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Short communication

Synthesis and characterization of *N*-propyl-*N*-methylene phosphonic chitosan derivative

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ABSTRACT

A simple methodology for the preparation of a new chitosan derivative called *N*-propyl-*N*-methylene phosphonic chitosan (PNMPC) is proposed. Introduction of a propyl chain onto a modified chitosan (*N*-methylene phosphonic chitosan) offers the presence of hydrophobic and hydrophilic branches for controlling solubility properties of the new derivative. Its chemical identity was determined by FT-IR, ^{1}H, ^{13}C and ^{31}P NMR spectroscopy. The degree of propyl substitution estimated by elemental analysis was 0.64. Furthermore derivative molecular weight is about 60×10^3, X-ray diffraction and SEM showed certain degree of crystallinity and homogeneous surface with a rather packed structure. This derivative opens new perspectives in food, pharmaceutical and cosmetic fields.

1. Introduction

Chitosan, the deacetylated derivative of chitin, is a natural biopolymer consisting of β-1-4 linked *N*-acetyl glucosamine (GlcNAc) and glucosamine (GlcN) repeating units. It has a high molecular weight resulting in its low solubility in most solvents and shows bioactivity only in acidic medium, these reasons limit its applications especially in medicine and food industry.

To increase aqueous solubility and to improve biological, chemical and physical properties, many derivatives of chitosan have been synthesized (Alves & Mano, 2008; An, Dung, Thien, Dong, & Nhi, 2008; Dung, Milas, Rinaudo, & Desbriéres, 1994; Ma et al., 2008; Mourya & Inamdar, 2008; Rinaudo, 2006; Sui, Wang, Dong, & Chen, 2008).

In a previous work we described 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, one step, reaction system for the purpose of creating a chitosan derivative that allowed solubility in water under neutral conditions (Heras, Rodríguez, Ramos, & Agulló, 2001). Lately a methodology was developed for the preparation of a derivative carrying alkyl and phosphonic groups (LMPC). On the LMPC the addition of alkyl groups seems to weaken the hydrogen bond and provides good solubility in organic solvents (Ramos, Rodríguez, Rodríguez, Heras, & Agulló, 2003). Moreover, the new derivative proves to be an amphiphilic system in which the hydrophobic moiety counterbalances the electrostatic repulsion but it also gets more tensioactive properties.

In this paper, we report the successful preparation of an *N*-alkyl derivative of the water soluble NMPC using a reductive *N*-alkylation with propyl aldehyde to obtain a new amphiphilic hybrid material of synthetic and natural polymer so-called *N*-propyl-*N*-methylene phosphonic chitosan (PNMPC).

2. Materials

2.1. Preparation of chitin and chitosan

Chitin was isolated from shrimp shells waste (*Pleoticus mülleri*). It was homogenized and rinsed with water to remove the organic material, then treated with 9% (w/w) NaOH at 65 °C for 90 min, to remove proteins and demineralized with 10% (v/v) HCl at 20 °C for 15 min, washed until neutral pH and dried.

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

2.2. Synthesis of N-methylene phosphonic chitosan (NMPC)

A solution of phosphorous acid/water (1:1 w/w) was added dropwise with stirring, at room temperature for 1 h to chitosan 2% (w/v) in glacial acetic acid 1% (v/v). The temperature of the reaction vessel was raised to 70 °C and one part of formaldehyde 36.5% (by weight) was added dropwise over 1 h with reflux and left overnight at the same temperature. Solution was dialyzed against

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demineralized water for 48 h or until pH was raised to 6.8 in dialysis tubing with a cut off value of 12,400 Da. Finally solution was freeze-dried (Heras et al., 2001).

2.3. Synthesis of N-propyl-N-methylene phosphonate chitosan (PNMPC)

NMPC (1 g) was suspended in 100 mL distilled water–methanol (1:1 v/v), propyl aldehyde (1.5 g) was added and stirred for 30 min. Reduction was carried out with an excess of sodium borhydride which was added for 2 h and then left stirring overnight at room temperature. PNMPC sodium salt was obtained by dialyzing the reaction mixture against demineralized water for 48 h or until a water pH of 6.8 (dialysis tubing with a M_W cut off value of 12,400). The solution was freeze-dried.

