Synthesis and Characterization of Phosphorylcholine-Substituted Chitosans Soluble in Physiological pH Conditions

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A polymer analogous synthesis involving the reductive amination of phosphorylcholine (PC)−glyceraldehyde with primary amines of deacetylated chitosan ($M_w \approx 57000$ g mol^{-1}) was used to prepare phosphorylcholine-substituted chitosans (PC−CH) with a degree of substitution (DS) ranging from ∼11 to ∼53 mol % PC-substituted glucosamine residues. The PC−CH derivatives were characterized by ^{1}H NMR spectroscopy, FTIR spectroscopy, and multiangle laser light scattering gel permeation chromatography (MALLS-GPC). The pK_a of the PC-substituted amine groups (p$K_a \approx 7.20$) was determined by ^{1}H NMR titration. The PC−CH samples (1.0 g L^{-1}) were shown to be nontoxic using an MTT assay performed with human KB cells. Aqueous solutions of PC−CH samples (4.0 g L^{-1}) of DS ≥ 22 mol % PC-substituted glucosamine residues remained clear, independently of pH (4.0 < pH < 11.0). The remarkable water solubility and nontoxicity displayed by the new PC−CH samples open up new opportunities in the design of chitosan-based biomaterials and nanoparticles.

Introduction

Chitosan, a linear polysaccharide composed of β-(1→4)-linked 2-amino-2-deoxy-D-glucopyranose and 2-acetamido-2-deoxy-D-glucopyranose, is a nontoxic, biocompatible, and biodegradable polymer obtained via de-*N*-acetylation of chitin, which is the second most abundant natural polysaccharide after cellulose. Chitin is extracted readily from the exoskeleton of numerous arthropods.[1,2] It remains a widely underutilized natural resource, although currently, chitin and its derivatives are employed or assessed as building blocks of new functional materials in various fields, including environmental[3,4] and biomedical[5] engineering, drug delivery,[6] and gene therapy.[7] Chitosan dissolves readily in acidic aqueous solutions, but it is insoluble in water and in most organic solvents. This limited solubility restricts the range of chitosan applications, in particular in therapeutics. It is anticipated that the impact of chitosan-based materials in the biological and physiological domains would increase dramatically with the availability of chitosans soluble under physiological pH conditions (pH 7.2−7.4).

Several strategies have been devised to increase the neutral solubility of chitosans. They involve careful control of the molecular weight characteristics and of the relative amounts of *N*-acetylglucosamine and glucosamine residues, coupled to chemical derivatization, such as carboxymethylation,[8,9] sulfatation,[10] *N*- and/or *O*-acylation,[11−13] regiospecific introduction of branched oligosaccharide moieties, such as α-mannosides,[14,15] and grafting of poly(ethylene glycol) (PEG).[16−21] The latter approach is particularly promising since PEG chains not only enhance the water solubility of chitosans, but are also exceptionally effective in preventing protein adsorption, a property exploited in the design of chitosan-based drug delivery systems.[22] Phosphorylcholine (PC) groups also exhibit remarkable protein-repelling properties,[23−26] a feature taken to advantage in the production of biocompatible and hemocompatible interfaces via grafting of PC moieties or coating with organosoluble copolymers of [(methacryloyloxy)ethyl]phosphorylcholine.[27,28] In addition, solutions, hydrogels, and nanoparticles composed of PC polymers are used or are under evaluation as delivery vehicles in pharmaceutical formulations, cosmetics, and nonviral carriers for gene therapy.[29−31]

We described recently a route toward PC-based acrylamides whereby PC groups are linked to a preformed polymer via reductive amination of phosphorylcholine−glyceraldehyde (PC−CHO) by primary amine groups linked to the polymer.[32] The synthesis, which proceeds in high yield and allows great flexibility in polymer architecture and composition, was applied to the preparation of copolymers of *N*-isopropylacrylamide and shown to be useful in the preparation of PC-modified natural polymers.[33] Linking PC groups to poorly water soluble polymers results in a significant enhancement of their water solubility even in solutions of high ionic strength, a direct consequence of the zwitterionic nature of the PC group.

