Colloid Polym Sci (2008) 286:1387–1394
DOI 10.1007/s00396-008-1908-9

ORIGINAL CONTRIBUTION

Spherical aggregates obtained from N-carboxymethylation and acetylation of chitosan

Silvana Gonçalves Ferreira Felicio · Maria Rita Sierakowski · Carmen Lucia de Oliveira Petkowicz · Joana Lea M. Silveira · Adriana F. Lubambo · Rilton Alves de Freitas

Received: 1 May 2008 / Revised: 3 July 2008 / Accepted: 6 July 2008 / Published online: 2 August 2008

Abstract Chitosan is a biopolymer with great industrial potential. However, solubility in aqueous solutions limits some applications. In this paper, the N-carboxymethylation (CM) of chitosan generated five derivatives namely, *N*-carboxymethylchitosan (NCMC) 1, NCMC2, NCMC3, NCMC4, and NCMC5, from 10.1% to 80% of CM. Afterwards, a new NCMC6 (~60% of CM) was acetylated to produce the NCMCAc derivate. The modifications were confirmed by ^{13}C nuclear magnetic resonance. A globular conformation to NCMC derivatives was confirmed by association of static and dynamic light scattering in which the form factor (ρ) is ~1.3 in phosphate buffer (pH 7.4). The NCMCAc showed a ρ of 0.75, which is characteristic of dense spheres. Topographical analyses were performed by atomic force microscopy images in which dense spheres were observed. The critical aggregation concentration (CAC) was determined using pyrene as a hydrophobic fluorescent probe. For NCMC1–6 and NCMCAc, a CAC of 1.0 and 0.1 mg mL^{-1} were, respectively, observed. NCMC1–6 and NCMCAc showed a globular and dense spherical conformation, respectively.

Keywords Chitosan · N-Carboxymethylchitosan · Acetylation · Aggregation

S. G. Ferreira Felicio · R. Alves de Freitas (✉)
Curso de Farmácia, Mestrado em Ciências Farmacêuticas,
Núcleo de Investigações Químico-Farmacêuticas (NIQFAR),
Universidade do Vale do Itajaí—UNIVALI,
Itajaí, SC 88302-202, Brazil
e-mail: rilton@univali.br

M. R. Sierakowski · A. F. Lubambo
Laboratório de Biopolímeros, Departamento de Química, UFPR,
Curitiba, PR 81531-990, Brazil

C. Lucia de Oliveira Petkowicz · J. L. M. Silveira
Departamento de Bioquímica e Biologia Molecular, UFPR,
Curitiba, PR 81531-990, Brazil

A. F. Lubambo
Departamento de Física, UFPR,
Curitiba, PR 81531-990, Brazil

Introduction

Particulate colloidal carriers (liposomes, nanospheres, or nanocapsules) were developed and are now proposed as a new approach for drug administration and vaccines [1]. In nanotechnology, a good strategy to increase the efficacy of pharmacological treatment is the use of hydrophobic or hydrophilic nanoparticles as drug delivery agents.

In literature, the use of hydrophobic nanoparticles show a preferential distribution to the liver, spleen, and lungs [2]. However, in treatments where prolonged drug liberation is necessary, hydrophilic nanoparticles could be used. These also will depend of the size of nanoparticle, and usually, smaller nanoparticles could be filtered by nephrons (<10 nm) than bigger ones recognized by the reticuloendothelial system (>200 nm) [2–6]. In some cases, the increase in nanoparticle lifetime in blood circulation induces a backlog in the solid tumor, increasing also the pharmacological efficacy of the treatment [7, 8].

Another important factor is that nanoparticles with 100 nm or lower show advantage on the drug delivery, due to its small size allowing the penetration in smaller capillary vessels and proportioning interaction with specific sites of the body [5, 9]. The increase in drug incorporation and drug release control, associated with a drug protection against physical and chemical agents, is an important

justification for the development of new nanoparticle systems [9].

The research of chitosan-carboxymethylated nanoparticles focus on the *O*-carboxymethylchitosan (OCMC) derivate, due to its good water solubility and biocompatibility [10, 11]. However, another usual method to generate a water-soluble chitosan derivative can be achieved by means of a carboxymethylation (CM) reaction known as *N*-carboxymethylchitosan (NCMC). NCMC obtained by this method is not only soluble in water but has unique chemical, physical, and biological properties, such as high viscosity, large hydrodynamic volume, low toxicity, biocompatibility, and film- or gel-forming capabilities, all of which make it an attractive option for its use in food products and cosmetics [12–18]. Chemically, NCMC is a β-(1→4)-glucan, with 2-amino-2-desoxy-D-glucose, 2-acetamide-2-desoxy-D-glucose, and 2-carboxymethylamino-2-desoxy-D-glucose [13, 14].

