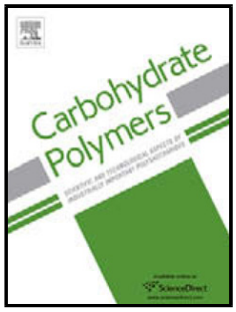

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Synthesis, characterization and evaluation of reactional parameters on substitution degree of *N*-hexyl-*N*-methylene phosphonic chitosan

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Highlights

- N-hexyl-N-methylene phosphonic chitosan (HNMPC) is synthesized under mild conditions.
- The influence of reactional parameters on substitution degree was evaluated.
- HNMPC was thoroughly characterized by FT-IR, ^{1}H, ^{13}C, $^{1}H^{13}C$-HSQC NMR, XRD and SEM techniques.
- HNMPC forms transparent films.
- Stability of emulsions evaluated makes HNMPC promising to industrial applications.

Abstract

N-methylene phosphonic chitosan (NMPC) is treated with hexyl aldehyde to give an imine, which is easily converted into *N*-hexyl-*N*-methylene phosphonic chitosan (HNMPC) under mild conditions. The structure of this new chitosan derivative is characterized by FT- IR, ^{1}H, ^{13}C, ^{31}P, $^{1}H^{13}C$-HSQC NMR, SEM and XRD. The influence of reactional parameters on the substitution degree (DS), evidenced that a mol ratio 1.50:1.00 (hexyl aldehyde: free amino groups); a reaction time of 1 h. and 45ºC of temperature afford the best DS. HNMPC molecular weight is 12,768.62 Da. It shows good emulsifying properties giving o/w emulsions with high stability in time.

Microscopic observation as well as particle size distribution show an unimodal droplet size distribution with low droplet diameters. Preliminary tests lead us to believe that this new polymer has good film forming properties.

Keywords: *N*-hexyl-*N*-methylene phosphonic chitosan, characterization, NMR, degree of substitution, films, emulsifying capacity.

1. Introduction

The sugar backbone of chitosan consists of β-1,4-linked D-Glucosamine with a variable degree of *N*-acetylation, a structure very similar to that of cellulose, except that the hydroxyl group on the C-2 position is replaced by an acetyl amino group. Chitosan possesses -OH and -NH_2 groups that can give rise to hydrogen bonding or can be exploited for production of derivatized chitosan (Dung et al. 1994; Rinaudo, 2006, Ma et al. 2008; An et al. 2008; Alves & Mano, 2008; Mourya & Inamdar, 2008; Badawy et al. 2012; Badawy et al. 2013). From a chemical standpoint, the incorporation of new chemical groups or moieties provides versatile materials with specific functionalities and modified physical and biological properties. These derivatives are non-toxic, have a wide range of applications and the raw material sources are unlimited.

Chitosan has been processed in different ways to get modified physicochemical properties. Some of them, with the aim of improving its solubility, chemical and even biological properties (Kumar, Muzzarelli, Muzzarelli, Sashiwa & Domb, 2004; Kurita, 2001; Mourya & Nazma, 2008, Kumar, Dutta & Dutta 2009, Badawy et al. 2014, Hu et al, 2016, Zhang et al., 2017). It is largely known for its activity against a wide

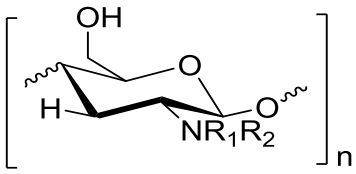
range of microorganisms, in which the most acceptable antimicrobial mechanism is found to include the presence of charged groups in the polymer backbone and their ionic interactions with bacteria wall constituents (Goy et. Al, 2016).

Degree of substitution (DS) is a parameter that plays a very important role on the applications of chitosan derivatives and its determination has been well documented in the literature (Kurita et al., 2002; Santos et al., 2005a; Santos, Dockal, & Cavalheiro, 2005b, Pestov et al., 2016).

The successful preparation of *N*-methylene phosphonic chitosan (NMPC) was opportunely reported, as well as, the effect of preparation methods on its properties (Heras et al. 2001, Ramos et al. 2003). NMPC proved to have film forming properties like chitosan, but with the improvement of an increased solubility over an extended pH range. This derivative was also modified and grafted with alkyl chains to obtain amphiphilic properties that have potential applications in cosmetics (Ramos et al., 2003). Hydrophobicity is adjusted according to the length of the alkyl chain or the substitution degree on amine function (Desbrières, 2004; Desbrières et al 1996; Keisuke et al. 2002; Sashiwa & Shigemasa 1999; Uragami et al. 1997, Aranaz et al. 2010; Philippova & Korchagina 2012; Viegas de Souza et al. 2013). Reductive amination of carbonyl compounds was carried out to obtain *N*-lauryl- (LNMPC) and *N*-propyl-*N*-methylene phosphonic chitosan (PNMPC) (Ramos et al, 2003; Zúñiga et al. 2010). Although successful preparation and characterization of these novel biopolymeric derivatives was informed, any effort in order to maximize the extension of substitution degree has yet to be described.

The main object of the present work is to inform the synthesis and characterization of *N*-hexyl-*N*-methylene phosphonic chitosan (HNMPC) (Fig.1), as well as the influence of some reactional parameters (mol ratio of aldehyde: free amino groups,

reaction temperature and time) on DS in the preparation of the biopolymeric derivative. Solubility, film forming properties and potential emulsifying capacity of the new derivative are analyzed.



[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	$-CH_2-(CH_2)_4-CH_3$
F	$-CH_2-(CH_2)_4-CH_3$	$-CH_2-(CH_2)_4-CH_3$

Figure 1.Chemical structure of *N*-hexyl-*N*-methylene phosphonic chitosan (HNMPC)

2. Materials

Chitin was isolated from shrimp shells waste from (*Pleoticus muelleri*) Ingeniero White Port, Bahía Blanca, Argentina. The material was homogenized and the product was rinsed to remove the organic material. Afterwards, it was treated with 9% (w/w) NaOH at 65°C for 90 min to remove proteins and finally, demineralized with 10% (v/v) HCl at 20°C for 15 min. The product in the aqueous solution was then washed and dried.

