheme 2. Mechanism of the Zincke reaction.

intense red dianil salts in equilibrium;²⁷ the interconversion process of *trans* dianil salt to *cis* is a fundamental step preceding ring closure.²⁸ The *trans-cis-trans* isomer of the dianil salt can react by either nucleophilic addition or sigmatropic rearrangement of a zwitterionic intermediate to yield a cyclized intermediate.²⁸ The cyclization step seems to be the rate-determining of the reaction. The last step restores the aromaticity with the leaving of an amino group, and the novel pyridinium salt is formed.

Figure 3 shows the FTIR spectra of chitosan and **C1**. The main differences highlighted in the spectrum of **C1** in comparison with chitosan are: (i) the bands at 1604 and 1560 cm^{-1} , which can be attributed to the stretching of C=C bonds; (ii) the appearance of bands at 1490 and 1432 cm^{-1} , attributed to the stretching of C=N bonds; and (iii) a band at 836 cm^{-1} , related to the out-of-plane bending of C-H in the pyridine ring.²⁹ These changes indicate the introduction of the pyridine ring in chitosan. In addition, the absence of bands in the region of 1540 and 1340 cm^{-1} , attributed to the asymmetric and symmetric stretching vibration of NO_2 group,³⁰ in the spectrum of **C1**, indicates that this material is free of by-product 2,4-dinitroaniline.

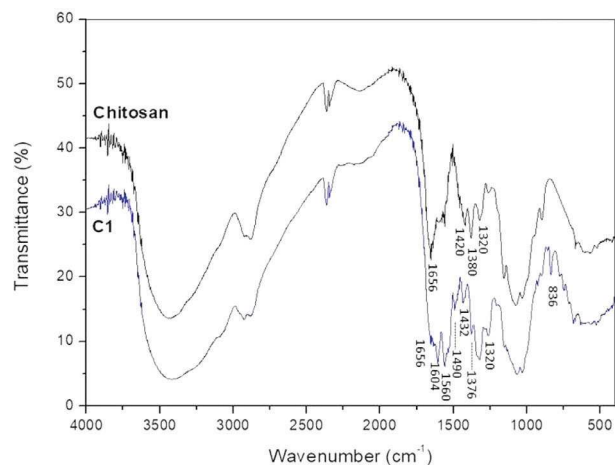


Figure 3. FTIR (KBr) spectra of chitosan and **C1**.

The solid-state ^{13}C NMR spectra of chitosan²⁸ and **C1** are shown in Figure 4. The spectrum of chitosan shows characteristic signals at 179.2 and 105.4 ppm corresponding to carbonyl and anomeric carbons, respectively. The signals at 57.3, 60.9, and 81.8 ppm are attributed to the carbon atoms C2, C6 and C4 in pyranose rings. The signal at 75.9 ppm can be assigned to the superposition of C3 and C5 resonances, and the signal at 22.5 is attributed to C8.³¹ After modification of chitosan with Zincke salt, new resonances above 120 ppm were observed. The signal at 147.0 ppm is assigned to Cc, an aromatic atom characteristically deshielded in pyridinium salts.²⁶ The resonances signals

at 129.0 and 142.0 ppm are expected and can be easily assigned to Cb and Ca, respectively. This set of signals is very important to confirm the presence of a quaternary pyridinium ring directly attached to the backbone of chitosan in **C1**.

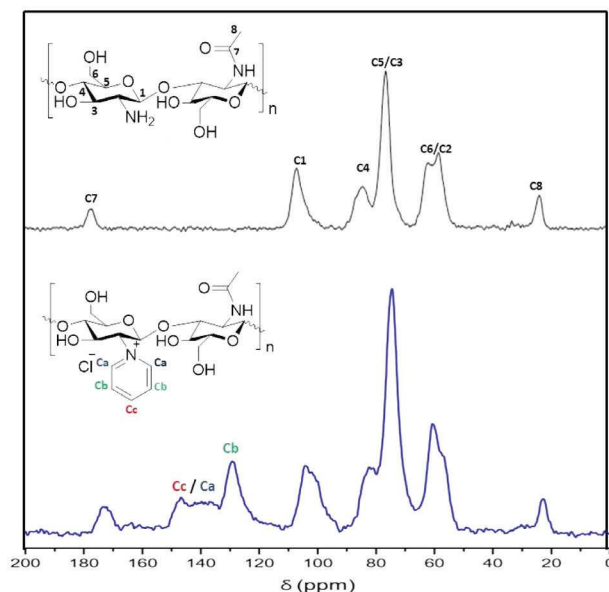


Figure 4. Solid-state ^{13}C NMR (100 MHz) of chitosan and **C1**.

Conclusions

The functionalization of chitosan using the Zincke reaction produced a novel water insoluble polycationic chitosan-pyridinium derivative. A number of conditions were tried to carry out the reaction between the carbohydrate biopolymer and the Zincke salt and the use of water as solvent resulted in a high degree of chemical modification. A high percent weight gain (52%) was obtained using a reaction time of 48 h and a binary system of solvent with an excess of triethylamine and Zincke salt. **C1** was characterized with success using FTIR and solid state ^{13}C NMR spectroscopies. It is the first time that the Zincke reaction is used to functionalize chitosan, incorporating the nitrogen atom of the amino group into a pyridinium cycle.

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