We describe here a facile preparation of PC-substituted chitosans (PC−CH) which exhibit remarkable solubility in physiological pH conditions. The polymers were obtained

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Table 1. Preparation Conditions and Physicochemical Properties of Phosphorylcholine-Substituted Chitosans

polymer	PC−CHO/NH_2 molar ratio	PC content[a] (mol %)	d*n*/d*c*[b] (mL/mg)	M_w[c] (g mol^{-1})	M_n[c] (g mol^{-1})	M_w/M_n
CH			0.1761 (±0.0013)	5.7×10^4	4.4×10^4	1.29
PC10−CH	0.15	11 ± 1	0.1611 (±0.0017)	5.1×10^4	3.9×10^4	1.32
PC20−CH	0.25	22 ± 1	0.1539 (±0.0022)	6.2×10^4	3.7×10^4	1.66
PC40−CH	0.50	40 ± 2	0.1537 (±0.0014)	6.0×10^4	2.8×10^4	2.17
PC50−CH	1.00	53 ± 1	0.1491 (±0.0022)	4.0×10^4	2.5×10^4	1.61

[a] Average of the values obtained from ^{1}H NMR measurements, acid−base titration, and colorimetric assay for phosphorus (see the Experimental Section) expressed in mole percent PC-substituted glucosamine residues. [b] Solvent: aqueous acetic acid (0.3 M)/sodium acetate (0.2M) buffer, pH 4.5. [c] From GPC-MALLS (see the Experimental Section).

through reductive amination of PC−CHO by the amine groups of fully de-*N*-acetylated chitosan. A combination of ^{1}H NMR and FTIR spectroscopy, turbidimetry, and potentiometry was employed to characterize the solution properties of the polymers. Moreover, the cytotoxicity of the polymers was evaluated, confirming the nontoxic nature of chitosan and its PC derivatives.

Experimental Section

Materials. Chitosan (Wako-10, degree of deacetylation (DD) 85%) was purchased from Wako Chemical Co. Sodium cyanoborohydride, magnesium nitrate, sodium hydroxide, sodium acetate, and acetic acid were purchased from Aldrich Chemical Co. The ion-exchange resins AG 501-X8 (D) and DOWEX 2X8-400 were obtained from BioRad Laboratories and Supelco, respectively. Spectra/Pore membranes (Spectrum) were employed for dialysis. Phosphorylcholine−glyceraldehyde was prepared as described previously,[32] starting from l-glycerophosphorylcholine obtained from Sigma Chemical Co. All solvents were of reagent grade and used as received. Water was deionized using a Milli-Q water purification system (Millipore).

Instrumentation. ^{1}H NMR spectra were recorded on a Bruker ARX-400 400 MHz spectrometer. FTIR spectra were recorded on a Perkin-Elmer MIR instrument using KBr pellets of the samples. Five scans were recorded for each sample with a resolution of 1 cm^{-1}. UV/vis spectra were measured with a Hewlett-Packard 8452A photodiode array spectrometer or a Cary 100 spectrophotometer equipped with a Peltier system. Gel permeation chromatography (GPC) analyses were carried out on a GPC system consisting of an Agilent 1100 isocratic pump, a Dawn EOS multiangle laser light scattering detector (Wyatt Technology Co.), an Optilab DSP interferometric refractometer (Wyatt Technology Co.), and a TSK-GELPW (Tosoh Biosep, serial number G0014) column eluted with a pH 4.5 acetic acid (0.3 M)/sodium acetate (0.2 M) buffer, injection volume 100 μL, flow rate 0.5 mL min^{-1}, temperature 25.0 °C. The d*n*/d*c* values of the polymers were measured at 690 nm with the same refractometer used in the off-line mode. The sample solution pH was determined using an Orion pH meter.

Deacetylation of Chitosan. A solution of chitosan (4.0 g, 2.45×10^{-3} mol of monosaccharide units) in aqueous acetic acid (200 mL, 2 wt %) was added dropwise to aqueous NaOH (100 mL, 50 wt %) at room temperature under magnetic stirring and an atmosphere of nitrogen. At the end of the addition, the suspension was refluxed for 1 h. It was poured into stirred water (4 L) preheated to 80 °C. The precipitate was decanted, washed five times with water, and separated by filtration. The solid was dissolved in acetic acid (200 mL, 2 wt %) and subjected to the same procedure one more time to achieve a higher deacetylation degree. The resulting polymer was purified by dialysis against water for 3 days and isolated by lyophilization (yield 3.2 g, 80%).

Preparation of PC-Substituted Chitosans. Chitosans with varying amounts of grafted PC were prepared by the procedure reported by Miyazawa et al.[32] The procedure is illustrated below for the synthesis of PC40−CH. A solution of PC−CHO (0.4 g, 1.64 mmol) in methanol (10 mL) was added dropwise to a solution of deacetylated chitosan (0.5 g, 3.1 mmol of monosaccharide residue) in aqueous acetic acid (20 mL, 2 wt %) kept at 0 °C. At the end of the addition, the solution was stirred for 30 min at 0 °C. The pH of the reaction mixture was adjusted to 6.5 by adding aqueous NaOH (1.0 M). The reaction mixture was stirred for 1 h at room temperature. It was cooled to 0 °C, and a solution of sodium cyanoborohydride (0.5 g, 8 mmol) in water (10 mL) was added dropwise under stirring. Thereafter, the reaction mixture was warmed to room temperature and stirred for 20 h. The reaction mixture was dialyzed (membrane of MWCO 12000−14000) first against water for 2 days, then against aqueous NaOH (0.05 M) for 1 day, and finally against water for 2 days. The polymer PC40−CH was isolated by lyophilization (yield 0.53 g). Other PC-substituted chitosans were prepared under identical conditions except for the initial PC−CHO/NH_2 molar ratio (Table 1).