Chitosan was prepared directly by heterogeneous deacetylation of chitin at 136°C with 50% (w/w) NaOH for one hour. Its characteristics were: molecular weight 52,980.96 Da; acetylation degree 11.8%; moisture 12%; ash 0.39%; viscosity 63mPas (1% w/w in 1% acetic acid at 25°C) measured with a Brookfield model DV-IV+ viscometer at 50 RPM with spindle 21 (Brookfield, Stoughton, Ma.).

2.1 Synthesis of *N*-methylene phosphonic chitosan (NMPC)

Phosphorous acid dissolved in water (1:1 w/w) at room temperature for one hour was added dropwise with stirring to a 2% chitosan solution (w/v) in glacial acetic acid 1% (v/v).After that, the temperature of the reaction vessel was raised up to 70°C and one part of formaldehyde 36.5% (by weight) was added over 1 h with reflux and left overnight at the same temperature. The obtained solution was dialyzed against 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, the solution was freeze-dried (Heras et al.2001). NMPC characteristics were: moisture 10.9%; ash 4.0%; viscosity 80 mPa.s (1% w/v at 25°C). NMPC substitution degree (DS) was determined by elemental analysis as 1.54.

2.2 Syntesis of *N*-hexyl-*N*-methylene phosphonic chitosan (HNMPC)

NMPC (1 g) was suspended in distilled water-methanol in a ratio 1:1 (45 mL). This reaction mixture was let to react under different experimental conditions:

- Mol ratio of hexyl aldehyde and free amino groups of 1.00:1.00, 1.10:1.00, 1.50:1.00 and 2.00:1.00.The mixtures were stirred during 30 minutes at 25ºC (free amino groups of NMPC were determined by ^{1}H NMR).
- Reaction time of 1, 2 and 3 hours. The ratio of 1.50:1.00 hexanal: free amino groups was kept and stirred at room temperature.
- Reaction temperatures of 25º, 35º, 45º and 55°C. The ratio of 1.50:1.00 hexanal: free amino groups was maintained stirring for 1 hour.

Then, reduction was carried out with an excess of sodium borohydride which was added dropwise and then left stirring overnight at room temperature. The reaction mixture was dialyzed against demineralized water until it reached a pH of 6.8 (dialysis tubing with a MW cut off value of 12,400). The solution was freeze-dried and HNMPC was obtained in a good yield (88%).

3. Methods

3.1 X-Ray Diffraction Spectrometry

X-Ray Diffraction data was collected using a Rigaku D-Máx. III C Diffractometer (Cu $K\alpha$, λ = 1.5406 Å) irradiated at 35 kv-15 ma. DATASCAN was the acquisition data program used. Data was analyzed using JADE 8 and the PDF4 database of the International Centre for Diffraction Data (ICDD).

3.2 NMR Spectroscopy

^{13}C, ^{1}H, ^{31}P and $^{1}H^{13}C$-HSQC NMR spectra were recorded on a Varian VNMRS-400 instrument spectrometer at 70°C. HNMPC (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. VNMRJ 3.0 was used for data acquisition and MNova software for data processing.

3.3 FTIR Spectroscopy

Fourier Transform Infrared (FTIR) spectrum was performed ranging from 4000 cm^{-1} to 400 cm^{-1} using a Nicolet FT-IR instrument. KBr discs were prepared by blending anhydrous KBr with HNMPC (1%). The spectrum was collected using 32 scans with

a resolution of 4 cm^{-1}. Data analysis was performed using the spectral analysis software OMNIC 8.0 (Thermo Fisher Scientific, USA).

3.4 Solubility Tests

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

3.5 Molecular Weight Determination (M_w)

Measurements were performed using an automatic viscosity measuring unit (Visco-Clock, Schott-Geräte, Germany) and an Ubbelohde capillary viscometer at constant temperature (25ºC) using 0.3 M acetic acid- 0.2 M sodium acetate solution (Brugnerotto, Desbrières, Heux, Mazeau & Rinaudo, 2001) and capillary model 525.20II, diameter 1.03 mm. The M_w was calculated from the intrinsic viscosity values using the Mark-Houwink equation constants a= 0.76 and Km= 0.69 $x10^{-3}$ dL/g.

3.6 Elemental Analysis

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

3.7 Differential Scanning Calorimetry (DSC)

Thermal properties of HNMPC were studied using a DSC Q20 TA Instrument. The baseline was obtained using empty aluminium pans. HNMPC sample (5 mg) was encapsulated in a pan and heated at 20°C/min. from 30 to 400° in a N_2 gas

atmosphere (50 mL/min.). Universal Analysis 2000 software, version 4.5A was used to analyze the data.

3.8 Scanning Electron Microscopy (SEM)

Scanning electron microscope LEO mod. EVO 40 XVP was used to characterize the surface of the HNMPC derivative. Data analysis was performed using the corresponding software LEO-32 V04.00.10.

3.9 Emulsion Preparation and Creaming Index (CI)

Emulsion stability of oil/HNMPC solutions (10/90) at different aqueous concentrations of HNMPC derivative (0.04, 0.20 and 1%) in acetic acid 2% was assessed to determine Creaming Index (CI), at 24 h and after 15 days. Droplet size diameter (D(4,3)) was evaluated using a HORIBA LA-950V Laser diffraction particle sizer. The emulsions were prepared by stirring at 13,600 rpm for 1 minute with a Braun Food Processor MR5550 CA. Emulsions were stored in 100 mL graduated glass cylinders covered with stoppers for 24 hours at room temperature. The creaming behavior of the samples was monitored by visual observation. The CI is defined as the Hs/Ht ratio, in which Hs is the height of the upper concentrated emulsion after creaming and Ht the total height of the solution in the test tube.

The extent of creaming was characterized using the following equation:

$CI = 100x\ H_s / H_t$

The tests were run in triplicate and with a dilution of 1/100 with distilled water.

3.10 Microscopic Observations

An Olympus optical microscope (BH2-UMA, Olympus America Inc.) equipped with a Sony CCD IRIS/RGB photographic camera was used to capture images of the emulsions.

Brookfield viscometer (Model DV-II+, Brookfield Engineering Inc., USA) was used to measure the viscosity of the emulsions at 25°C.