3. Methods

3.1. Characterization of PNMPC

3.1.1. X-ray diffraction spectrometry

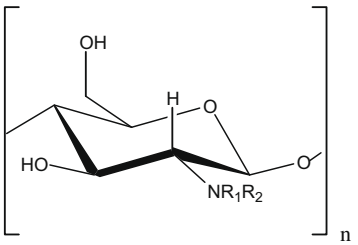
X-ray diffraction spectrometry data were collected using a Rigaku D-Máx. III C diffractometer (Cu Kα) irradiated at 35 kv–15 ma.

3.1.2. NMR spectroscopy

^{13}C, ^{1}H and ^{31}P NMR spectra were recorded on a Varian VNMRS-400 instrument spectrometer at 70 °C. PNMPC (23 mg) was dissolved in 0.5 ml of 5% (w/w) DCl/D_2O at 70 °C. Chemical shift values were recorded downfield from trimethylsilyl propionate sodium salt (TSP) as standard and PO_4H_3 (85%) for the ^{31}P NMR spectrum. The heterocorrelation was done with a Bruker DMX-500 instrument.

3.1.3. IR spectroscopy

The spectrum was recorded on a Nicolet FT-IR instrument. The KBr discs were prepared by blending anhydrous KBr with PNMPC (1%).



[A-/-B-/-C-/-D-/-E-/F…]n

	R_1	R_2
A	-H	$-COCH_3$
B	-H	-H
C	-H	$-CH_2-PO_3H_2$
D	$-CH_2-PO_3H_2$	$-CH_2-PO_3H_2$
E	-H	$-CH_2-CH_2-CH_3$
F	$-CH_2-CH_2-CH_3$	$-CH_2-CH_2-CH_3$

Fig. 1. Chemical structure of *N*-propyl-*N*-methylene phosphonic chitosan.

3.1.4. Solubility test

Solubility of PNMPC in different solvents was evaluated. Solutions of 10 mg of the polymer in 5 mL of each solvent were prepared.

3.1.5. Molecular weight determination

Weight-average molecular weight (M_w), number-average molecular weight (M_n) and molecular weight dispersion (M_w/M_n) were determined by a Waters-Breeze gel permeation chromatograph (Model 1525), connected to a Waters 2414 (Mod.410) Differential refractometer and a Dawn DSP Light scattering detector. A set of five columns connected in series Ultrahydrogel, Waters 7.8 × 300 mm of 120, 250, 500, 1000 and 2000 Å size pore were used. The temperature was maintained at 30 °C. The eluent was CH_3COOH/CH_3COONa pH 4.8, standards were Pullulans (Shodex Standard P-82. No. 30901-Showa Denko).

3.1.6. Elemental analysis

Elemental analysis were carried out with a Carlo Erba 1108 instrument. Gas separation was done by a gas chromatograph with a variable length Porapak column and a TCD detector.

