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N-methylene phosphonic chitosan: a novel soluble derivative

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Abstract

A novel water-soluble chitosan derivative carrying phosphonic groups was synthesized using a one-step reaction that allowed homogeneous modifications without any sharp decrease in its properties, such as filmogenic capacity (this compound and its synthesis have been protected with a patent). The ^{1}H NMR, ^{13}C NMR assignments and ^{1}H–^{13}C NMR correlation permitted the identification of the structure by the subtituent distribution of the product, which is partly *N*-monophosphonomethylated (0.24) and *N*,*N*-diphosphonomethylated (0.14) and *N*-acetylated (0.16) without modification of the initial degree of acetylation. The identity of the *N*-methylene phosphonic chitosan was confirmed by FT-IR spectrometry, X-ray diffraction and elemental analysis.Taking advantage of the known chelating ability of the phosphonic groups, especially for calcium, this new derivative opens new perspectives as biomedical material.

Keywords: *N*-methylene phosphonic chitosan; Phosphonomethylation; Chitosan soluble derivative; ^{1}H NMR; ^{13}C NMR

1. Introduction

Chitosan is a potential polysaccharide resource owing to its specific structure and properties (Muzzarelli, 1977). The cationic nature of chitosan limits the versatility of aqueous solutions because certain acids in excess quantity are required to form water soluble chitosan salts (Roberts, 1992a,b). Many efforts to prepare functional derivatives of chitosan by chemical modifications, in order to increase the solubility in water, have been reported (Muzzarelli, 1988; Muzzarelli, 1992; Muzzarelli & Ilari, 1994; Muzzarelli, Ilari & Tomasetti, 1993; Muzzarelli, Tanfani, Emanuelli & Mariotti, 1982).

One of the reasons for the intractability of chitosan lies in the rigid crystalline structure and the acetamido or primary amino group residues that have an important role in the formation of conformational features through intra and/or intermolecular hydrogen bonding (Nishimura, Kohgo, Kurita & Kuzuhara, 1991). Removal of the two hydrogen atoms of amino groups of chitosan and the introduction of some hydrophilic nature by chemical modification result in solubility improvement in aqueous solvents.

In addition to solubility, reactivity is another important requisite to make possible versatile molecular designs leading to novel functional materials. Chitosan and many of its derivatives have chelating ability towards transition metal ions but do not exhibit the same ability towards alkali metal ions (Muzzarelli & Tubertini, 1969); for instance, chitosans carrying carboxyl groups might chelate calcium (Muzzarelli, 1988; Muzzarelli & Zattoni, 1986; Muzzarelli et al., 1998).

On the other hand, among the organic phosphorous compounds, biphosphonates and phosphonic acids play an important role. Based on the report by Schwarzenbach, Ackermann and Ruckstuhl (1949) phosphonic complexing agents have been considered to be as effective as or even more effective than those containing carboxylic groups (Hendrickson, 1967). The diphosphonic acids have particular characteristics; Fleisch, Graham and Francis (1969) and Jung, Bisaz and Fleisch (1973) studied the behavior of biphosphonates and their high affinity for the calcium ion, when used as regulators in calcium metabolism (Germany Patent, 1980).

The presence of an amine group, as in the case of chitosan, in the molecule to obtain $NH_2–CH_2–PO_3^{2-}$, combines its strong donor effect with a monodentate ligand as $–PO_3^{2-}$, thus increasing the metal-binding abilities (Westerback & Martell, 1956; Westerback, Rajan & Martell, 1965).