Gel Permeation Chromatography Analysis. Solutions for GPC analysis were prepared by dissolving an exact amount of polymer in an acetic acid (0.3 M)/sodium acetate (0.2 M) buffer of pH 4.5, to achieve a concentration between 0.5 and 1.0 mg/mL. The polymer solutions were stirred for 3 days and filtered through a 0.45 μm membrane before analysis. For d*n*/d*c* measurements, stock solutions of each polymer (1.0 mg/mL) in a pH 4.5 acetic acid (0.3 M)/sodium acetate (0.2 M) buffer were diluted with the same buffer to obtain solutions of concentration ranging from 0.2 to 1.0 mg/mL. The solutions were kept at room temperature for 3 days under gentle stirring prior to the measurements.

Potentiometric Titrations. Solutions for titration were prepared by adding an excess of HCl (0.10 N) to a solution of polymer (CH or PC−CH, ~40 mg). Titration curves for all polymers were obtained by monitoring the pH changes upon addition of aqueous NaOH (0.10 M, increments of 50−250 μL). Titration curves were recorded in the pH range of 2−12. All titrations were conducted in duplicate.

Colorimetric Quantification of the PC Content of PC−CH. A solution of $Mg(NO_3)_2$ in ethanol (0.030 mL, 10 wt %) was added to an aqueous solution of PC−CH (0.020 mL, 1.0 g/L) placed in a long test tube. After mixing, the sample was heated over a flame to remove the solvent and ash the polymer. Aqueous HCl (0.30 mL, 0.5 N) was added to the cooled tube to dissolve the residue. The tube was capped, heated in a 100 °C water bath for 15 min, and cooled to room temperature. Aqueous ascorbic acid (0.70 mL, 10 wt %) and aqueous ammonium molybdate (0.50 mL, 0.42 wt % in 1 N H_2SO_4) were added to the tube contents. The resulting solution was mixed and kept in a 100 °C water bath for 5 min. The phosphate concentration was determined from the absorbance at $\lambda = 820$ nm of the cooled solution, using a calibration curve established with KH_2PO_4.[32] Tests were conducted in triplicate, and the average value was used to calculate the phosphate concentration.

Determination of the Degree of Substitution (DS) by ^{1}H NMR Spectroscopy. Polymer solutions (10 mg/mL) were prepared in D_2O/DCl (100/1, v/v). Their ^{1}H NMR spectra were recorded at 70 °C. The DS was determined by two methods: (1) from the areas (I_{CH_2}) of the signal at δ 4.9 ppm attributed to the resonance of the methylene group of $NHCH_2CH_2OP$ (signal α, Figure 1) and of the signals due to the anomeric protons of PC-substituted and unsubstituted glucosamine residues, H1s and H1, respectively (Figure 1), using eq 1

$$\mathrm{DS} = \frac{(1/2)I_{\mathrm{CH_2}}}{I_{\mathrm{H1}} + I_{\mathrm{H1s}}} \qquad (1)$$

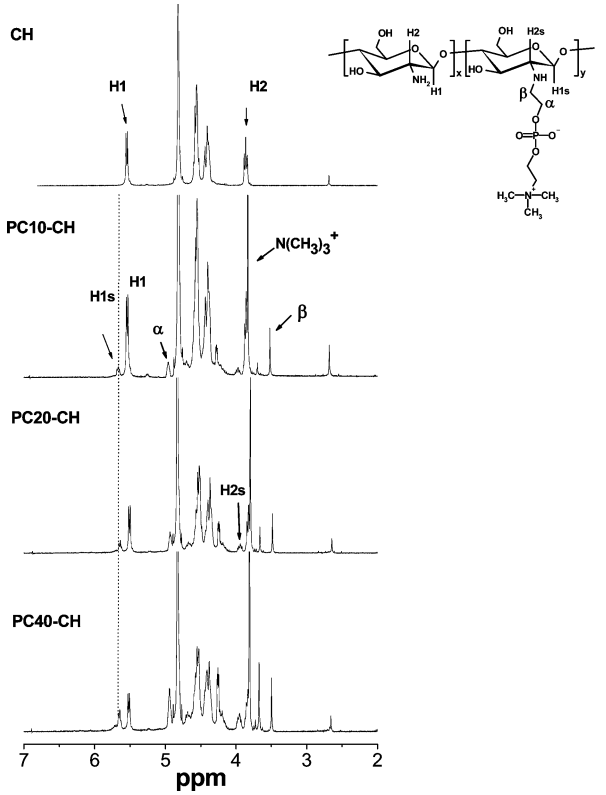


Figure 1. ^{1}H NMR spectra of deacetylated CH and PC-modified chitosans, temperature 70 °C, solvent D_2O/DCl (1%).