3.11 Film Characterization

Films were prepared by dissolving HNMPC (0.2 g) in 20 mL of 1%. acetic acid solution at room temperature. The solution was casted in a Petri dish and was dried for 48 h at 37°C, with light presence to form the desired film. Complete drying was avoided as some moisture is required for films to remain flexible and not to crack. The film was finally removed (by peeling) from the dish. Film thickness measurements were performed with a Filmetrics F20-UV thin film measurement system (Filmetrics Inc.) with a regulated deuterium and tungsten halogen high-power UV-Vis fiber light source (Hamamatsu Inc.) over a wavelength range from 200 to 1100 nm with the incident light normal to the sample surface. Five measurements were taken at random positions around the film samples.

4. Results and discussion

4.1 The effect of reactional parameters on Substitution Degree (DS)

Elemental analysis allowed us to determine C/N ratio and substitution degree (DS) of HNMPC under different experimental conditions.

The reaction was initially carried out at room temperature with stirring for 30 minutes, using different molar ratios of hexyl aldehyde and NMPC free amino groups (Tab. 1).

Table 1. Effect of Mol Ratio

Mol ratio[a]	C/N	DS
1.00:1.00	8.31	0.062
1.10:1.00	8.69	0.085
1.50:1.00	**8.72**	**0.090**
2.00:1.00	8.67	0.082

[a] Hexyl aldehyde: NMPC free amino groups al 25ºC after 30 minutes of reaction

These results show that the molar ratio leading to the higher DS is 1.50:1.00.

The increase of hexyl aldehyde to NMPC ratio (2.00:1.00) leads to a significant decrease in DS of HNMPC.

The influence of the reaction time on the degree of substitution was also studied (Table 2).

Table 2. Effect of Reaction Time

Reaction time (h)[b]	C/N	DS
1	**8.72**	**0.090**
2	8.69	0.085
3	8.31	0.082

[b] Mol ratio 1.50:1.00 (hexyl aldehyde:NMPC free amino groups), at room temperature

As it can be seen, the ideal reaction time is 1 h. Longer exposures cause a marked decrease in the DS.

Table 3 shows the effect of the temperature on the reaction carried out using a 1.50: 1.00 molar ratio with a stirring of 1 h.

Table 3. Effect of Reaction Temperature

Reaction temperature (ºC)[c]	C/N	DS
25	8.52	0.056
35	8.38	0.033
45	**8.82**	**0.106**
55	8.62	0.073

[c] Mol ratio 1.50:1.00, reaction time of 1 h.

Temperature is the reactional parameter that leads to the most remarked effect in the DS of HNMPC.

These results confirm that higher temperatures or a prolonged time was not effective to further improve the DS (Wang et al., 2009, Illy et al., 2014).

From these experimental results, it can be affirmed that DS in the preparation of HNMPC from NMPC and hexyl aldehyde, can be increased since optimized conditions are used during the reaction. The influence of reactional parameters on the substitution degree (DS), evidenced that a mol ratio 1.50:1.00 (hexyl aldehyde: free amino groups); a reaction time of 1 h. and 45ºC of temperature afford the best DS, without waste of aldehyde, in a short time and at a reaction temperature which prevents chain hydrolysis.

4.2 FT-IR Spectroscopy

The IR spectrum of HNMPC is shown in Fig.2 and the following characteristics IR bands are identified on wavenumbers: v(-OH) 3600-3000 cm^{-1} (intensive wide band) overlapped with v(-NH) of medium intensity, v_{as} y v_s(-CH) 2925 cm^{-1} and 2851 cm^{-1} (-CH_3 y -CH_2, strong intensity), v (-C=O) 1634 cm^{-1} (amide I band, medium intensity), δ (-NH) 1560 (amide II band, weak intensity), δ_{as} (-CH) 1466 cm^{-1}

(medium intensity) and δ_{sy} (-CH) 1388 cm^{-1}, v (-P=O) 1066 cm^{-1} (strong intensity), δ $(CH_2)_n$, n≥4 853 cm^{-1} (weak intensity).