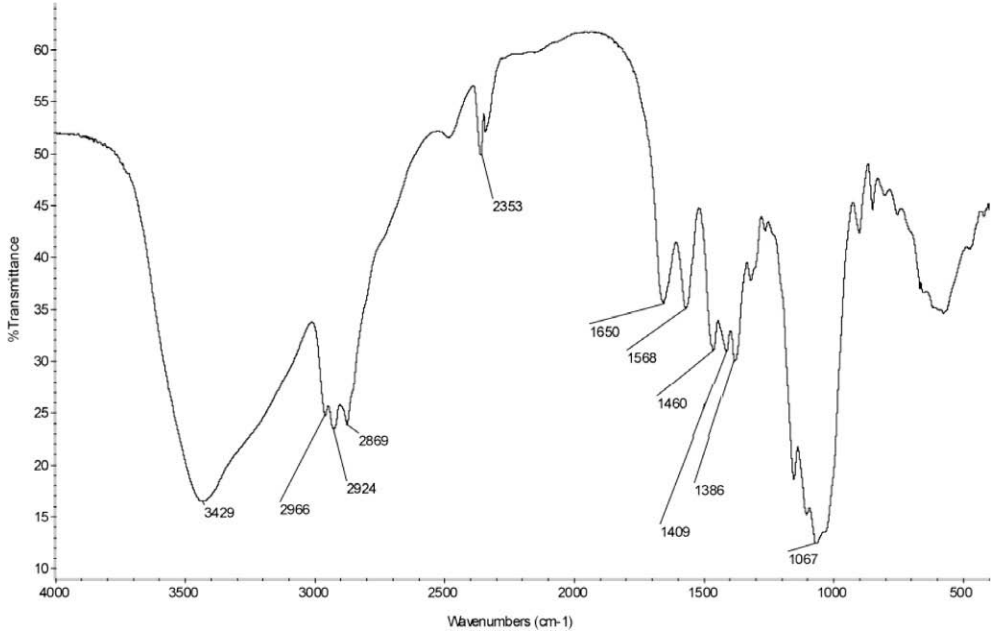


Fig. 2. FT-IR spectrum of PNMPC.

Table 1
^{13}C NMR chemical shifts of PNMPC.

Carbon	C_1 (C)	C_1 (F)	C_1 (D + E)	C_2	C_3	C_4	C_5	C_6	CH_2 (D)	CH_2 (E)
δ (ppm)	100.37	99.49	98.00	70.81 71.49	71.07 72.52	79.69 80.90	77.64	63.89	51.70	45.24

Signals at 45.24, 22.24 and 13.17 correspond to $-CH_3$ and $-CH_2$ of the propyl group.

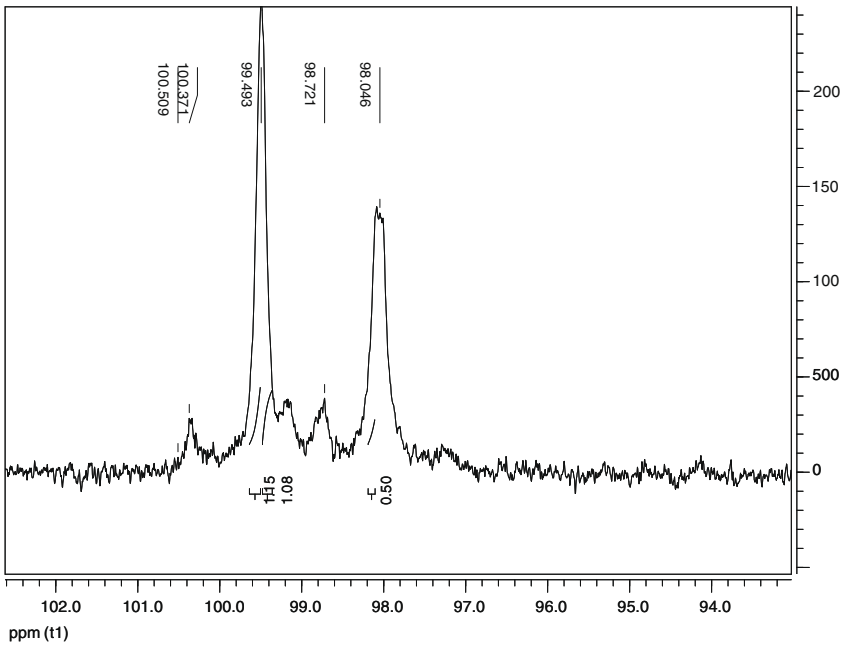


Fig. 3. ^{13}C NMR PNMPC spectrum expansion.