and (2) from the areas of the signals due to the two types of anomeric protons, H1s and H1, using eq 2. The two methods gave values that agreed with each other within ±2.0%.

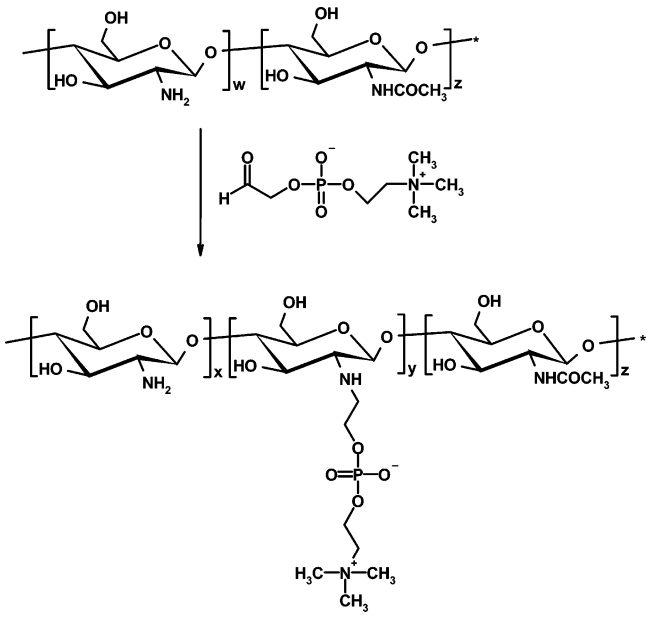
$$\mathrm{DS} = \frac{I_{\mathrm{H1s}}}{I_{\mathrm{H1}} + I_{\mathrm{H1s}}} \qquad (2)$$

Determination of the pK_a of PC−CH by ^{1}H NMR Spectroscopy. Spectra were recorded for solutions kept at 25 °C. Chemical shifts were determined with respect to an internal standard, sodium 3-(trimethylsilyl)propionate-d_4 (TSP), obtained from Sigma-Aldrich Chemical Co. (10 μL of a 1% stock solution). A solution of the polymer (12 mg, PC50−CH) in D_2O (1 mL) was brought to pD ≈ 3 by addition of DCl (0.1 M). A ^{1}H NMR spectrum was collected. Thereafter, the solution pD was increased stepwise in pD intervals of ∼0.2 unit by adding aqueous NaOD (0.1 M). After each pD adjustment, a ^{1}H NMR spectrum was collected. This process was continued until the chemical shift of the signals of interest did not change with pD (up to pD ≈ 10).

Turbidity Measurements. Changes of the transmittance at 566 nm of aqueous CH−PC solutions (4.0 g/L) were monitored as a function of pH. Samples were dissolved in HCl (0.1 M) and titrated with an aqueous NaOH solution (0.1 M). The solution pH was measured with a Digimed DM21 pH meter equipped with a Corning-spear gel combo electrode.

Cytotoxicity Assay. Human KB cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and 1% penicillin−streptomycin in a 5−95% CO_2−O_2 atmosphere at 37 °C. The cells were seeded in triplicate in 24-well culture plates at a density of approximately 5×10^5 cells/mL in 1 mL of cell culture medium per well. The cells were cultured for 24 h at 37 °C. Thereafter, they were exposed to a solution of PC−CH (0.10 or 1.0 g/L) in PBS buffer (pH 7.4) followed by an incubation period of 24 h. Cell viability was evaluated using the MTT colorimetric assay.[34] The assay is based on the reduction of MTT by mitochondria in viable cells to water-insoluble formazan. The absorbance was measured at 570 nm with an EL800 universal microplate reader (Bio-Tek Instruments Inc.).