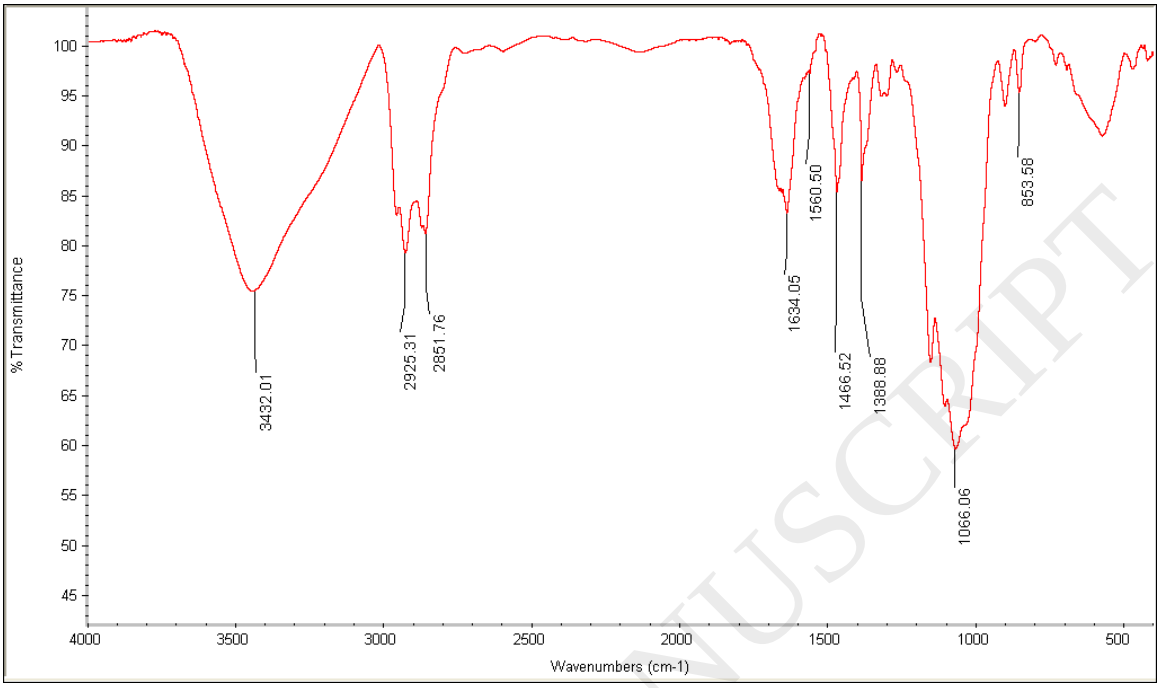


Figure 2. FTIR Spectrum of HNMPC

The FTIR spectrum of HNMPC is characterized by absorption bands arising from chitosan, which shows basic characteristic peaks at 3432 cm^{-1} (overlapped stretching vibrations between –OH and –NH groups), 2925 and 2851 cm^{-1}(-CH stretch), 1634 and 1560 cm^{-1}(amide II band due to C-O and N-H stretching, respectively), 1066 (skeletal vibrations involving C-O stretch) (Demetgül C. & Beyazit N. , 2018, Ignjatović et al, 2014, Kumar et al, 2012). The analysis of Fig. 2 reveals some differences compared to the spectrum of chitosan. Sharp signals at 2925 cm^{-1} and 2851 cm^{-1} emerge from C-H stretching due to chitosan and they are assigned to symmetrical and antisymmetric -CH stretching of $-CH_3$ and $-CH_2-$ of the hexyl substituent. Bands at 1458 (δ_{as}-CH), 1367 cm^{-1}(δ_s-CH) and the band of weak intensity at 853 cm^{-1}, due to $(-CH_2)n$ with n≥ 4, confirm the successful incorporation of the alkyl group on chitosan skeleton. The strong band at 1066 cm^{-1} is assigned to

-P=O stretching of -PO_3H_2 group. Its intense broadening can imply that hydrogen bonding may be formed between phosphonic groups and -NH of chitosan (Zhou et al, 2007).

4.3 ^{1}H NMR Spectroscopy

^{1}H NMR spectrum show the following chemical shifts (Fig.3):

δ (ppm)= 5.15 (H_1, E); 5.08 (H_1,C); 4.20-3.70 (H_3,H_4, H_5 and H_6, C and E); 3.25 (-NH-C**H**$_2$-PO_3H_2), 3.05 (H_2, E); 2.90 (H_2, C); 2.10 (-NH-COC**H**$_3$); 1.72 (-NH-C**H**$_2$-$(CH_2)_4$-CH_3); 1.3 (-NH-(C**H**$_2$)$_4$-CH_3; 0.8 (-NH-$(CH_2)_5$-C**H**$_3$).

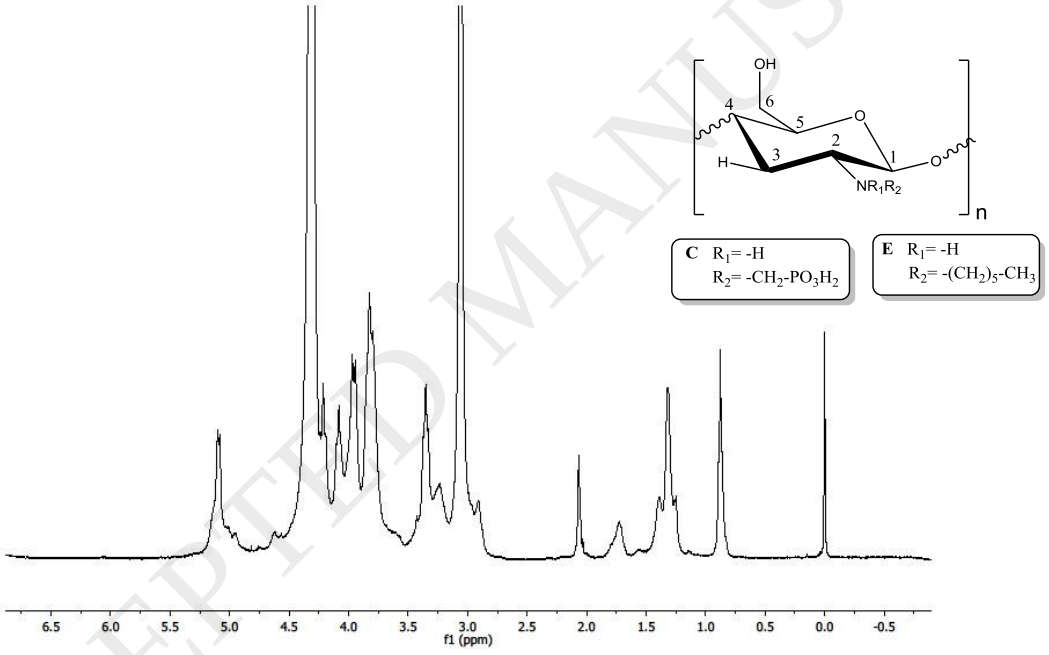


Figure 3. ^{1}H NMR Spectrum of HNMPC

^{1}H NMR spectrum of HNMPC is rather similar to the one of its precursor NMPC (Heras et al. 2000). The signals at 5.15 ppm and 5.08 ppm are assigned to anomeric protons (H_1), which correspond to monoalkylated HNMPC (E) and to the product of N-monophosphomethylation (C), respectively (Fig.1). Non anomeric

protons (H_3 to H_6) usually appear between 3.00 to 4.00 ppm (Dang et al, 2018). Fig. 3 presents split signals between 3.7 to 4.2 ppm corresponding to H_3, H_4, H_5 and H_6 of HNMPC (C) and (E), respectively. The singlet at 3.25 ppm is due to -CH_2- group of -NH-$CH_2PO_3H_2$ substituent. The signal at 2.10 ppm corresponds to the hydrogens of the methyl moieties belonging to the acetamido group. Peaks from 1.3 to 1.7 ppm are attributed to the methylene hydrogens of the -CH_2- groups while a typical peak at 0.8 ppm corresponds to the methyl protons at the terminal groups -CH_3, both belonging to the -C_6H_{13} aliphatic chain (Ma G. et al, 2009, Wang J. et al, 2016).

4.4 ^{13}C NMR Spectroscopy

Furthermore the chemical shifts assignments for ^{13}C NMR are: δ (ppm)= 177.30 (-NH-**CO**-CH_3); 104.00 (C_1, C); 98.31 (C_1, E); 79.63 (C_4), 77.88 (C_5), 71.34 (C_3), 71.20 (C_2), 63.64 (C_6), 44.95 (-NH-**C**H_2-$(CH_2)_4$-CH_3, E), 44.92 (-NH-**C**H_2-$(CH_2)_4$-CH_3, E); 33.11(-**C**H_2, E); 28.03(-**C**H_2, E), 25.05(-**C**H_2, E) , 24.37(-**C**H_2, E), 15.93 (-NH-CH_2-$(CH_2)_4$-**C**H_3, E).