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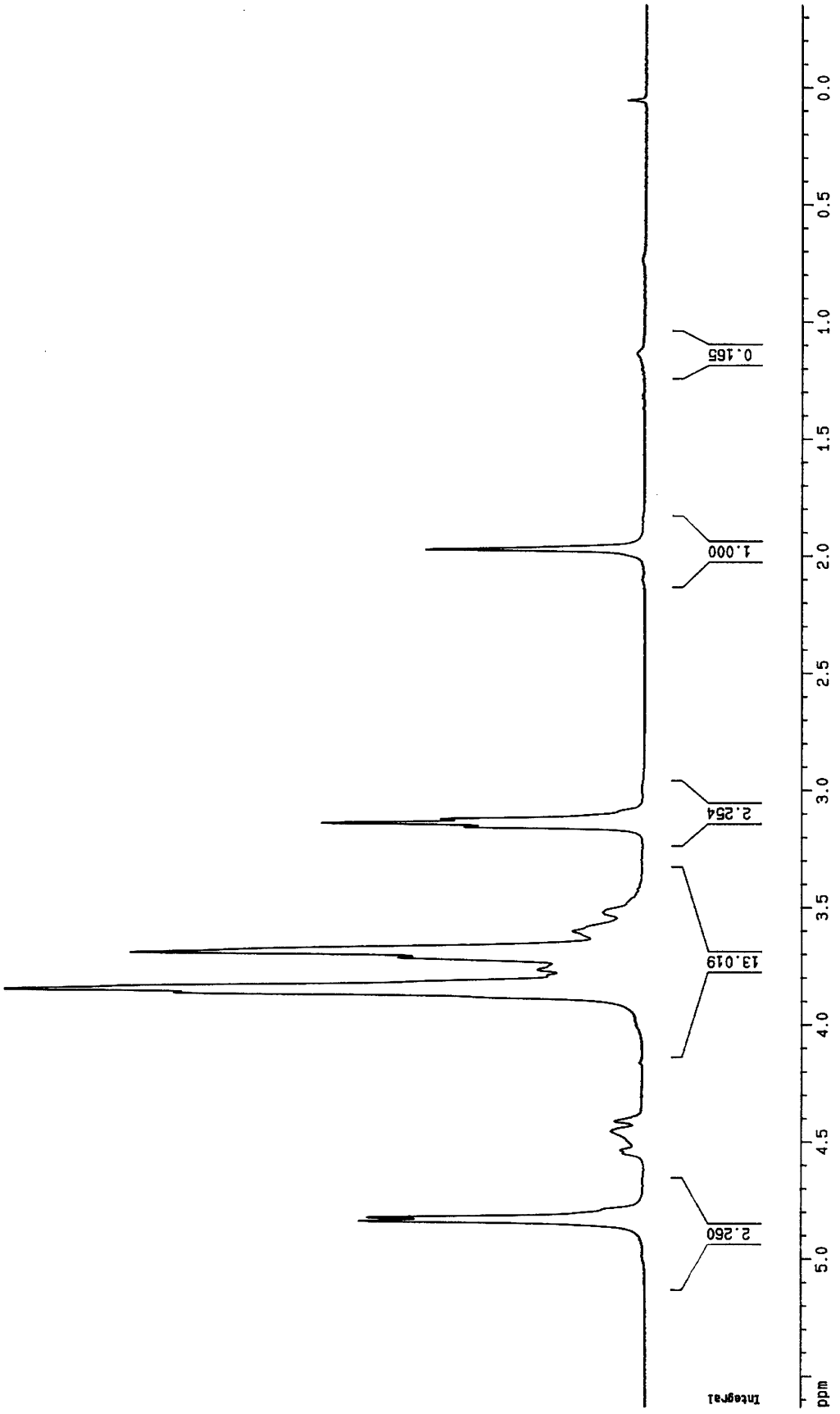


Fig. 1. ^{1}H NMR spectrum of chitosan dissolved in 2% (w/w) DCl/D_2O. Polymer concentration 20 g/l.