Scheme 1



Results and Discussion

To achieve regiospecific functionalization of chitosan, we selected to introduce the PC groups via chemical modification of the C2 amine group, thus leaving intact all the hydroxyl groups which play an important part in the biological activity of chitosan derivatives. The synthetic strategy involves two reactions carried out in sequence, without isolation of the intermediate (Scheme 1): (1) reductive amination of phosphorylcholine−glyceraldehyde by the C2 primary amine groups of chitosan and (2) reduction of the resulting imine groups with $NaCNBH_3$, a reagent extensively used in various modifications of chitosan.[35−37] Note that, overall, the reaction path converts primary amines into secondary amines, a transformation that will affect, yet maintain, the polyelectrolyte properties of native chitosan.

The transformation was carried out starting from a fully deacetylated chitosan, obtained via deacetylation of a commercial chitosan sample with a nominal DD of 85%, following a known procedure.[38] The deacetylation was carried out under continuous bubbling of nitrogen to prevent degradation of the polymer. Analysis by GPC confirmed that the molar mass of chitosan was not affected significantly. It changed from a value of $M_w = 61000$ g mol^{-1} ($M_w/M_n = 1.65$) for the starting material to a value of $M_w = 56700$ g mol^{-1} ($M_w/M_n = 1.23$) for the product (CH). The slight sharpening of the molar mass distribution may be a consequence of the extensive purification by dialysis of the deacetylated material. The degrees of deacetylation, expressed in $-NH_2$ mole percent, determined from the ^{1}H NMR spectra of the starting material and the fully deacetylated sample, were 86.0 and 98.1 mol %, respectively. These values were obtained from the areas of the doublet at 5.5 ppm, due to the resonance of the anomeric proton (H1), and of the singlet at 2.7 ppm attributed to the acetamido methyl protons.[39] Potentiometric and conductometric titrations conducted on solutions of CH confirmed that the deacetylation occurred with high efficiency, yielding DD values of 97.7 and 97.5 mol %, respectively.

The fully deacetylated chitosan was treated first with PC−glyceraldehyde and, second, with $NaCNBH_3$ (Scheme 1). The degree of substitution was varied by setting the initial molar ratio of PC−glyceraldehyde to glucosamine units to values

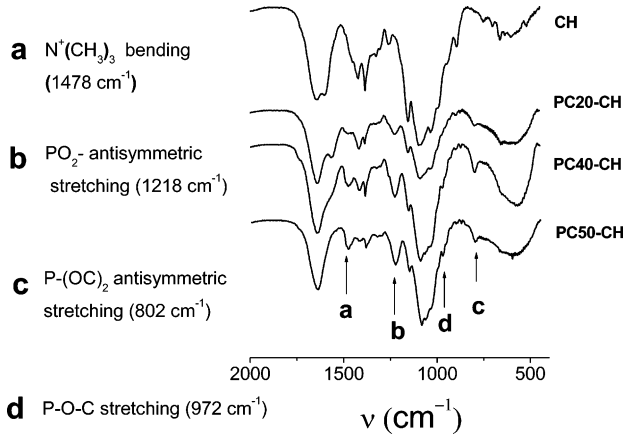


Figure 2. FTIR spectra of deacetylated CH and PC-modified chitosans. The arrows point to bands assigned to the vibrations of the phosphorylcholine group.

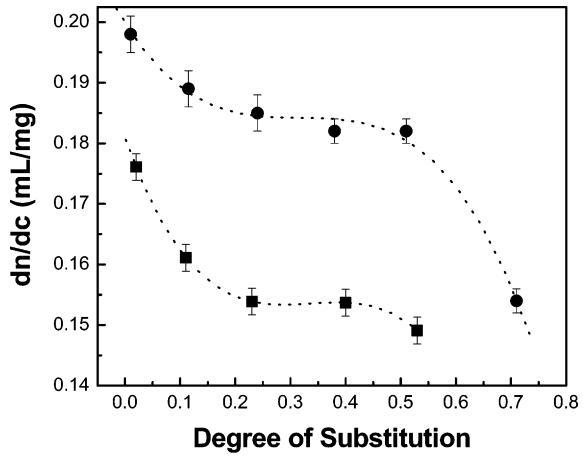


Figure 3. Changes of the refractive index increment (d*n*/d*c*) as a function of the degree of substitution (expressed as the fractional content of PC-substituted glucosamine residues) for PC-modified chitosans (■) in a pH 4.5 acetic acid/sodium acetate buffer ($\mu = 0.20$ M), $\lambda = 690.0$ nm, and for *N*-acetylated chitosans (●) with data taken from ref 47.