As described above, few works in the literature use the NCMC derivative as the subject of research, and actually, the author focused on the OCMC. But, the expressive publications of OCMC properties do not represent the macromolecular and hydrodynamic properties of NCMC due to differences in the CM sites.

In this work, the objective is to evaluate the aggregation properties of N-carboxymethylated and a N-carboxymethylated and acetylated derivative of chitosan and the formation of a hydrophobic domain, to be used in future studies as a drug carrier agent. The effect of the chemical modifications of chitosan under the aggregation behavior was confirmed using atomic force microscopy (AFM) and static and dynamic light scattering. The hydrophobic site was identified in the aggregates using fluorescence assay, using pyrene as the hydrophobic probe. The cell L929 biocompatibility assay was also performed.

Materials and methods

Chitosan, NCMC, and NCMCAc

The chitosan used was commercially obtained from China (Shangyu Biotech), and the chitin used to produce chitosan was derived from Shrimp-Shell. The product was commercially imported by Purifarma®.

The N-CM procedure was previously described [13–16] and summarized here. Every 10 g of chitosan in 1 L of acetic acid solution (0.05 M; Nuclear) after 24 h of stirring was added with a molar relation of 50% glyoxylic acid solution (Sigma-Aldrich) to obtain derivatives with theoretical CM of 5%, 25%, 50%, 75%, and 100%, named, respectively, NCMC1, NCMC2, NCMC3, NCMC4, and NCMC5. The mixtures were kept in a mechanical stirrer for 24 h. The pH was adjusted to 8.0 with NaOH solution 0.1 M (Nuclear) and was added with 2% solution of $NaBH_4$ (Nuclear). The reaction was kept further 24 h while stirring. The pH was adjusted between 6 and 8, and the system was filtered (cloth nylon), under vacuum, to remove products in suspension like chitosan. The filtered product (NCMC) was precipitated with 3 L of ethanol (Dinamica) and dried at room temperature (25 °C) under vacuum.

After the initial characterization of derivatives NCMC1, NCMC2, NCMC3, NCMC4, and NCMC5, a new derivative NCMC6, with theoretical CM of 55%, was synthesized (due the lower amount of NCMC1–5 samples), for posterior modification by acetylation, as described below.

Acetylation of NCMC was performed as previously described [19]. Briefly, 2 g of NCMC (60% CM) was dispersed in 200 mL acetic acid (Nuclear) 0.1 M. Sequentially, the sample was diluted with 250 mL of methanol (Biotec Reagents), and the desired molar amount of acetic anhydride (Nuclear) was added, diluted in 50 mL of methanol. After 24 h, the polymer was precipitated with 1 L of ethanol (Dinamica), filtered by cloth nylon, and vacuum-dried at room temperature (25 °C).

Chemical analysis

The level of chitosan deacetylation was assayed as described by Broussignac [20] and Muzzarelli [21]. The chitosan was dissolved in a know excess of acid HCl 0.1 M, and the solution was titrated with 0.1 M sodium hydroxide solution. a curve with two inflexions points was obtained, and the difference of the volumes corresponds to the acid consumed for the salification of amine groups and allows the determination of degree of acetylation (DA) of chitosan. The degree of substitution of NCMC was evaluated by an alkalimetric curve, constructed by dissolving 0.25 g of a NCMC, in 100 mL of water, bringing the solution to pH ~2 with 0.1 M HCl (Dinamica) and titrating with 0.1 M NaOH (Nuclear) [22, 23].

Characterization of NCMC

The ^{13}C nuclear magnetic resonance (NMR) spectra were obtained using a Bruker AVANCE-DRX-400 instrument. The solvent used was D_2O (Sigma-Aldrich) and NCMC samples at 20 mg mL^{-1} in the salt form. The analysis was carried out at 70 °C, and the chemical shifts were determined in parts per million.

The Fourier transform infrared (FTIR) analysis was performed in a Hartmann and Braumn MB-series equipment, using KBr for support. The acquisition spectra range was from 400 to 4,000 cm^{-1}, with a resolution of 2 cm^{-1}. Prior to the FTIR, the derivatives were suspended in 80% ethyl alcohol aqueous solution (1:100), then hydrochlori-

dric acid (3.7%; Dinamica) was added and stirred for 30 min. Then, the solid was centrifuged at 10,000×g and rinsed in 90% ethanol (Dinamica) at neutral pH. The resulting product was the H^+ form of CM chitosan [24].