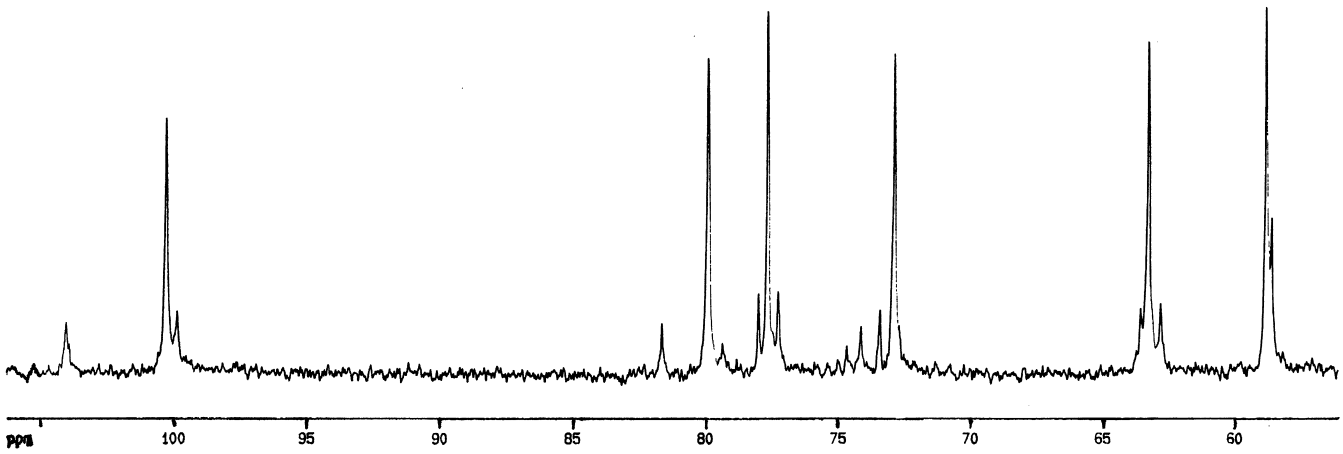


Fig. 2. ^{13}C NMR spectrum of chitosan dissolved in 2% (w/w) DCl/D_2O. Polymer concentration 20 g/l.

The present article describes the synthetic strategy of a novel soluble chitosan derivative: the *N*-methylene phosphonic chitosan (NMPC) by the transformation through an additional functional group in a homogeneous reaction system for the purpose of creating finely designed biomedical material.

2. Materials

2.1. Preparation of chitin

Chitin was isolated from shrimp shells waste (*Pleoticus müller*i). The material was homogenized and the product was rinsed in order to remove the organic material. After this it was treated with 9% (w/w) NaOH at 65°C for 90 min to remove proteins, demineralized by treatment with 10% (v/v) HCl at 20°C for 15 min, washed, then dried.

2.2. Preparation of chitosan

Chitosan was prepared directly by heterogeneous deacetylation at 136°C with 50% (w/w) NaOH for 1 h.

2.3. Synthesis of N-methylene phosphonic chitosan

Chitosan solution 2% (w/v) in glacial acetic acid 1% (v/v) was prepared. One part (by weight) of chitosan was used and one part of phosphorous acid (by weight) dissolved in water was added drop-wise with continuous stirring for 1 h. Then the temperature of the reaction vessel was raised to 70°C and one part of formaldehyde 36.5% (by weight) was added drop-wise over 1 h with reflux. Heating was protracted for 6 h at the same temperature.

The clear pale yellow solution was dialyzed against demineralized water for 48 h or until the pH of water was raised to 6.8 in dialysis tubing with a cut-off value of 2500 Da. Finally, the solution was frozen and freeze-dried.

3. Methods

3.1. Characterization of chitosan

Degree of deacetylation. This was determinated by the titration method (Wojtasz-Pajak, Brzeski & Malesa-Ciecwierz, 1994), NMR (Hirai, Odani & Nakajima, 1991) and first-derivative UV spectrophotometry (Muzzarelli, Rocchetti, Stanic & Weckx, 1997).

Determination of viscosity. The measure of viscosity of chitosan 1% (w/v) in acetic acid 1% (v/v) was made on a Broockfield viscometer at rotational velocity of the spindle of 50 rpm at 25°C.

Determination of molecular weight (M_v). The solvent system used was 0.1 M HOAc–0.2 M NaCl. The values for the Mark–Houwink equation constants K and a were 1.81×10^{-3} and 0.93, respectively (Roberts, 1992a,b).