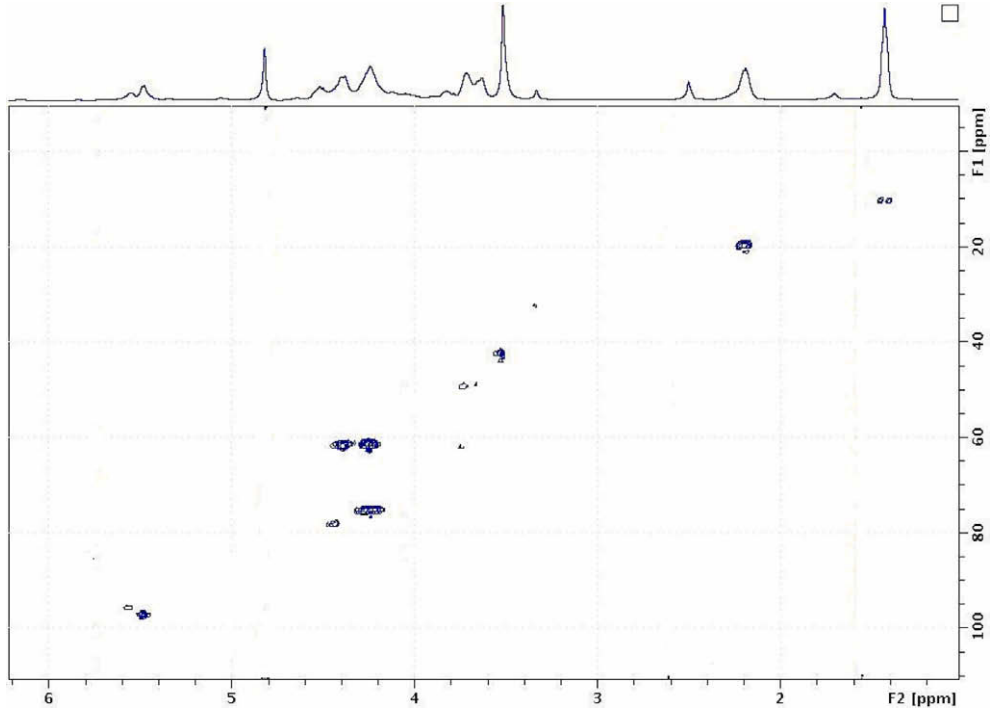


Fig. 4. $^{1}H-^{13}C$ NMR spectra.

3.1.7. Scanning electron microscopy

Scanning electron microscopy were carried out with a Scanning electron microscope LEO mod. EVO 40 XVP.

4. Results and discussion

The introduction of C3 chain onto NMPC's free amino groups by a reductive amine reaction leads to *N*-propyl-*N*-methylene phosphonic chitosan (PNMPC). The incorporation of *N*-methylene phosphonic group afforded the hydrophilic moieties and alkyl group the hydrophobic one.

Fig. 1 shows its chemical structure with the possible combinations of R_1 and R_2 substituents.

The FT-IR spectrum (Fig. 2) shows the axial stretching of OH group in 3429 cm^{-1} which appears superimposed to the NH stretching band, an amide band (amide I) at 1568 cm^{-1} and an axial deformation band due to C–N at 1409 cm^{-1}.

The alkyl chain shows signals at 2966, 2924 and 2869 cm^{-1} corresponding to CH_3 and CH_2 groups, besides the characteristic deformation signals at 1460 and 1386 cm^{-1}.

The carbonyl group due to NH–$COCH_3$ group appears in 1650 cm^{-1}.

This derivative also presents two phosphonic group characteristics bands, one at 2353 cm^{-1} (splitted) due to P–H stretching and the other at 1067 cm^{-1} corresponding to *v* P–OH.

The assignments and chemical shift of ^{1}H NMR signals are:

δ = 5.09 (C and F, H_1), 5.02 (E, H_1); δ = 4.31 (CH–N=C imine); δ = 4.06–3.80 (H3, H4, H5, H6), δ = 3.38–3.28 (H_2); δ = 3.27 [N–CH_2–P, (D)] δ = 3.07 [CH_3–CH_2–**CH_2**–N, N–CH_2–P (C)]; δ = 2.06 (–NHCO**CH_3**); δ = 1.75 (CH_3–**CH_2**–CH_2–N); δ = 0.99 (**CH_3**–CH_2–CH_2–N).