The structure of HNMPC is confirmed by ^{13}C NMR (Fig.4). Two anomeric carbons may be identified at 104.00 ppm and 98.31 ppm which are assigned to the C and E forms of HNMPC derivative. Chitosan amine group replacement to obtain HNMPC derivative showed only one distinguishable form that was assigned to the monoalkyl substituted amine (δ_{C1}= 98.31ppm), which is confirmed by ^{1}H NMR (δ_{H1}= 5.15 ppm). ^{13}C NMR chemical shift values observed at 79.63 ppm (C_4), 77.88 ppm (C_5), 71.34 ppm (C_3), 71.20 ppm (C_2), and 63.64 ppm (C_6) are due to chitosan skeleton (Jothimani et al, 2017, Demetgül C.& Beyazit N., 2018). Signals between 44.92 ppm and 15.93 ppm belong to the methylene groups of the hexyl substituent.

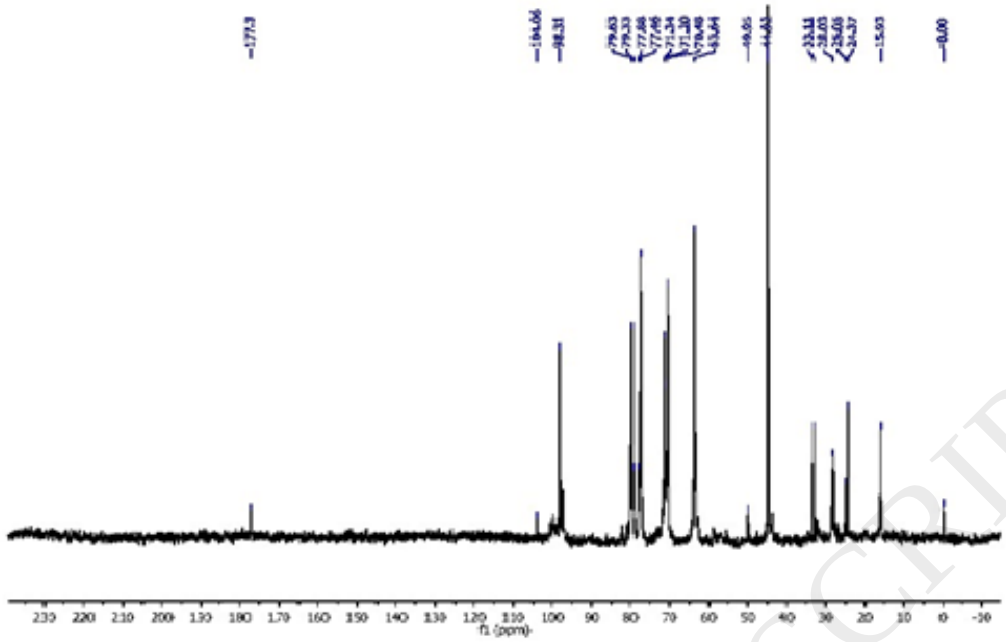


Figure 4. ^{13}C NMR Spectrum of HNMPC

4.5 $^{1}H^{13}C$ NMR Spectroscopy

Assignments of ^{1}H and ^{13}C NMR spectra were confirmed by two-dimensional $^{1}H^{13}C$-HSQC of HNMPC (Fig.5).

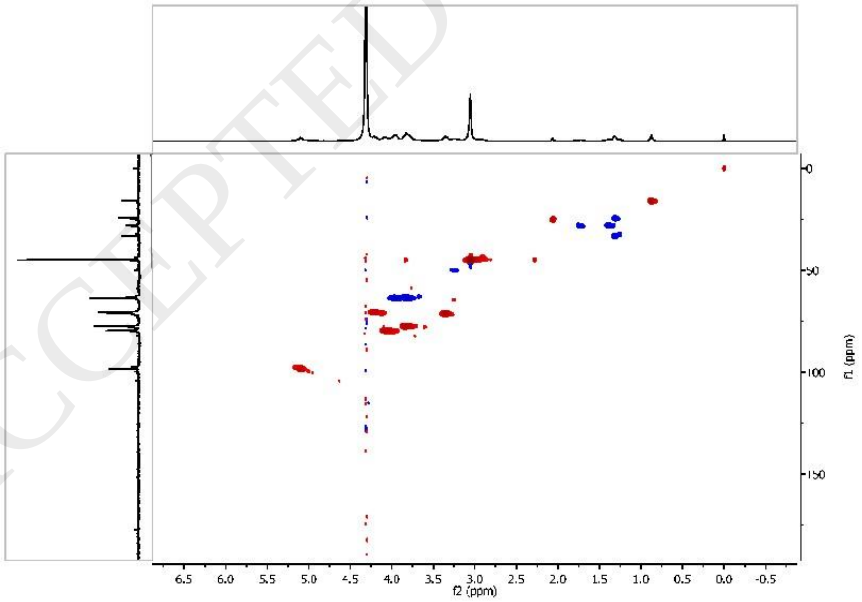


Figure 5. $^{1}H^{13}C$-HSQC NMR Spectrum of HNMPC

4.6 ^{31}P NMR Spectroscopy

^{31}P NMR spectrum (Fig.6) shows a signal centered at 10.57 ppm, which confirms the presence of -NH-CH_2-PO_3H_2 moiety in HNMPC derivative. This chemical shift is coherent with values previously reported for aminoalkyl phosphonic acid oligo-chitosans (Illy et al., 2014).