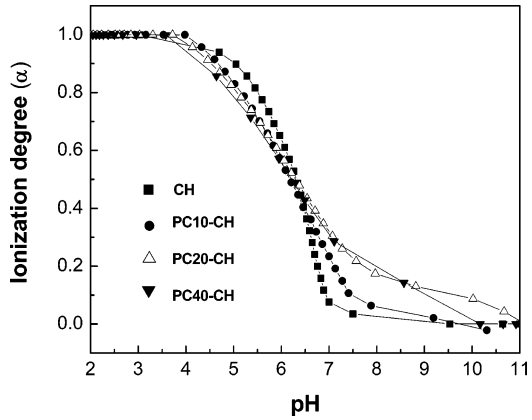


Figure 4. Changes of the degree of ionization (α) of CH and PC-substituted chitosans as a function of the solution pH.

ranging from 0.15 to 1.00. The amount of reducing agent added in the second step was changed accordingly, while keeping the PC−glyceraldehyde/$NaCNBH_3$ molar ratio constant. The ^{1}H NMR spectrum of PC-modified chitosan exhibited a singlet at δ 3.8 ppm, attributed to the resonance of the trimethylammonium protons of the phosphorylcholine moiety, and signals at δ 4.25 and 4.9 ppm, corresponding to the resonances of the methylene protons of $PO_4CH_2CH_2N^+(CH_3)_3$ and $NHCH_2CH_2$-PO_4, respectively (Figure 1). The attachment of PC groups to the chitosan framework brings further changes in the ^{1}H NMR spectrum of chitosan, most notably in the resonances of the protons in close proximity to the substitution site: the anomeric proton H1 and the proton H2 linked to C2 of the glucosamine unit. The anomeric proton signal undergoes a downfield shift, from δ 5.50 ppm to δ 5.65 ppm, while the multiplet at δ 3.83 ppm shifts to δ 3.90 ppm. Similar deshielding of H1 and H2 upon C2 derivatization of chitosan has been reported in the case of chitosan-*N*-sulfate[40] and chitosan carrying oligosaccharide branches.[14] FTIR spectroscopy provided further evidence for the successful incorporation of PC moieties on the chitosan backbone. The IR spectra of PC−CH samples (Figure 2) present the four-band signature of the phosphorylcholine group, with bands at 1478 cm^{-1} corresponding to the bending of $N^+(CH_3)_3$ and at 1218, 962, and 790 cm^{-1} due to the antisymmetric stretching of PO_2^-, the stretching of P−O−C, and the antisymmetric stretching of $P(OC)_2$ moieties, respectively (Figure 2).[31,41,42]

The DS of the PC−CH samples was estimated from ^{1}H NMR data and by a phosphorus-specific colorimetric assay (see the Experimental Section). The DS values ranged from 12 to 53 mol % PC-substituted glucosamine residues (Table 1). They are of the same order as those of other modified chitosan derivatives of enhanced water solubility, such as CH−PEG,[43] CH−sialic acid,[44] and CH−lactose.[35] The molar masses of the samples obtained by MALLS-GPC analysis are reported in Table 1. The number-average molar masses of the polymers tend to decrease upon PC substitution, especially in the case of the more substituted samples, an indication that main chain degradation took place during the chemical modification of CH. The weight-average molar masses are not greatly affected, in view of the increase in the average molecular weight of the monomer units upon PC substitution.[45]

The d*n*/d*c* values of the CH−PC samples in an acetic acid buffer of pH 4.5 vary as a function of DS, as shown in Figure 3. For polymers of low DS, the d*n*/d*c* values are high. They decrease with increasing DS. Solutions of polymers with DS > 20% have the same d*n*/d*c* value, which lies within the range characteristic of neutral polysaccharides, such as pullulans or dextrans, in aqueous solvents.[46] One may note that the trends of the changes in d*n*/d*c* values of CH−PC are similar to those reported by Schatz et al.,[47,48] for chitosans with degrees of acetylation ranging from 0 to 70% (Figure 3). The changes in d*n*/d*c* with DS reflect changes of solvent quality. The pH 4.5 buffer employed is a good solvent for unmodified chitosan and samples of low DS, which in this solvent behave as true polyectrolytes. The quality of the solvent worsens for polymers with higher DS, which exhibit antipolyelectrolyte behavior due to the zwitterionic nature of the PC groups.