Viscosity-averaged molecular mass (Mv)

Accurately weighed chitosan was dissolved in 0.3 M acetic acid/0.2 M sodium acetate (Nuclear) in purified water (MilliQ system) to yield concentrations of 0.10, 0.07, 0.05, 0.025, 0.01, and 0.005 g dL^{-1}. The intrinsic viscosity was determined using a Cannon-Fenske (no. 75) apparatus, suspended in a temperature water bath maintained at 25 °C. The efflux times were recorded in triplicate, and each assay was repeated up to a variation of 0.5%. The viscosity average molecular mass (Mv) of the chitosan was calculated using the Mark–Houwink equation [25]: $[\eta] = K_m Mv^a$, where $K_m = 76 \times 10^{-5}$ and $a = 0.76$

Ultrasonication

The ultrasonication was performed in a Sonics Vibra Cell, Mode VCX 750, probe 1, from 0 to 30 min, 30 W, in an ice batch. The sample of NCMCAc at 1 mg mL^{-1}, in purified water (MilliQ System), was filtered through 0.22 μm cellulose acetate filter, and the dynamic light-scattering analysis was performed immediately after the ultrasonication process.

Static light scattering

A multiangle laser light-scattering apparatus from Brookhaven Instruments (New York, USA) BI 9000 was used with a He–Ne laser (632.8 nm) as the light source. Instrument alignment was checked to be satisfactory, with an error less than 0.3%. The sample solution (1, 0.1, or 0.01 mg mL^{-1}) in phosphate buffer (pH 7.4) was filtered through 0.22 μm cellulose acetate filter directly into a cylindrical vial of 25 mm in diameter, which was immersed in a decalin bath at 25 °C temperature. The measurements were carried out over an angular range of 20° to 155°, and data were analyzed by the Guinier plot.

Dynamic light scattering

The dynamic light-scattering study was performed on the same apparatus and the same sample as the static light-scattering measurements. The measurement was made at 25, 30, 35, and 40 °C, using a decalin bath and angle of 90° as reference. The results were evaluated by the Dynamic light scattering software (Brookhaven Instruments), and the CONTIN method was applied, due to the statistical parameters, characterizing the particle distribution. The preparation of the samples was the same as the static light scattering (2.6) but using solvents like water, phosphate buffer (pH 7.4), and $NaNO_3$ 0.1 M [26]

Atomic force microscopy

Topographical images analyses of NCMC derivatives were obtained in a microscope AFM Shimadzu SPM09500J3, using dynamic mode and phase imaging. A 5 μl drop of 1 mg mL^{-1} of NCMC3, NCMC6, and NCMCAc was deposited on mica, left during 1 min for incubation and then washed three times with 30 μl of saline buffer at pH 7.4. Mica sheets were bought from SPI supplies®, cleaved with a scotch tape, and immediately used for the adsorption experiments. The samples were then left to dry during 24 h at 23 °C and 45% of relative humidity before image acquisition. AFM images were acquired with 256×256 pixels and a scan rate of 1 Hz. After data acquisition, flattening correction was applied.

Fluorescence assay

The fluorescence assays were performed with a F-4500 spectrofluoremeter, using pyrene as hydrophobic marker at final concentration of 2 μM and polymer concentration of NCMC1–6 and NCMCAc from 0.001 to 10 mg mL^{-1}, with excitation wavelength at 343 nm and emission wavelength ranging from 360 to 500 nm. The changes in the vibratory bands I and III, at 373 and 384 nm, respectively, were evaluated to understand the hydrophobic domains in the polymer.

Biocompatibility

Cell culture experiments were carried out using L929 mouse fibroblasts (Cell Bank of Rio de Janeiro BCRJ, Brazil). Cells were grown in Eagle's minimal essential medium (E-MEM) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine at 37 °C, 5% CO_2, with 95% relative humidity. The NCMC6 and NCMCAc samples were previously treated by dialysis against distilled water, for 48 h, prior to the biocompatibility studies, and resolved in phosphate-buffered saline (PBS) buffer at pH 7.4 and autoclaved at 120 °C by 20 min.

Cell viability was realized using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described below. Briefly, L929 cells were seeded into 96-well microtiter plates at a density of 20,000 cells per well. After 4 h, the medium was replaced by E-MEM without FCS with serial dilutions of polymer/polymer extract stock solutions of E-MEM without FCS (n=8) and incubated by 24 h, and 20 μl MTT (5 mg mL^{-1} in PBS) was added. After an incubation time of 4 h, the unreacted dye was removed by aspiration,

and the purple formazan product was dissolved in 200 μl per well dimethyl sulfoxide (DMSO; Merck, Germany) and quantified by plate reader (Asys Hitech) at 570 nm. Relative cell viability (%) related to control wells containing the cell culture medium without polymer was calculated by [Absorbance] test/[Absorbance] negative control×100. DMSO was used as positive control and the medium without the polymer as the negative one.

