Carbohydrate Polymers 77 (2009) 217–222

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Microspheres of chitosan/carboxymethyl cashew gum (CH/CMCG): Effect of chitosan molar mass and CMCG degree of substitution on the swelling and BSA release

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ARTICLE INFO

Article history:
Received 26 May 2008
Received in revised form 8 December 2008
Accepted 22 December 2008
Available online 14 January 2009

Keywords:
Chitosan
Carboxymethyl cashew gum
Molar mass
BSA release
Polyelectrolyte complexes (PEC)

ABSTRACT

Chitosan/carboxymethyl cashew gum microspheres (CH/CMCG) were prepared with carboxymethyl cashew gum with two different degrees of substitution (DS) and loaded with bovine serum albumin (BSA). In water, for microspheres formed using low molar mass chitosan (LCH) sample swelling was observed for both CMCG samples and CMCG sample with higher DS showed greater swelling. Using high molar mass chitosan (HCH) sample swelling was observed only for microsphere with high DS of CMCG (DS = 0.44). At pH 7.4, the HCH sample led to a lower degree of swelling. The diffusion coefficients D_v were higher for the higher DS of CMCG in both media and the HCH sample had a lower D_v than LCH one. Faster BSA release rates were observed for beads prepared with the higher DS, whereas those prepared with DS = 0.16 took twice the time to reach similar release profiles. All microsphere systems investigated had a non-Fickian BSA release mechanism.

1. Introduction

The importance of biocompatible and biodegradable carbohydrate polymers is continuously increasing in pharmaceutical applications because of their propensity to form crosslinked three-dimensional network hydrogels that tend to swell in water or biological fluids (Shariati & Peters, 2003). Several polymers have been used in the pharmaceutical industry, and of these chitosan (CH) plays a very important role (Reverchon, Porta, De Rosa, Subra, & Letourneur, 2000) owing mainly to its well accepted and largely reported biocompatibility (Lawrie et al., 2007). Polyelectrolyte complexes (PECs) result from complete or partial ionic condensation between oppositely charged polymers. PECs based on chitosan have been proposed for many applications including scaffolds for pulp cell regeneration and matrixes for protein carriers and antibiotics (Chen & Fan, 2007; Sarmento, Ribeiro, Veiga, Ferreira, & Neufeld, 2007). Chitosan in aqueous media can interact with anionic polysaccharides, such as carboxymethyl Konjac (Du, Dai, Liu, & Dankovich, 2006), carboxymethyl cashew gum (Maciel, de Paula, Paula, Miranda, & Sassaki, 2006) dextran sulfate (Sarmento et al., 2007), alginate (Fundueanu et al., 1998; Li et al., 2002; Vasiliu, Popa, & Rinaudo, 2005), carrageenan (Hugerth, Caram-Lelham, & Sundelof, 1997), chondroitin sulfate (Denuziere, Ferrier, Damour, & Domard, 1998) and carboxymethyl cellulose (Chen & Fan, 2007; Yan, Qian, & Zhu, 2001).

The particle size, swelling and drug delivery behavior of PECs can be affected by the molecular parameters of oppositely charged polyelectrolytes. Lee, Park, and Ha (1997) investigated the effect of the deacetylation degree of chitosan on the composition of chitosan/alginate PECs. As the degree of deacetylation increased, PECs with lower alginate content were formed. Bechéran-Marón, Peniche-Covas, and Arguelles-Monal (2004) showed that the composition of a chitosan–alginate polyelectrolyte complex was independent of alginate chemical composition as well as of the molar mass of chitosan. The stoichiometry and particle size of polyelectrolyte complexes formed by chitosan and dextran sulfate have been investigated by Schatz et al. (2004a), Schatz, Domard, Viton, Pichot, and Delair (2004b) and Drogoz, David, Rochas, Domard, and Delair (2007). Drogoz et al. (2007) reported that the nature of the polymer in excess strongly affected the mechanism in which colloid particles were formed. The existence of various complexation mechanisms depending on the kind of polyelectrolyte in excess was attributed to the differences in chemical reactivity of the ion in excess and to the conformation and flexibility of the macromolecular chains. When the chitosan was in excess, the molar mass of this polysaccharide had a great influence on the submicrometer particle size. The increase in chitosan molar mass resulted in an increase in the particle diameter, whatever the size of the dextran sulfate and degree of acetylation of the polycation

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doi:10.1016/j.carbpol.2008.12.037

(Schatz et al., 2004a). Vasconcelos et al. (2006) investigated the effect of poly(methacrylic acid) (PMAA) molar mass on the formation of a colloidal dispersion of a chitosan/PMAA polyelectrolyte complex. The authors found that an increase in PMAA molar mass inhibited the formation of insoluble complexes, while an increase in ionic strength first favored the formation of the complex and then inhibited it at higher concentrations.

Cashew gum (CG) is an exudate polysaccharide from *Anacardium occidentale* trees and has been previously characterized (de Paula & Rodrigues, 1995). The gum is composed of galactose (72%), glucose (14%), arabinose (4.6%), rhamnose (3.2%) and glucuronic acid (4.7%) (de Paula, Heatley, & Budd, 1998; de Paula & Rodrigues, 1995). The polysaccharide has been modified by carboxymethylation with monochloroacetic as an etherifying agent, resulting in samples with degrees of substitution (DS) between 0.10 and 2.21 (Silva et al., 2004). Chitosan/CG gels have been prepared by re-acetylation of chitosan with acetic anhydride, characterized by infrared spectroscopy, thermal analysis and X-ray diffraction. Addition of CG to the CH gel was found to decrease the pilocarpine release rate in the medium. The release of pilocarpine from CH gel was found to be dependent on pH values (Maciel et al., 2006).