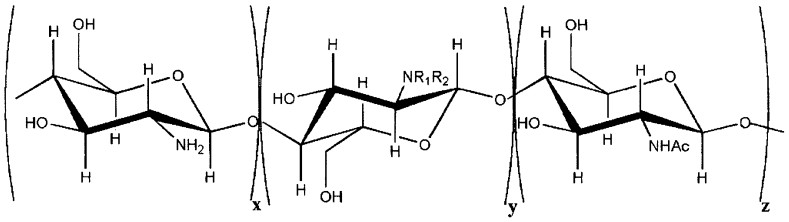
X-ray diffraction spectrometry. The material in the powder form was submitted to X-ray diffraction spectrometry by using a vertical powder diffractometer; the source was a rotating anode generator Rigaku Denki RU-300 and Ni-filtered CuK_α radiation ($\lambda = 0.154$ nm).

NMR spectroscopy. ^{13}C and ^{1}H NMR measurements were performed on an AMX500 Brukker NMR spectrometer under a static magnetic field of 125 and 500.13 MHz, respectively, at 70°C. For those measurements, 10 mg of the sample was introduced into a 5 mm ∅ NMR test tube, to which 0.5 ml of 2% (w/w) DCl/D_2O solution was added, and finally the tube was kept at 70°C to dissolve the polymer in solution.

3.2. Characterization of N-methylene phosphonic chitosan

Solubility test. The sample (10 mg) and 5 ml of solvent were placed in a test tube and stored at 20°C for 7 days.

Film casting. NMPC powder (1.0 g) was dissolved in 100 ml of water. Films could be cast by evaporation from 10 ml of this solution in polystyrene Petri dishes at 37°C (overnight). The thickness of the film was measured using a micrometer.



$R_1 = H$, $R_2 = CH_2PO_3H_2$
$R_1 = R_2 = CH_2\text{-}PO_3H_2$

Fig. 3. Chemical structure of *N*-methylene phosphonic chitosan.

IR spectroscopy. NMPC powder samples have been obtained between 400 and 4000 cm^{-1} on a Nicolet 20-SX FT-IR spectrometer (DTGS detector) equipped with a Spectra Tech. Multiple Internal Reflectance (DRIFT) accessory. It was filled with a mixture of a small amount of sample grounded with anhydrous KBr.

Scanning electron microscopy (SEM). A Philips SEM 505 scanning electron microscope was used to characterize the surface of NMPC particles. The samples were prepared by gold coating.

Determination of viscosity. The measure of viscosity of NMPC (1% w/v) was made on a Broockfield viscometer at rotational velocity of the spindle of 50 rpm at 25°C.X-ray diffraction spectrometry and NMR spectroscopy were described before.