All data were obtained at least in octuplicate and presented as means±standard deviation. One-way analysis of variance at a significance level of $P<0.05$ with Dunnett's post-test was performed for the biocompatibility tests.

Results and discussion

Preparation of NCMC derivatives and product characterization

The chitosan showed a deacetylation degree of 87±1.4% by the titration method [20, 21], and the intrinsic viscosity (η) and the viscosity-averaged molecular mass (Mv) for chitosan were 4.5±0.01 dL g^{-1} and 0.79×10^5 g mol^{-1}, respectively.

Varying molar ratio of chitosan and glyoxylic acid, it was possible to generate different NCMC derivatives. The degree of CM was determined using an alkalimetric curve method [22, 23], and the results obtained were 10.1, 31.6, 50.7, 62.5, and 80.0 for the derivatives, respectively, named NCMC1, NCMC2, NCMC3, NCMC4, and NCMC5. The CM proposed was very similar, when compared to the theoretical and experimental results.

The new lot of NCMC (NCMC6) was generated, with a CM of 60% and characterized by FTIR and ^{13}C-NMR. The NCMC6 was used to generate three lots of an acetylated derivative, named NCMCAc, to evaluate whether the synthesis generates the same pattern of aggregates. The structures by ^{13}C-NMR of the three derivates NCMCAc are equivalent, with the same chemical shift (data not shown). In Fig. 1b, only a representative sample of NCMCAc (second lot) is rendered. The modification by N-CM (NCMC6) and acetylation (NCMCAc) of chitosan was confirmed by ^{13}C NMR as presented in Fig. 1a,b, respectively.

In Fig. 1a, the ^{13}C-NMR signals attributed to NCMC6 are 179.1 and 52.0 ppm for COO^- and N–CH_2, respectively. The presence of only one chemical shift (δ) at lower field (179.1 ppm) confirms that the CM occurs only in the