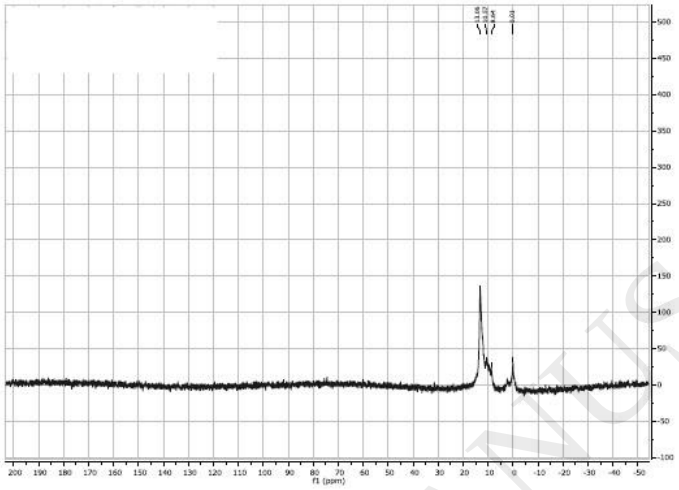


Figure 6. ^{31}P NMR Spectrum of HNMPC

4.7 Molecular Weight (M_w)

Polymer intrinsic viscosity and molecular weight were determined based on viscometric constants in the Mark–Houwink Sakurada equation. HNMPC Molecular weight (M_w) is 12.768,62 Da. The phosphonometylation reaction on chitosan and further incorporation of hydrophobic moieties into the backbone of NMPC, via reductive alkylation, caused a decrease in the molecular weight of the original chitosan (M_w 52,980.96 Da). This M_w reduction suggests that numerous backbone cleavages are occurring during the successive steps (Ramos et al, 2003, Sajomsang et al, 2008).

4.8 Differential Scanning Calorimetry (DSC)

DSC Thermogram shows a broad endothermic peak at 100 °C and a sharp one at 298°C (Fig.7). The presence of the endothermic peak may be explained by elimination of the matrix moisture and the exothermic one may be attributed to sample decomposition. Alkyl chain introduction results in a displacement of the exothermic peak from 230ºC (NMPC) to 298°C in HNMPC.

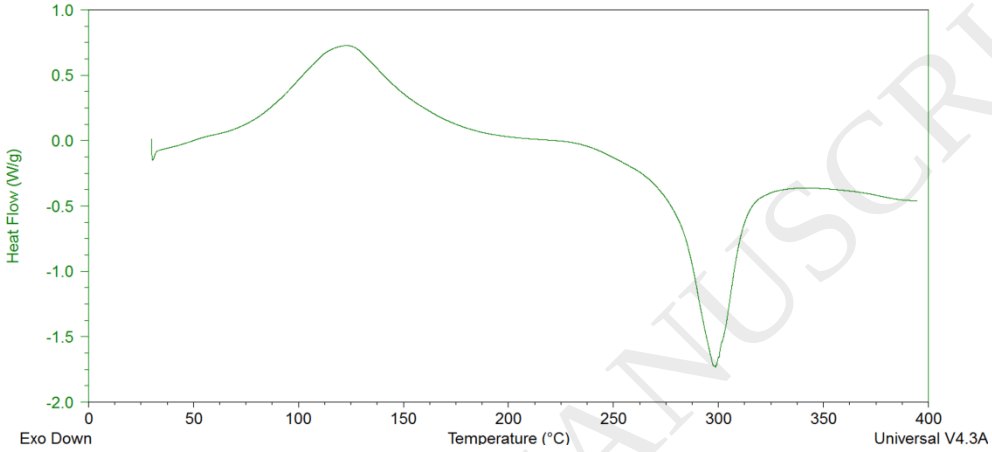


Fig. 7 DSC of HNMPC

4.9 X-Ray Diffraction (XRD), Solubility and SEM

X-ray diffraction analysis was conducted for chitosan, NMPC and HNMPC to further evaluate their crystallization behaviors. The HNMPC X-Ray diagram shows two reflections, one that falls at 2θ: 19º40´ and a more intense peak at a 2θ value lower than 4° (Fig. 8). Peak shape evidences a certain degree of crystallinity as a result of alkyl chain introduction. Comparison between lauryl, hexyl and propyl NMPC derivatives confirm that the first peak is shifted to lower 2θ values as the length of the n-alkyl group increases (Ramos et al., 2003, Zúñiga et al., 2010, Muzzarelli et al, 1983).

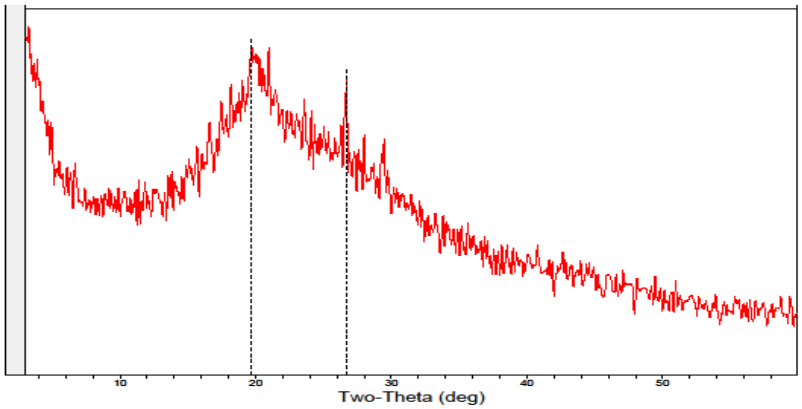


Fig. 8 XRD Analysis of HNMPC

4.10 Solubility of HNMPC was studied at room temperature (Table 4).

Table 4. Solubility of HNMPC at room Temperature

Acetic Acid	Water	Ethanol	Acetone	HCl 0.1M	HCl 1%	NaOH 1%	NaOH 0.1M	DMSO	DMF
++	-	-	-	++	++	-	-	+	-

++ soluble /+ partially soluble /- insoluble

At 25°C it proves to be soluble in Acetic Acid, HCl solutions (0.1M and 1%) and partially soluble in DMSO. The *N*-methylene-*N*-lauryl phosphonic and *N*-methylene-*N*-propyl phosphonic derivatives, previously synthesized by our group, showed a rather similar behavior when its solubility was evaluated in the same solvents.

HNMPC surface morphology is characterized by SEM (Fig. 9). A rugged structure can be observed with holes scattered regularly all over the surface of the new derivative. The introduction of the new groups destroy the original hydrogen bonds so the surface becomes uneven and raised (Fan et al., 2018). This structure could be favorable for embedding metal ions.