On the ^{1}H and ^{13}C NMR spectra the replacement of the free amino group of chitosan to obtain the NMPC derivative showed two forms distinguishable: one was assigned to the monophosphonic secondary amine and the other one to the tertiary diphosphonic amine, showing that the time of reaction enhances the degree of phosphonomethylation (Heras et al., 2001).

The signal at 5.09 ppm is the evidence of dialkylation of the amine group which is rather increased due to the presence of a minor percentage of the *N*-methylene phosphonic moiety. Desbrières, Martínez, and Rinaudo (1999) noted an identical chemical shift for a similar propyl derivative.

The signal at 5.02 ppm, of higher intensity, is assigned entirely to the monopropyl form showing that it is the predominant group.

At 4.54 ppm there is a little signal corresponding to the *N*-acetylglucosamine unit.

Table 1 shows the signals assignment of the ^{13}C NMR spectrum. The chemical shift at 99.49 ppm is due to the introduction of one alkyl group. Signals at 45.24, 22.24 and 13.17 correspond to –CH_3 and –CH_2 of the propyl group.

An expansion of the spectrum allowed us to find that in 98.00 ppm there are evidences of a splitting which is the result of the overlapping of the dialkylated moiety and the tertiary diphosphonic amine. Furthermore there is a small signal due to the monophosphonic secondary amine at 100.37 ppm (Fig. 3).

To confirm the presence of the phosphonic group a ^{31}P NMR spectrum was registered. The ^{31}P NMR chemical shifts of α-aminomethylphosphonic acids are greatly dependent upon the acidity of the solution in which they are measured, such effect causes downfield shifts (Moedritzer & Irani, 1966). In this study the polymer was dissolved in D_2O/DCl and shows a signal centered at −0.004 ppm Assignments of ^{1}H and ^{13}C NMR spectra were confirmed by two-dimensional heteronuclear shift correlation spectroscopy (Fig. 4).

The substitution degree (SD) of the PNMPC was calculated by comparing the C and N molar ratio obtained from the elemental analysis in different derivatives. It can be observed an increase in the molar ratio as a consequence of the introduction of the propyl group. The degree of phosphonomethylation and alkylation were stipulated to be 1.56 and 0.64, respectively (Table 2).

X-ray diffraction analysis (Fig. 5) showed that PNMPC white powder has a certain degree of crystallinity. Two reflections fall

Table 2
Elemental analysis and substitution degree (SD) of chitosan and its derivatives.

	C	N	C/N*	DS
Chitosan	40.87	7.61	6.30	
NMPC	34.68	5.25	7.86	1.56
PNMPC	43.63	5.23	9.78	0.64

* Molar ratio C/N.