4. Results and discussion

4.1. Chemical identity

The chitosan used for the synthesis of NMPC was

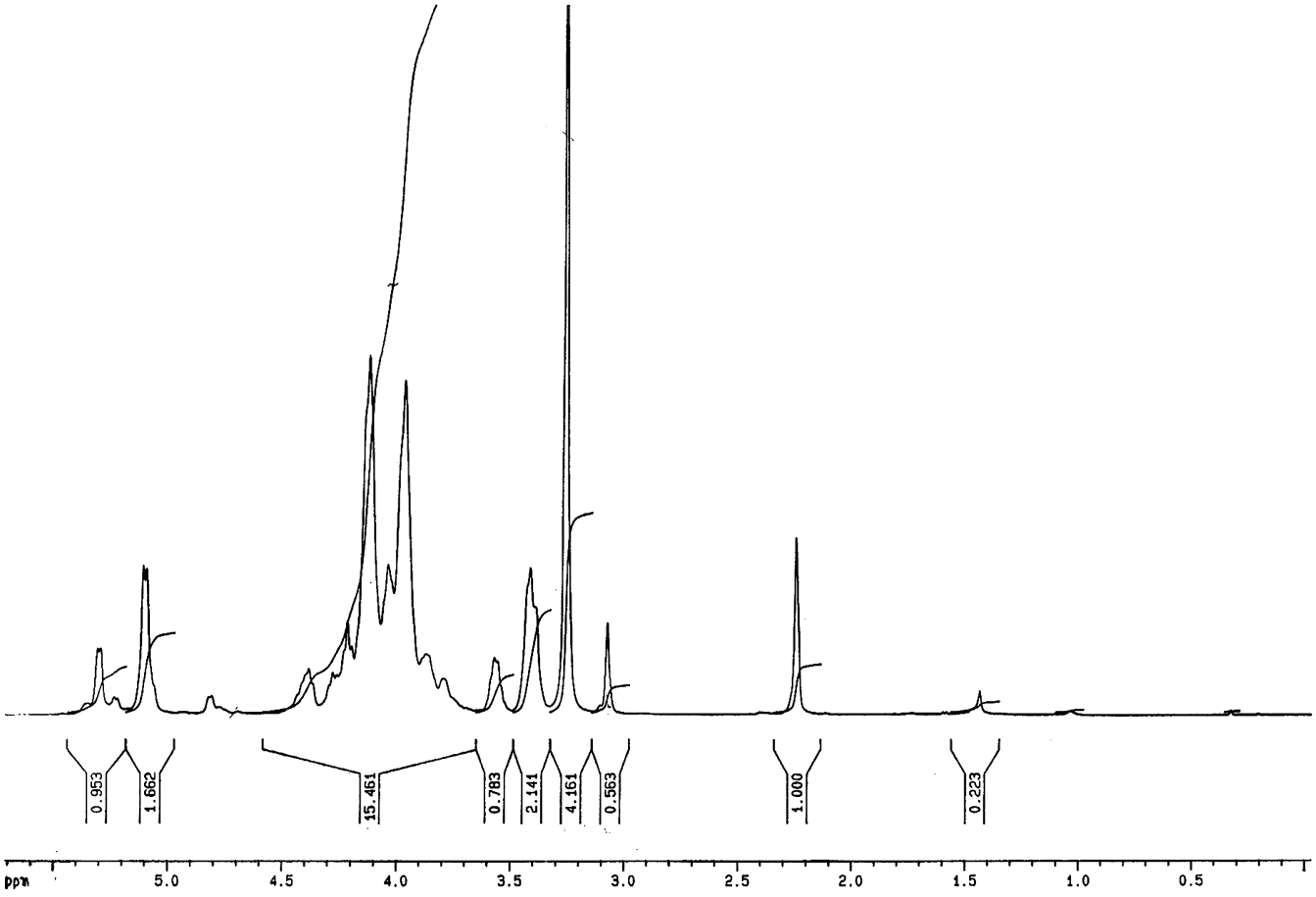


Fig. 4. ^1H NMR spectrum of NMPC dissolved in 2% (w/w) DCl/D_2O. Polymer concentration 20 g/l.