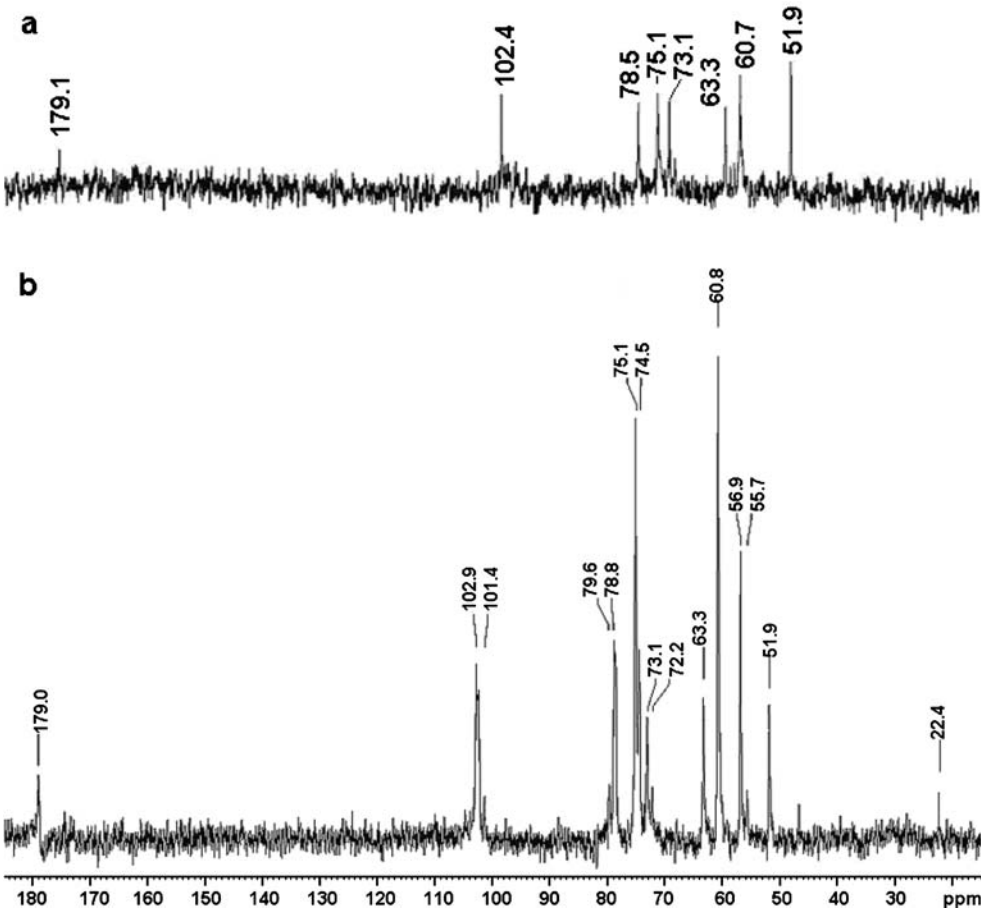

Fig. 1 ^{13}C-NMR of **a** N-CMC6 and **b** N-CMCac2, 20 mg mL^{-1}, using D_2O as solvent, at 70 °C

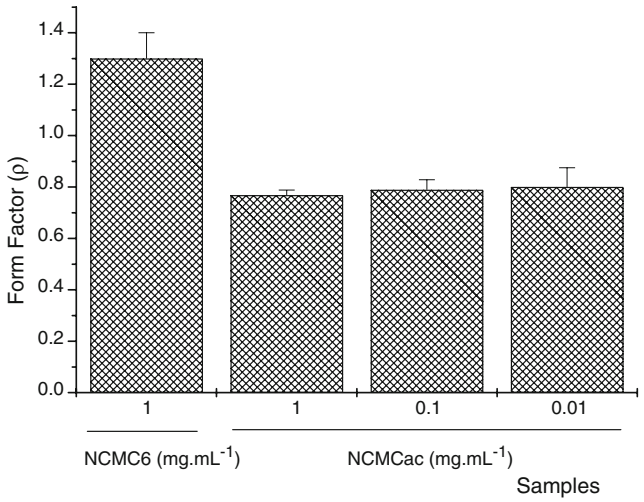

Fig. 2 Form factor as a function of the concentration for the NCMCAc1 samples, using saline buffer, pH 7.4, as solvent, at 25 °C (mean±SD of three samples)

C2 amine group. A downfield shift was observed for the carbon bearing the NCMC (C2) with respect to the corresponding carbon of unmodified residues (60.8 ppm). Chemical shifts at 102.4, 78.7. 76.1, 73.2, and 60.8 ppm related to carbons 1, 4, 5, 3, and 6, respectively, were also observed. These results are in agreement with those published [16, 23]. The chemical shifts for the other derivatives are similar (data not shown).

The chemical shift for the NCMCAc in Fig. 1b, at δ 22.4 ppm, is referent to the acetyl group. This is a good evidence of chemical modification by acetylation.

Dynamic and static light scattering

To observe the NCMCAc structure formed, a comparative study of the radius of gyration and hydrodynamic radius of the particles generated was performed. Dynamic light scattering was used to determine the hydrodynamic diameter and radius (nm). The mean hydrodynamic diameter (D_h; nm) was determined as 64.6±1.5, 62.4±1.6, 78.0±0.8, 44.4±1.5, and 63.4±1.4 nm, for NCMC1, NCMC2, NCMC3, NCMC4, and NCMC5, respectively. The method used to determine the D_h and R_h was the non-negative constrained least square (regularized; Contin), and the polydispersion of the samples was at least 0.333, which shows a highly polydispersed system. Janes et al. [27] reported chitosan particles with a diameter of 132±16 nm, and this is a good indication of intramolecular aggregation of NCMC derivates in solution, reducing the hydrodynamic macromolecular diameter.

Using the same dynamic light-scattering solutions, the static light scattering analysis was performed, by batch model, to obtain the radius of gyration (R_g), using the Guinier method.

The static light-scattering measurements were evaluated by Guinier's method. This method should be used whether the polymer solution contains globular structures [28–30]. The R_g, determined by the Guinier plot, was of 48.8±0.7, 46.4±0.8, 47.6±0.2, 31.6±0.7, and 44.9±0.6 nm, for the NCMC1, NCMC2, NCMC3, NCMC4, and NCMC5, respectively. The R_g was similar to chitosan in acid solutions, as described previously by Hu et al. [31] and Augsten and Mader [32].

The form factor (ρ), which represents a relation of the R_g and R_h, was 1.5±0.1, 1.5±0.07, 1.2±0.01, 1.4±0.05, and 1.4±0.03, respectively. Apparently, in phosphate buffer (pH 7.4) at 1mg mL^{-1}, the ρ of NCMCs showed a behavior between a hollow sphere (vesicle) or a highly branched globular shape molecule, which is characteristic of molecules with ρ of approximately 1 and polydisperse linear random coil molecules (ρ of 2.1 in good solvents). The derivative NCMC6 used to prepare the NCMCAc samples showed a ρ of 1.3±0.1 (Fig. 2).