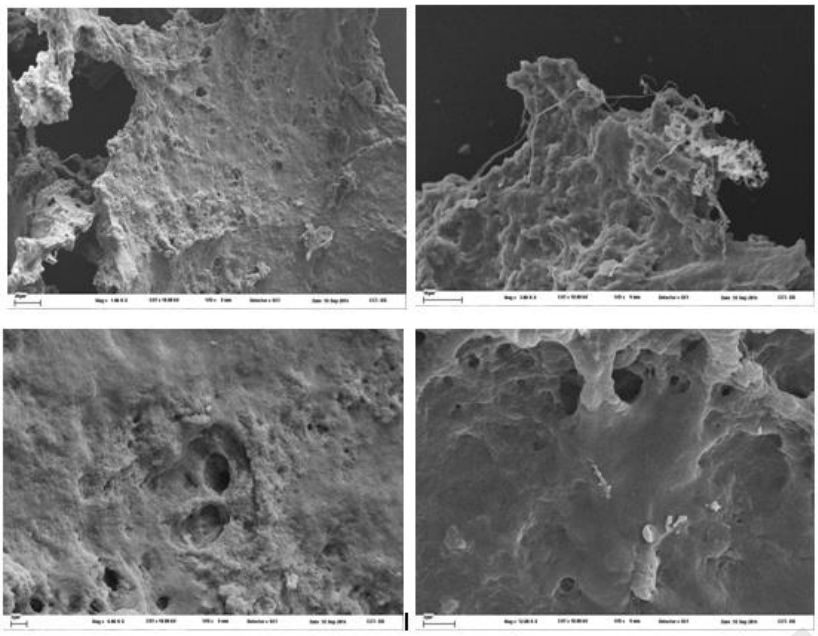


Figure 9. SEM Images of HNMPC

4.11 Film Thickness

As well as its precursor chitosan, HNMPC forms transparent films. The films were obtained by drop casting, film thickness measurements were performed and the mean value of five samples analyzed was 35 μm. These results are relevant achievements in relation to the potential production of biodegradable packaging with antimicrobial activity.

4.12 Emulsion Preparation and Creaming Index (CI)

With the aim of studying the properties of the emulsions formed with this derivative, the optimal oil/HNMPC solution ratio was evaluated. At first, a set of emulsions with sunflower oil/HNMPC solution w/w ratio of 80/20, 70/30, 60/40, 50/50, 30/70, 20/80 and 10/90 emulsions with a fixed solution concentration were made and evaluated. The better stabilization over time was observed for the one with the 10/90 ratio, which was used to study the characteristics of the derivative with regard to emulsion formation varying the aqueous phase concentration.

Different solutions of HNMPC in 0.2% acetic acid were studied (0.04, 0.2 and 1%). After storing emulsions for one hour, it was observed that the most stable one was obtained with HNMPC 1%. On ageing, emulsions separate into two layers: an optically opaque “cream” layer at the top and a transparent “serum” layer at the bottom.

Emulsion stability of oil/HNMPC solutions (10/90) at different aqueous concentration of HNMPC derivative in terms of Creaming Index (CI) and Droplet size diameter (D(4,3)) are reported in Table 5. The CI of HNMPC solutions at different concentrations was observed within 24 h and until a maximum value was reached (15 days).

The CI is defined as the Hs/Ht ratio, in which Hs is the height of the upper concentrated emulsion after creaming and Ht the total height of the solution in the test tube. The D(4,3) diameter is defined as the volume mean diameter and is given by the instrument (Horiba Laser Diffractometer) software.

Table 5. Creaming Index (CI) of HNMPC Emulsions and Droplet Diameter

Concentration (%)[a]	CI (24 h)	CI (15 days)	D(4,3) (μm)
0.04	12.50	13.90	6.50
0.20	7.80	15.60	4.20
1.00	5.50	12.30	3.70

[a] In aqueous solution of acetic acid (0.2%)

In the emulsions containing 0.04% HNMPC (0.2 % acetic acid) rapid creaming was observed in 24 h. and it slightly increased with time until a maximum value was reached after 15 days. On contrary, emulsions with 0.2 and 1% HNMPC expressed low creaming index after the first 24 h., while further storage led to an increase until maximum values of 15.60 and 12.30 % were reached.

Size distribution of its droplets is the most important parameter to characterize any emulsion. It influences its properties in different ways, such as degradation rates, long-term stability, resistance to creaming, texture and optical appearance, viscosity, physiological efficiency, and chemical reactivity. The distribution of particle size is represented in the plots shown below. An unimodal distribution for all concentrations studied can be observed (Fig. 10a, 10b and 10c).