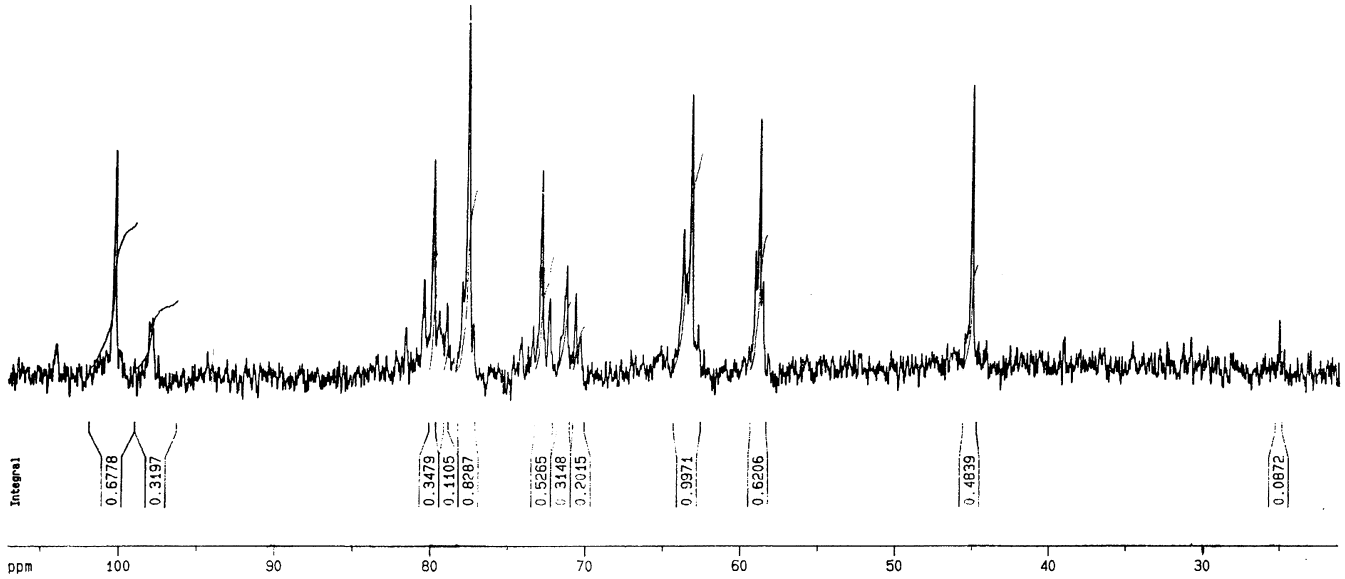


Fig. 5. ^{13}C NMR spectrum of NMPC dissolved in 2% (w/w) DCl/D_2O. Polymer concentration 20 g/l.

characterized and the results were: acetylation degree 16%, moisture 11%, ash 0.38%, viscosity 50 mPa s, molecular weight (M_v) 223,872 and elemental analysis (%)—C, 39.48; O, 46.3; H, 6.93; N, 7.29 (Muzzarelli, 1985; Wojtasz-Pajak et al., 1994).

The assignments and chemical shifts of the ^1H and ^{13}C NMR signals of chitosan are given in Figs. 1 and 2, respectively. Chitosan: ^1H NMR (DCl/D_2O) $\delta = 4.82$ (H_1), $\delta = 3.13$ (H_2), $\delta = 3.59$–3.84 (H_3 H_4 H_5 H_6) and $\delta = 1.97$ ($NCOCH_3$). Chitosan: ^{13}C NMR (DCl/D_2O) $\delta = 100.3$ (C_1), $\delta = 58.8$ (C_2), $\delta = 73.1$ (C_3), $\delta = 79.9$ (C_4), $\delta = 77.9$ (C_5), $\delta = 63.3$ (C_6). These attributions are in agreement with the literature (Dung, Milas, Rinaudo & Desbrieres, 1994; Hirai et al., 1991; Rinaudo, Dung, Gey & Milas, 1992).

The introduction of the phosphonic acid function in the chitosan macromolecule via the Moedritzer and Irani (1966) reaction modified by us, as described above, with phosphorous acid and formaldehyde yielded a chitosan derivative (Fig. 3) with different properties from chitosan. Our hypothesis is the formation of the monophosphonic secondary amine (I) and of the tertiary diphosphonic amine (II).

Chitosan–NH–CH_2–PO_3H_2
(I)

Chitosan–N(–CH_2–PO_3H_2)$_2$
(II)

In the case of NMPC the ^1H NMR spectrum shows the modification due to the introduction of NH–CH_2–PO_3H_2 group replacing the free amino group. The two forms (I and II) are distinguishable on the spectrum (Fig. 4) because of two different chemical shifts of H-1, which appear at 5.08 and 5.3 ppm.

From Fig. 5 and especially the H-1 integrals (Table 1), the yields of species I and II are estimated to be 64 and 36%, respectively. The substituents give characteristic signals between 3.00 and 3.6 ppm.

The suggested structure from ^1H NMR spectroscopy was confirmed by ^{13}C NMR spectrum, Fig. 5 (Table 2). The chemical shifts of C-1 are significantly affected by the phosphonomethylation. The assignments were mainly solved using ^1H–^{13}C correlations (Fig. 6). From this information the two new signals at 45 and 58 ppm are identified as –CH_2 from species I and II, respectively.

In addition to these results, elemental analysis made on the chitosan derivative gave an indication that the degree of acetylation was 0.16, the degree of substitution was 0.38 with *N*-monosubstitution of 0.24 and *N*,*N*-disubstitution of 0.14, and the rest of the amino groups were in the free form (0.37). The results of physical and chemical properties of NMPC were: moisture 10%, ash 12%, viscosity 22.5 mPa s, molecular weight (M_v) 616,595 and elemental analysis (%)—C, 32.66; O, 49.83; H, 6.79; N, 5.42; P, 5.3.