The sample NCMC6 was modified by acetylation, and to confirm whether the methodology to obtain nanospheres by acetylation of NCMC is reproducible, three samples were synthesized, generating the NCMCAc1, NCMCAc2, and NCMCAc3 samples, and the R_g was 50.4±0.5, 39.7±1.2, and 52.6±0.3 nm, and R_h was 66.3±1.2, 56.7±1.5, and

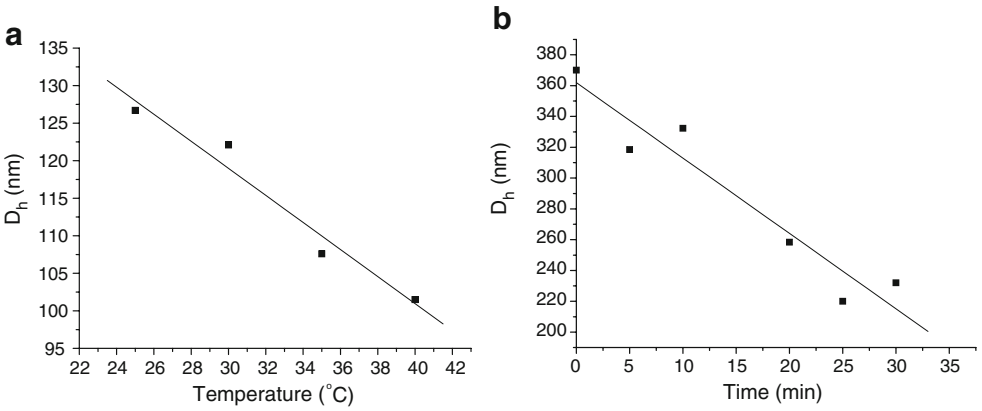

Fig. 3 Hydrodynamic diameter (nm) as a function of the temperature (°C), at 0.1 mg mL^{-1} in sodium nitrite 0.1 mol L^{-1} (**a**) and hydrodynamic diameter (nm) as a function of the sonication time at 1.0 mg mL^{-1} in water (**b**)

66.75±0.8, respectively. As observed, the samples obtained by three independent synthesis reveal a little variations in R_g and R_h. Nonetheless, the variation was proportional, generating a similar form factor of 0.76±0.02, 0.70±0.04, and 0.78±0.02 for NCMCAc1, NCMCAc2, and NCMCAc3, respectively.

The nanospheres generated by acetylation of NCMC6 are influenced by temperature. The hydrodynamic diameter of the nanospheres formed apparently shows a linear decrease in its hydrodynamic diameter with relation to temperature, and each 1 °C increase reduces the particle diameter by approximately 1.8 nm (Fig. 3a). Chen and Tsaih [33], studying chitosan, observed that increasing the solutions temperature rapidly results in a R_g and M_w decrease, resulting in increased chain flexibility and compactness of the molecule, which, in turn, causes also a decrease in the intrinsic viscosity. The reduction in the hydrodynamic parameter is due to decreasing hydrogen-bonded hydration of the intermolecular interaction, promoting the disengagement of the aggregated polymer chain and a decrease in specific volume [34, 35].

However, the temperature is not the only parameter that influences the hydrodynamic diameter: The solvent is an important influence as well. Solvents like phosphate saline buffer (pH 7.4), sodium nitrite (0.1 M), and purified water (MilliQ system), with the same NCMCAc1 concentration (0.1 mg mL^{-1}), significantly alter the hydrodynamic diameter (D_h)=127.4±1.5, 189.7±2.5, and 318.5±5.8 nm, respectively. Thus, another strategy to obtain spheres with smaller diameters was the use of phosphate saline buffer (pH) to disperse the nanoparticles in solution.

Although the following protocol was applied to evaluate the stability, it could also be considered as a different methodology to generate more homogeneous particles with smaller diameters: The samples were dispersed in water (higher diameter than other solvents tested) at 1 mg mL^{-1}, then they were submitted to ultrasonication from 0 to 30 min, using a probe of lower intensity, and ice-bathed during the process. As a result of the process aforementioned, a reduction in the hydrodynamic diameter (nm) was observed, and a straight line was obtained by the analysis of the diameter as a function of the sonication time (Fig. 3b) data, with R=0.96 (n=6, p<0.01). Each minute of sonication reduced the R_h of nanoparticles by approximately 2.5 nm. The formation of a more homogeneous particle also was obtained by sonication, due a reduction in polydispersion degree from 0.31 to 0.25.