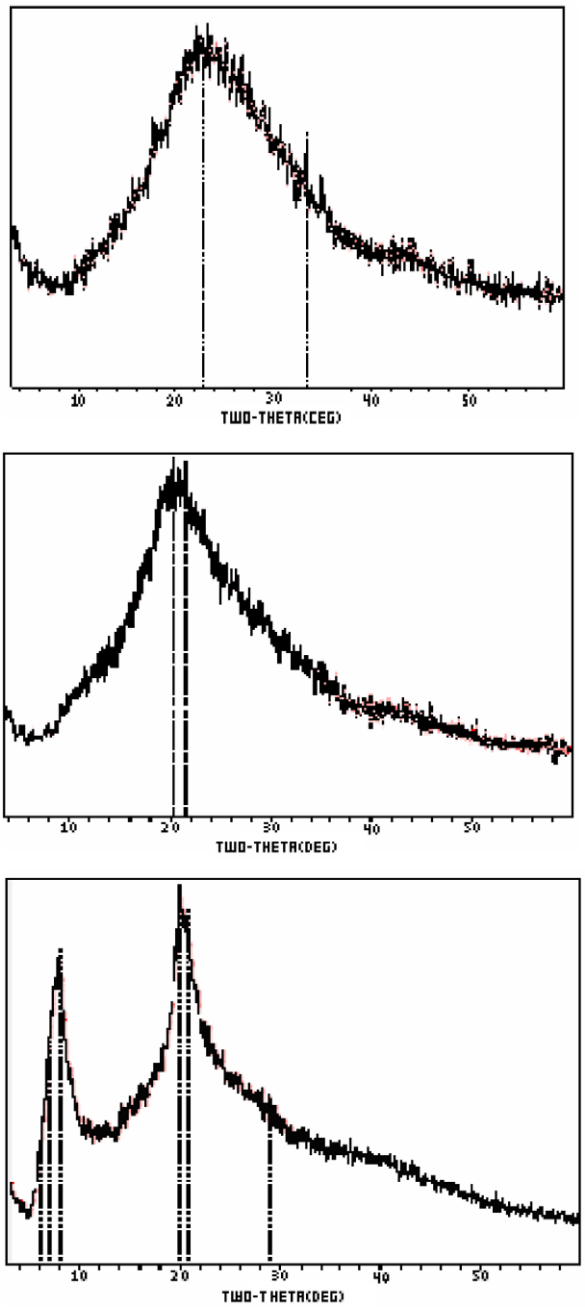


Fig. 5. X-ray diffraction pattern of Chitosan, NMPC and PNMPC.

at 2θ: 7°72′ and 20°03′ for the alkylated derivative with an evident increase of intensity of the second band, whereas chitosan and NMPC only showed one broad peak at 2θ: 21°44 and 22°72′ respectively. It may be concluded that the rise of crystallinity is a consequence of the introduction of the short alkyl chain.

The phosphonomethylation reaction proved to decrease the molecular weight of the original chitosan. This effect was more significant with longer reaction times (Ramos et al., 2003). Considering the experimental conditions we expected a molecular weight in the range of 50×10^3 Da. M_n, M_w and D (M_w/M_n) were determined by GPC. Figs. 6 and 7 show the signals obtained for PNMPC determined with a differential refractometer and a light scattering detector. Reproducibility of results obtained with both detectors at different concentrations proves to be acceptable, indicating that the main macromolecular structure of the derivative is about M_w 60×10^3 Da.

It is well known the effect of introducing hydrophobic branches on chitosan derivatives for controlling the solubility properties. Acetamido and primary groups of the polymer have an important role in the formation of intra- and/or intermolecular hydrogen bonding. The removal of the two hydrogens atoms of amino group of chitosan and the introduction of some hydrophobic nature group by chemical modification causes destruction of its crystalline structure resulting in the improvement of solubility in organic solvents.

Table 3 shows PNMPC solubility at different temperatures: at 60 °C it proves to be soluble in HCl solutions (0.1 M and 1%) and in organic solvents like acetone, ethanol, pyridine, DMF and DMSO.

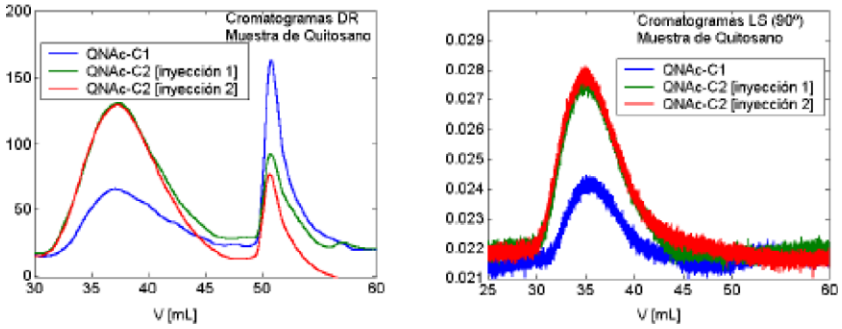
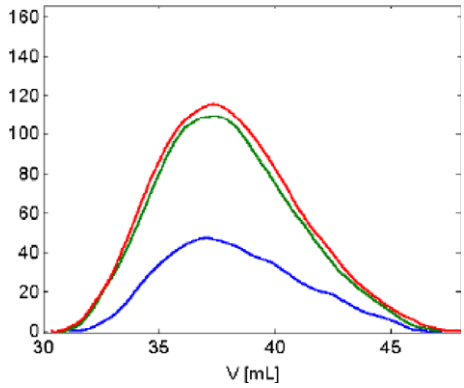