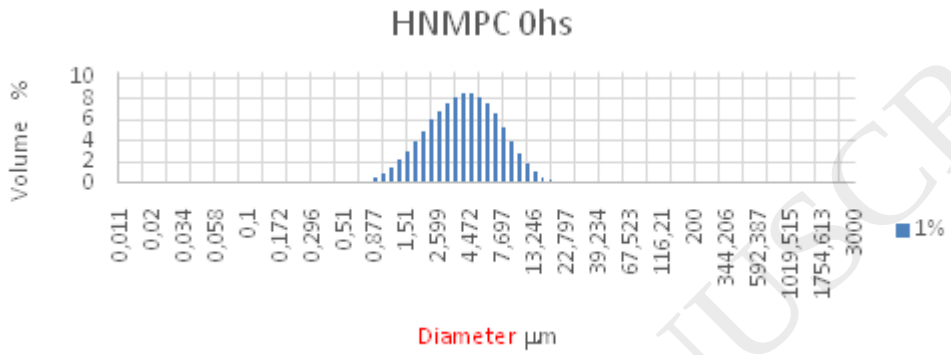


Figure 10 a. Distribution of HNMPC (1%) Particle Size

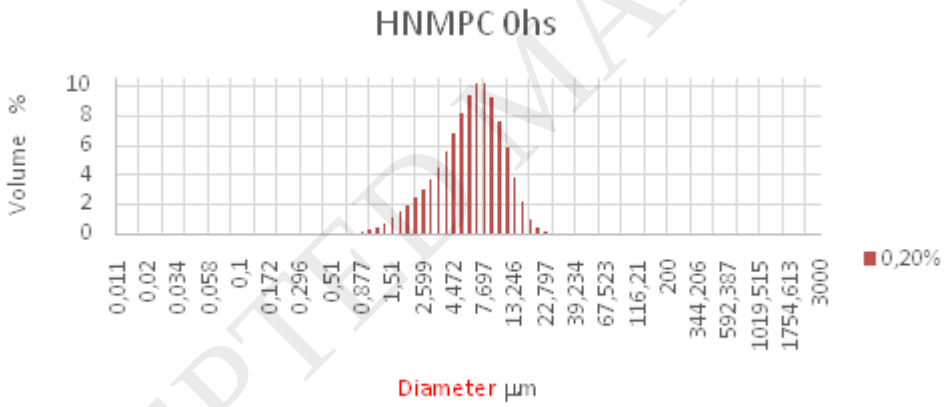


Figure 10 b. Distribution of HNMPC (0.2%) Particle Size

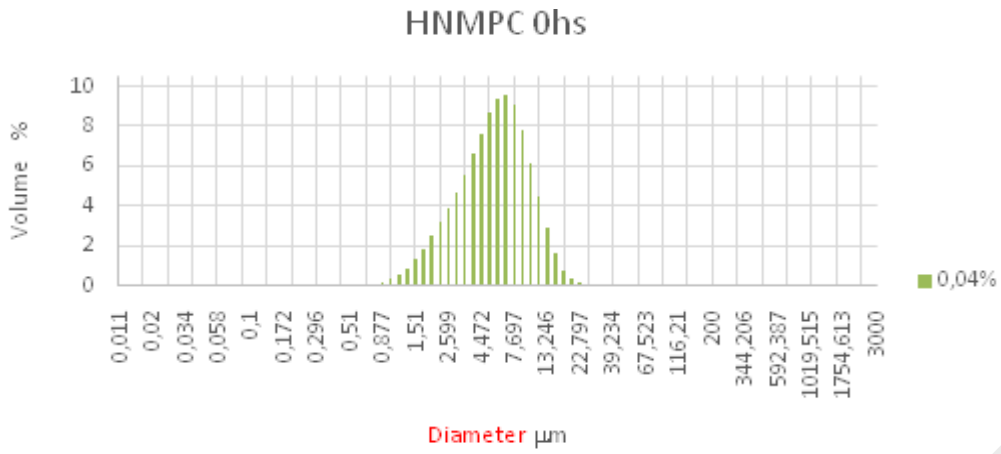


Figure 10 c. Distribution of HNMPC (0.04 %) Particle Size

Previous studies on N-methylene phosphonic chitosan (NMPC), which is HNMPC precursor, evidenced a similar behavior. The most stable emulsion in time was that with 1% acqueous sunflower oil/NMPC 10/90. A mainly oil in water emulsion (o/w) was formed in this case, but combined with some water/oil/water (w/o/w) droplets of a mean droplet diameter of 50 µm (Rodríguez et al., 2005).

Microphotographs of freshly prepared emulsions at different o/w concentrations (0.04%, 0.2 % and 1%) are presented in Fig.11. Emulsions were oil in water type with unimodal droplet size distribution with a viscosity of 30mPa/s, measured at 25°C with spindle 21 at 50 rpm.

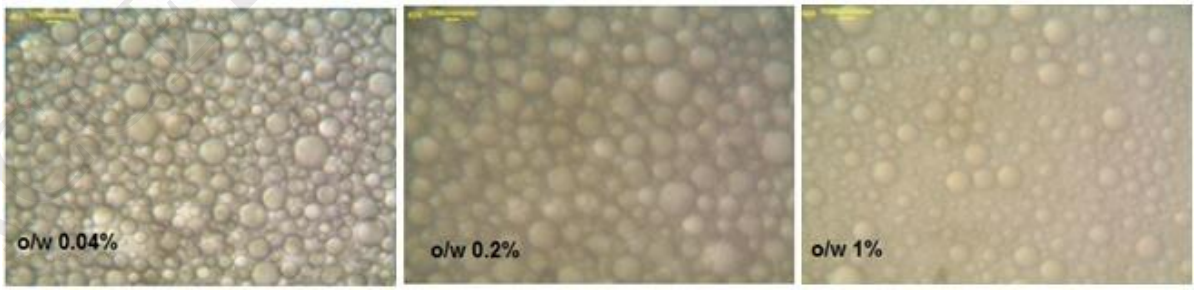


Figure 11. Microscopic Views of HNMPC Emulsions at Different o/w Concentrations

Emulsification properties, as well as emulsion stability, are proportional to chitosan concentration. Emulsions o/w (10/90) with HNMPC 1% (w/v) in an aqueous solution of acetic acid (0.2%) exhibited the best emulsifying properties within the test range.

5. Conclusions

N-hexyl-*N*-methylene phosphonic chitosan (HNMPC) was successfully prepared under mild conditions via reductive *N*-alkylation of NMPC. The present work provides information on the influence of the reaction conditions on the degree of substitution of HNMPC. It can be concluded that a mol ratio 1.50:1.00 (hexyl aldehyde: free amino groups); a reaction time of 1 h., and 45ºC of temperature are the conditions which afford the best DS without aldehyde waste, in a short time and at a reaction temperature which prevents chain hydrolysis.

FT-IR, ^{1}H, ^{13}C, ^{31}P and $^{1}H^{13}C$ HSQC NMR spectra were used to confirm the structure of the new derivative. ^{1}H and ^{13}C NMR information evidence that hexyl substituents allow only a monoalkylated form. Characterization of LNMPC proved to be consistent, in a previous work, with this result (Ramos et al., 2003). Instead, propyl substituents allow the formation of mono and dialkylated-NMPC on the amine group (Zúñiga et al., 2010).

A significant decrease in molecular weight (Mw: 12,768.62 Da) is observed due to derivatization.

As well as the precursor chitosan, the structure of HNMPC could be favorable for embedding metal ions. Moreover, it forms transparent films with thickness that proved to be regular all over the analyzed surface. This type of films can be used as coatings because of its biodegradability and probable antibacterial activity.

HNMPC shows good emulsifying properties giving o/w emulsions with high stability over time. The best behavior was obtained with 1% (w/v) HNMPC aqueous solution with the fixed ratio oil/derivative solution w/w of 10/90 . Microscopic observation as well as particle size distribution showed an unimodal droplet size distribution with low droplet diameters.

These results suggest that HNMPC shows a great potential as an emulsifying agent without adding any other surfactant. Besides, its biodegradability and non-toxic origin makes it a very useful material for applications such in food, cosmetics and pharmaceuticals emulsions.

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