Atomic force microscopy

AFM images of the chitosan derivatives (Fig. 4a) show a nonuniform particle aggregation, with the same globular shape as the one evaluated by light scattering in solution (NCMC3). For the sample NCMC6 (Fig. 4b), after acetylation, it was possible to evaluate a more homogeneous profile of nanospheres (NCMCAc1; Fig. 4c).

Subsequent AFM analysis confirms that the nanospheres were obtained by acetylation of carboxymethylchitosan (NCMC6). The observed dried structure deposited on mica is similar to the ones obtained in solution. Moreover, the three derivatives of NCMCAc show the same profile by

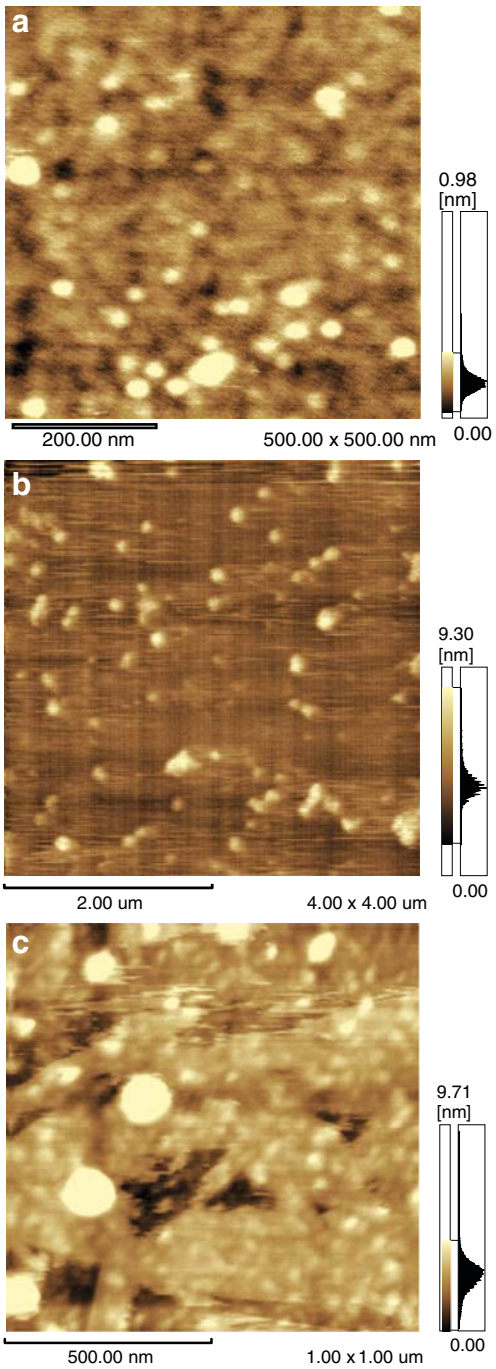

Fig. 4 AFM on dynamic mode of **a** NCMC3, **b** NCMC6, and **c** NCMCAc1 at 1 mg mL^{-1}, 25 °C, using saline buffer as solvent and mica as support

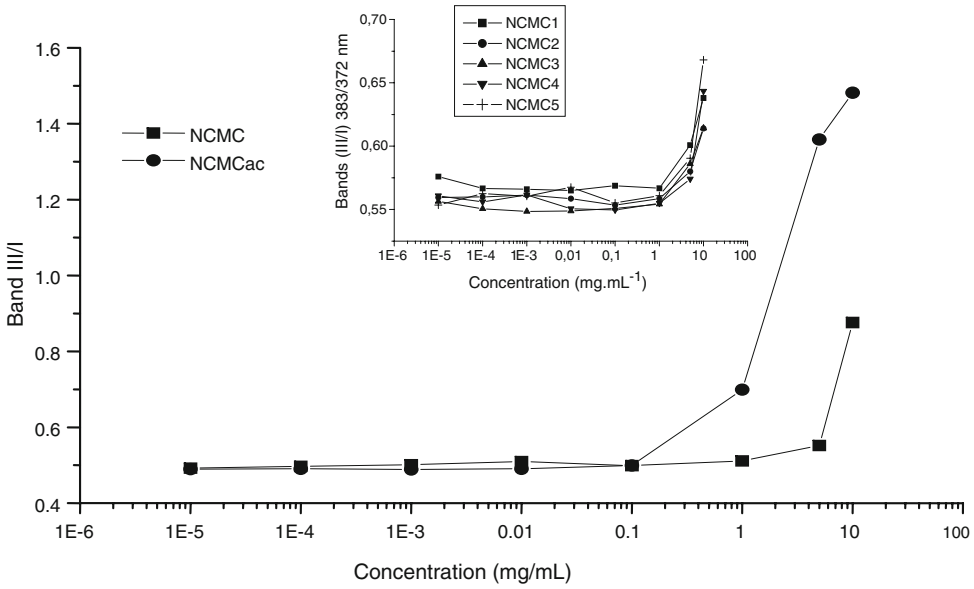

Fig. 5 Pyrene fluorescence ratio III/I as a function of the concentration for the NCMC6 and NCMCAc1 in NaCl 0.9%. In the *insert*, the NCMC1, NCMC2, NCMC3, NCMC4, and NCMC5 derivatives

AFM, confirming that the nanosphere pattern is also repetitious (data not shown).