Fig. 6. GPC/SEC-LS and DR signals for PNMPC.



PNMPC Samples	M_n	M_w	D	Mass [mg]	M P Volumen [mL]	Concentration [mg/mL]
QNAc-C1	25,900	57,800	2.23	5.6	5.00	1.12
QNAc-C2 Inyection 1	27,900	63,300	2.27	11.3	5.00	2.26
QNAc-C2 Inyection 2	26,600	63,400	2.38	11.3	5.00	2.26

Fig. 7. GPC/SEC-DR compared chromatograms.

Table 3
Solubility test of PNMPC derivative.

	NaOH 0.1 M	NaOH 1%	HCl 0.1 M	HCl 1%	Acetic acid 1%	DMF	DMSO	Acetone	Ethanol	Water	Pyridin
18 °C	–	–	+	++	–	–	–	–	–	–	+
40 °C	–	–	++	++	–	–	–	–	–	–	+
60 °C	–	+	++	++	–	+	++	+	+	+	+

Sample (10 mg) and 5 ml of solvent; ++, soluble; +, partially soluble; +–, swelled; –, insoluble.

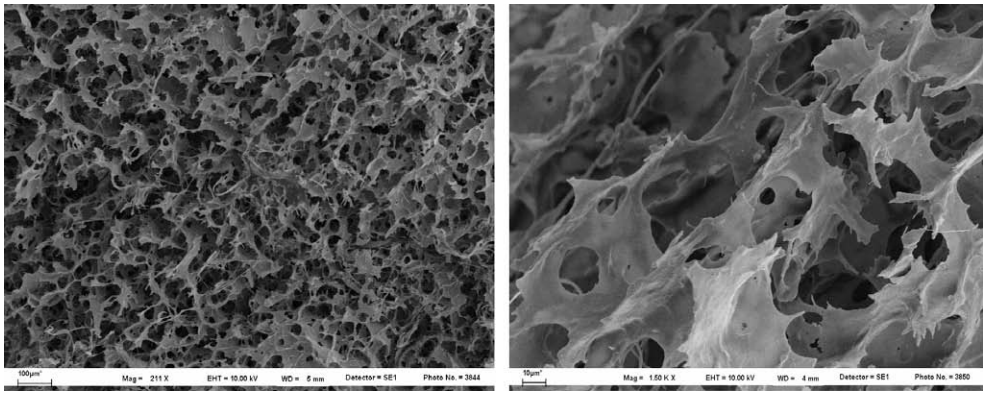


Fig. 8. SEM images of PNMPC derivative.

PNMPC showed a rather similar behaviour to LMPC derivative, synthesized previously by our group, when its solubility was evaluated with the same solvents (Ramos et al., 2003).

The SEM image presents a homogeneous surface with a rather packed structure (Fig. 8).

5. Conclusion

We developed the synthesis of a novel chitosan derivative which improved the properties of NMPC by adding a propyl group at the residual *N*-positions.

PNMPC has a predominant monoalkylated form, though it may be detected the dialkylated one. The introduction of the short chain slightly enhanced its water solubility as a consequence of the formation of the dialkyl moiety which increases the carbon contribution.

PNMPC M_w of 60×10^3 was acceptable for this kind of derivative and the X-ray proved that crystallinity increased respect to the original chitosan and NMPC derivative.

Acknowledgement

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