Viscosity. The solutions in the acidic pH range were clear, limpid and colorless; the viscosity of NMPC was practically

Table 1
^1H NMR chemical shifts and normalized integrals of NMPC (ΣH-1 = 100%)

Proton	H_1(I)	H_1(II)	H_2(I)	H_2(II)	H_3 H_4 H_5 H_6	–CH_2(I)	–CH_2(II)	N–$COCH_3$
δ (ppm)	5.10	5.32	3.42	3.58	3.8–4.3	3.25	3.08	2.25
Integral (%)	64	36	82	30	591	159	22	38

Table 2
^{13}C NMR chemical shifts and normalized integrals of NMPC (ΣC-1 = 100%)

Carbon	C_1(I)	C_1(II)	C_2	C_3	C_4	C_5	C_6	CH_2(I)	CH_2(II)
δ (ppm)	100.3	97.9	70.7–71.2	72.5–72.9	79.1–79.9	77.6	63.2	58.9	45
Integral (%)	68	32	51	53	46	83	99	62	48

independent of the pH value (22.5 mPa s) and these solutions at pH 6.8 became insoluble and precipitated as a white product.

Solubility test. Table 3 lists the solubility of chitosan and NMPC in aqueous acid solvents, alkali and organic solvents. As can be seen, the chitosan derivative shows a big solubility with respect to chitosan, especially in aqueous media over an extended pH range.

Film casting. The films obtained were translucent, brilliant and mechanically resistant and had a homogeneous aspect. The thickness of the film was 10.1 μm.

SEM. Even in the spongy freeze-dried NCMP, the tendency to form films is quite evident when the material is examined at the electron microscope. It shows a relatively homogeneous aspect with a tightly packed structure (Fig. 7).

X-ray diffraction spectrometry. Chitosan exhibits two main diffraction peaks centered around 9 and 19 2θ values, indicative of their structural and conformational features. In