Fluorescence spectroscopy

To determine the critical aggregation concentration (CAC), the methodology described [10] was performed, and pyrene was used as the hydrophobic probe in fluorescence spectroscopy.

As observed in Fig. 5, the NCMC6 shows CAC of 1 mg mL^{-1}; however, the NCMCAc1 shows a CAC of 0.1 mg mL^{-1}, ten times lower than the results observed for the above N-carboxylated derivative (NCMC). The same profile was observed for the NCMC samples 1 to 5 (Fig. 5—insert). This indicates that the modification by acetylation of NCMC increases the tendency of the molecule to aggregate in solution forming spherical-shaped particles. Furthermore, the aggregation observed generates a hydrophobic domain, which can be used to disperse liposoluble drugs.

Biocompatibility assay

The biocompatibility assay was realized to evaluate if the synthesis could form a toxic polymer. During the experiments, to avoid interaction between protein and polymer, as well as cellular growth during the acute cytotoxicity test, the test was performed in the absence of fetal bovine serum (FBS). This approach is commonly used to synchronize the cells in cell cycle Go [36], and can be applied for acute cytotoxicity tests (24 h) [37]. But, in the absence of FBS, also the biocompatibility test does not evaluate the cytotoxicity of compounds acting during the cellular cycle responsible for long-term toxicity (>24 h).

The first reaction process, the N-CM, generates a biocompatible polymer, as previously described in the literature. Nonetheless, the second reaction step, the acetylation of NCMC6, could affect the biocompatibility, which is not desirable. The biocompatibility of chitosan films was previously reported [38], and it was observed that films with low DA may be considered as very good biocompatible biomaterials, due to an increase in cell proliferation and adhesion, compared with films of chitosan with high DA. The cytotoxicity tests, using L929 cells, and at polymer concentration of 1,000 μg mL^{-1}, after 24 h of contact with the cells, are shown in Fig. 6.

As observed in Fig. 6, cell viability was not statistically modified by contact with a high concentration of polymer (1,000 μg mL^{-1}), when compared to the negative control.

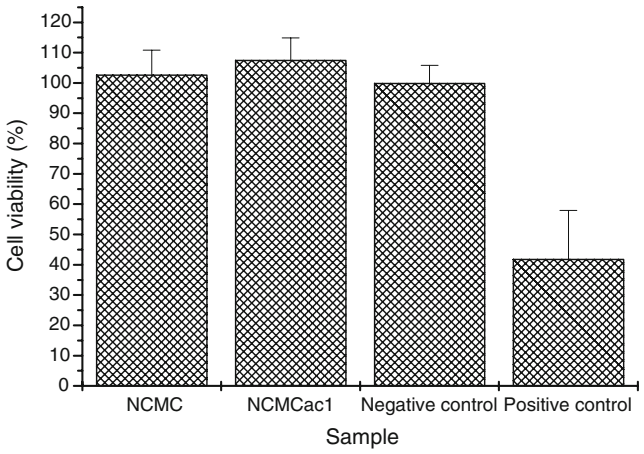

Fig. 6 Cell viability for the samples NCMC6 and NCMCAc1, after 24 h of contact with L929 cells. Cell culture medium (MEM) was used as the negative control and DMSO as the positive one

Summary

The specific modifications on chitosan to produce NCMC and NCMCAc derivatives showed development of globular nanoparticles and spherical shape, respectively, in phosphate buffer (pH 7.4). Treatments on these products using temperature and saline buffer phosphate can generate small nanoparticles, which were more homogeneous in size when each solution was submitted to sonication. The acetylated particles show a hydrophobic core with a potential as a drug carrier. Experiments using cell viability were not affected by contact with high concentration of biopolymers, indicating biocompatible nanoparticles.

Acknowledgements The authors are grateful to CNPq, Conselho Nacional de Pesquisa-Brasil, Rede de Nanoglicobiotecnologia (process number 55.5169/2005-7) for financial support.

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