Potentiometric Titration Curves. Potentiometric titrations of fully protonated PC−CH and CH in aqueous solutions were carried out by addition of aqueous NaOH to polymer solutions (2.5 g/L). The changes of α, the degree of ionization of the polymer, were plotted as a function of pH (Figure 4). We note significant differences among the curves over nearly the entire pH range ($4.0 < pH < 11$). Overall, the ionization degree decreases faster with increasing pH as the level of PC incorporation increases. These differences among the various samples may be ascribed to the presence along PC−CH chains of two types of amines: primary (unsubstituted) amines, expected to have a pK_a of ∼6.5, the value determined for CH, and secondary (PC-substituted) amines, expected to have a

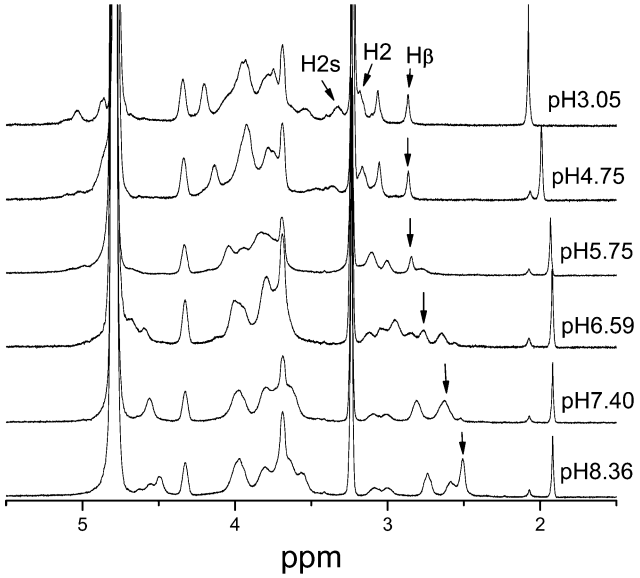


Figure 5. ^{1}H NMR spectra of the sample PC50−CH in D_2O solutions of pD ranging from 3.05 to 8.36. The arrows indicate the resonances of the protons H2s, H2, and Hβ (see the polymer structure in Figure 2), polymer concentration 12 g L^{-1}, solvent D_2O/DCl, temperature 25 °C.

slightly higher pK_a value, as commonly observed for aliphatic secondary amines, compared to the corresponding primary amines.[49]

The pK_a of the PC-substituted (secondary) amines was evaluated by ^{1}H NMR spectroscopy, a method employed successfully to determine the pK_a of various *N*-substituted chitosans. In most cases, the titration relies on the fact that the chemical shift of the chitosan C2 proton is not the same for protonated and neutral α-D-2-aminoglucopyranose residues. Thus, the signal due to the C2 proton undergoes an upfield shift of 0.3−0.4 ppm upon deprotonation of the C2 NHR moiety of *N*-substituted chitosans. We were not able to apply this protocol in the case of PC-modified chitosans because of an unfortunate overlap, in the spectra of deprotonated samples, of the signal due to the resonance of the C2 proton (H2s) and the strong singlet at δ 3.24 ppm due to the resonance of the phosphorylcholine trimethylammonium protons. This is exemplified in Figure 5, which presents ^{1}H NMR spectra of aqueous (D_2O) solutions of PC50−CH of various pD values. Under acidic conditions (pD 3.5) for which both the PC-substituted amines and the primary amines of the polymer are protonated, the C2 proton resonances of the substituted (secondary) amines (H2s) and unsubstituted (primary) amines (H2) are observed at δ 3.325 and 3.181 ppm, respectively. The resonances at δ 3.063 and 2.866 ppm (pD 3.05), attributed to the methylene protons β to the phosphorylcholine function (Hβ; see Figure 1), are also sensitive to changes in the protonation of the glucosamine residue. The signal at δ 2.866 ppm (pD 3.05) undergoes an upfield shift upon neutralization of the C2 amine group to a value of 2.474 ppm (pD 9.75). It can be monitored without interference throughout the pH range of interest. The corresponding titration curve is shown in Figure 6. The pK_a value (7.20) was taken as the inflection point of the sigmoidal curve. This value is not corrected for the difference between pD and pH. The pK_a value of the polymer in H_2O may be slightly lower. In previous reports of polysaccharide solutions by ^{1}H NMR, the correction pH = pD − 0.2 was applied to the pK_a value determined in D_2O.[14] The titration of acetic acid via ^{1}H NMR was performed under the same conditions, monitoring the pH-induced changes of the signal due to the resonance of the methyl

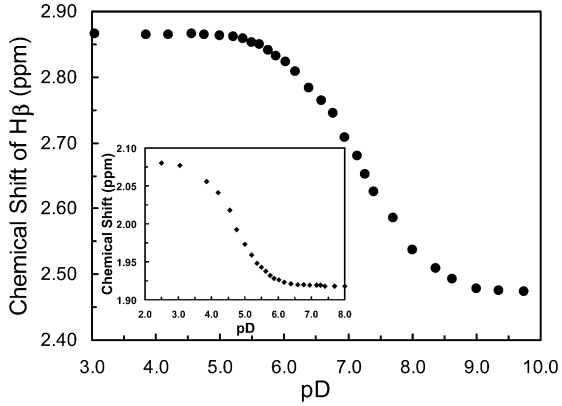


Figure 6. Titration curve for the Hβ resonance of PC50−CH obtained by ^{1}H NMR of a polymer solution (12 g L^{-1}) in D_2O/DCl, temperature 25 °C. Inset: titration curve for the methyl proton resonance of acetic acid added to the polymer solution.