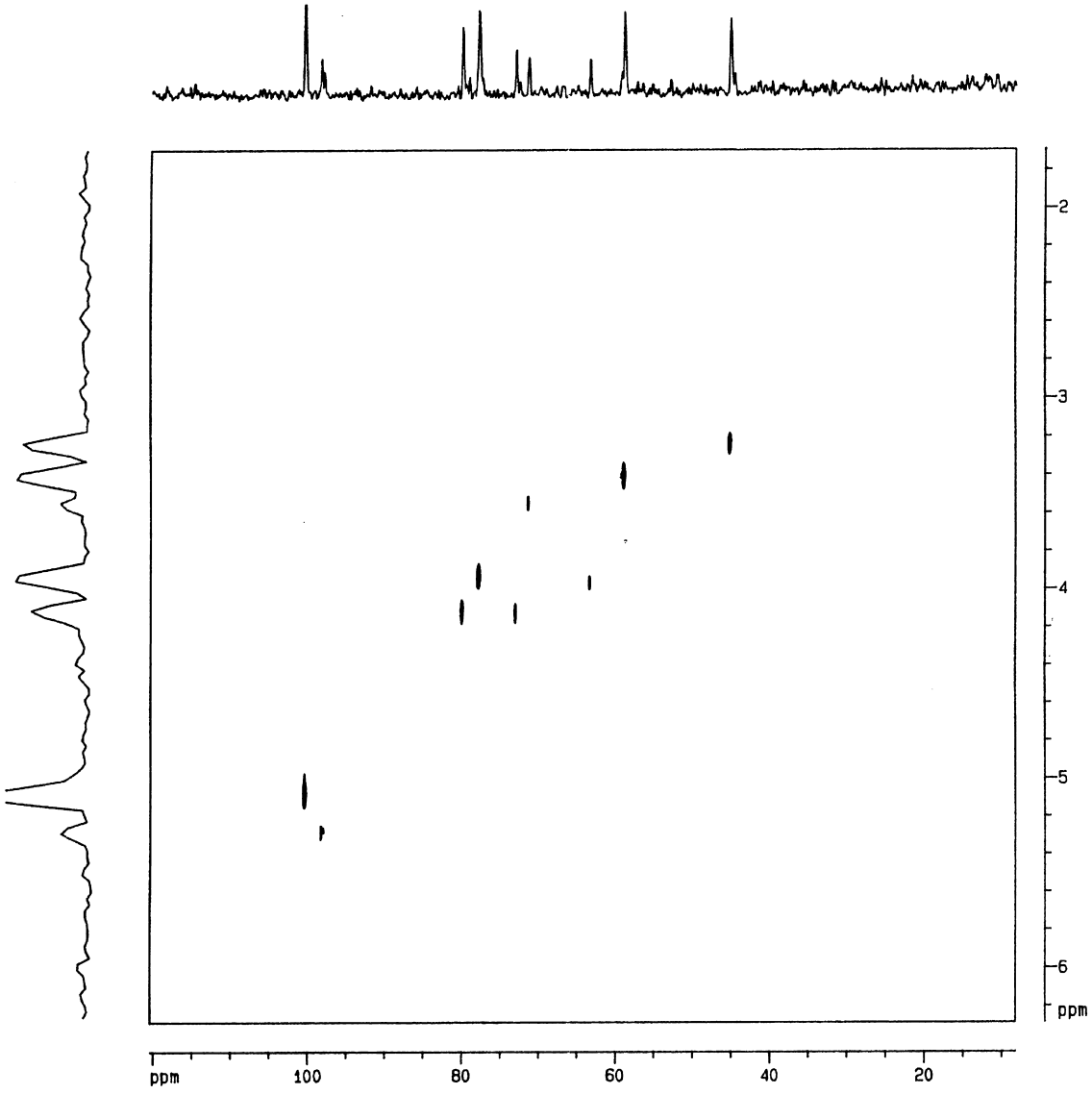


Fig. 6. $^{1}H-^{13}C$ NMR NMPC.

Table 3
Solubility of chitosan and NMPC

Solvent	Chitosan	NMPC
H_2O	Insoluble	Soluble (immediate)
NaOH (1%)	Insoluble	Low viscosity gel
HCl (1%)	Swelling	Soluble (immediate)
Acetic acid (1%)	Soluble (1 h)	Soluble (immediate)
Dimethylformamide	Swelling	High viscosity gel
Dimethylsulfoxide	Insoluble	Insoluble
Pyridine	Swelling	High viscosity gel
Acetone	Insoluble	Low viscosity gel
Ethanol	Insoluble	Low viscosity gel

the present case, the reflections at the highest 2θ values lie close to the values observed for chitosan and therefore, it could be speculated that these reactions on the amorphous chitosan restore in part the macromolecular order; however, the reflections at lower 2θ values were far from the values found in chitosan and were representative of a novel structure that accommodated the bulky substituents. At the X-ray analysis, the water-soluble derivative was amorphous; most of the diffraction bands were depressed or absent.

IR spectroscopy. The FT-IR spectrum taken on the modified chitosan shows the characteristic peaks at 2500–3500 cm^{-1} (P–OH), 1164 cm^{-1} (P=O), 1068 cm^{-1} (P–OH) and 918 cm^{-1} (P–O).

5. Conclusion

The chemically modified chitosan considered here has the filmogenic nature of its parent chitosan but with the improvement of an increased solubility over an extended pH range. The proposed preparation described here for the first time is simple and safe. The reactivity is so high that partially disubstituted chitosan is produced, even under mild conditions.

Fig. 7. Electron micrograph for NMPC freeze-dried sample, showing its film-forming ability (×198).

In fact, the chemical identity of the NMPC was assessed by FT-IR, ^1H and ^{13}C NMR spectrometry. It is thus demonstrated that it gives both *N*-monosubstitution and *N*,*N*-disubstitution without modification of the initial degree of acetylation. The two species formed (I and II) exist in a ratio of around 64/36. This work opens new perspectives by taking advantage of the introduction of phosphonic groups into the chitosan, providing water solubility and potential chelating ability.

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