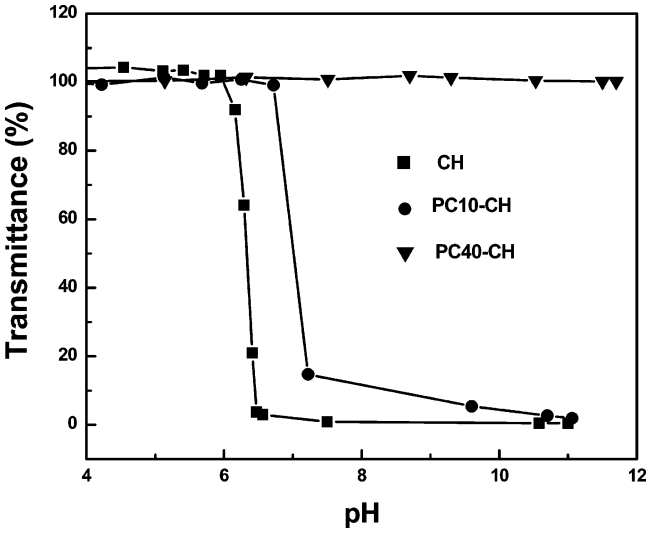


Figure 7. Changes with pH of the transmittance at λ = 566 nm of solutions of CH and PC-modified chitosans, polymer concentration 4.0 g/L.

protons of acetic acid also present in the PC50−CH solution. The pK_a of acetic acid obtained from the corresponding titration curve (inset, Figure 6) is 4.75, a value identical, within experimental error, to the value obtained by standard potentiometric methods.[50]

Solubility of PC−CH Samples in Neutral Aqueous Solutions. To assess the effect of the PC group substitution on the solubility of PC−CH samples in neutral solutions, we monitored the changes in transmittance at λ = 566 nm of aqueous polymer solutions within a wide pH range (4−11) (Figure 7). All PC−CH samples, as well as CH, are soluble in acidic conditions (pH < 4). Solutions of PC−CH samples of DS > 10 remained transparent to light (100% transmittance) independently of pH, whereas the transmittance of the CH solution decreased sharply for solutions of pH > 6.2, the threshold pH of insolubility in water of this polymer. The loss of solubility of the least substituted sample PC10−CH took place for pH > 7.

In Vitro Cytotoxicity Study. The cytotoxicity of PC−CH samples was examined using an MTT assay performed with human KB cells. The polymer composition dependence of cell viability after a 24 h incubation of cells with 1 g/L polymer solutions is presented in Figure 8. The polymers are nontoxic, regardless of the level of PC incorporation, leading the way to the use of PC-substituted chitosans in biomedical applications

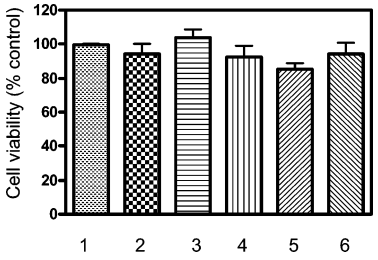


Figure 8. Effect of PC modification of chitosan on KB cell viability evaluated by the MTT assay following a 24 h incubation at 37 °C of the cells in the presence of polymer (1.0 g L^{-1}): 1, control; 2, CH; 3, PC10−CH; 4, PC20−CH; 5, PC40−CH; 5, PC50−CH.

requiring nontoxic materials soluble in neutral or alkaline aqueous environments.

Conclusions

Phosphorylcholine-substituted chitosans were prepared by means of a simple two-step one-pot procedure that allows one to control the level of PC incorporation to a desired value. The degree of PC substitution affects the pH dependence of the solubility of chitosan in water. As the degree of substitution increases, the solubility pH range increases, to an extent such that polymers of DS ≥ 23 mol % are soluble in water over the entire pH range. Trends toward increases in neutral solubility of chitosans have been reported previously as a result of *N*-substitution of the chitosan glucosamine residues. The attachment of branched oligosaccharides was found to be particularly effective in increasing the pH of the solubility threshold, yielding modified chitosans soluble in near-neutral conditions (pH 6.7). Our results demonstrate that introduction of the zwitterionic phosphorylcholine moiety even with modest degrees of substitution provides a new and effective route to nontoxic chitosans soluble in neutral and even alkaline conditions. The use of PC−CH in polyelectrolyte complexes for drug delivery and gene therapy is currently under investigation.

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