Also, chitosan/carboxymehtyl cashew gum complexes have been synthesized by "one-shot addition" using different proportions of chitosan (CH) and carboxymethyl cashew gum (CMCG). The thermal stability of the polyelectrolytes was investigated and the authors found that the activation energies followed the order: CMCG > CH > PECs samples (Maciel, Silva, de Paula, & Paula, 2005).

In this study we reported the effect of chitosan molar mass and the DS of CMCG on the swelling and BSA release of chitosan/CMCG microspheres. BSA was used as a model drug for protein release.

2. Experimental

2.1. Materials

The chitosan samples were donated by Prof. Cardenas of Quitoquimica. Two samples of chitosan with different molar mass were used in this study (HCH-high molar mass chitosan MM = 2.35×10^5 g/mol and degree of deacetylation (DD) = 83 ± 1% and low molar mass chitosan (LCH) MM = 7.82×10^4 g/mol and DD = 81 ± 1%). Bovine serum albumin (BSA) from Sigma.

Crude samples from cashew gum were collected from native trees in Fortaleza, Ceará, Brazil. They were purified as a sodium salt using a previously described method (de Paula & Rodrigues, 1995). Nodules free of bark were selected and dissolved in distilled water at room temperature to give a 5% (w/v) solution. The solution pH was adjusted to approximately 7.0 by addition of diluted aqueous NaOH. The clear solution was successively filtered through sintered glass and the polysaccharide precipitated with ethanol.

2.2. Carboxymethylation reaction

The carboxymethylation reaction was performed using the methodology reported by Silva et al. (2004) described as follows: purified gum (5 g, 0.0278 mol) was mixed with 5 ml of water until a homogeneous paste was formed. A 10 M NaOH solution was added and the mixture was kneaded for 10 min. After that, monochloroacetic acid (MCA) was mixed thoroughly with the paste. The mixture was maintained at 55 °C for 3 h. The system was neutralized with 1 M HCl and dialyzed against distilled water until all remaining reagents or added salt were eliminated (4–5 days). The solid carboxymethylated cashew gum (CMCG) samples were recovered by freeze-drying. The conditions applied to obtain samples with degrees of substitution (DS) of 0.16 and 0.44 were mol ratios of CG/MCA/NaOH of 1:1:1 and 1:1:2, respectively.

2.3. Degree of substitution (DS)

The absolute degree of substitution (DS_{abs}) was determined by potentiometric back-titration. In order to titrate all carboxymethylated groups, either in acid or salt form, 20 ml of the CMCG solutions (2.5% w/v) were passed through an Amberlite IR-120H$^+$ column and freeze–dried. The acid CMCG solution (10 mg/ml) was then titrated with 0.0182 M HCl after addition of a known amount of NaOH. The DS_{abs} value was calculated through the molar ratio of carboxymethyl acid groups to monosaccharide units, as follows:

$$DS_{abs} = \text{mol of} -CH_2COOH \text{groups/mol of monosaccharide unit} \quad (1)$$

The molar mass of galactose (180 g/mol), a major constituent of cashew gum (de Paula et al., 1998) was used to calculate the mols of monosaccharide units. An initial value for DS was then determined. The new molar mass of average monosaccharide units was then recalculated taking into account the addition of 58 g/mol (molar mass of CH_2COOH) for each DS increase of 1.0 unit. The method of successive approximation was applied to obtain the final absolute DS. Two samples with DS of 0.16 and 0.44 were obtained and the molar mass for both carboxymethylated samples were similar (1.0×10^4 g/mol).

2.4. Preparation of polyelectrolyte complex microspheres

Chitosan samples with different molar masses were dissolved in 2% acetic acid. The CH concentrations were 3% for HCH and 5% for LCH. The CH solution was added to a 3% solution of carboxymethyl cashew gum solution (1 M NaOH/ethanol (2:1), with different degrees of substitution forced by air flow (10 L/h) through a syringe equipped with a needle of 21 mm length and 0.8 mm diameter, as proposed by Tan, Hu, Jin, and Zhang (2003) for preparation of hyaluronate/chitosan beads. The amount of CMCG incorporated on the beads was determined using phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Roberts, and Smith (1956)).

2.5. Preparation of BSA-loaded CH/CMCG microspheres

BSA (1%) was added to carboxymethyl cashew gum solution (3%) in 1 M NaOH/ethanol (2:1) and microspheres were formed as described above by adding CH solution via a syringe.

In order to quantify the amount of BSA, the beads were grounded and BSA extracted in water for 24 h under stirring. The BSA content was analyzed by UV spectrophotometry at 280 nm and the protein percentage determined using a calibration curve.

The loading efficiency (LE) was calculated as:

$$LE(\%) = \frac{(\text{Total mass of BSA added} - \text{Residual mass of BSA})}{\text{Total mass of BSA}} \times 100$$

2.6. Optical microscopy

The swelling kinetics, shape and size of microspheres were determined using an Olympus CH30 optical microscope. Ten spheres were used to estimate the mean diameter of dry samples.

2.7. ^1H NMR spectroscopy

The presence of both polysaccharides in the microspheres was evaluated by NMR spectroscopy. ^{1}H NMR spectroscopy measurements were carried out on a Bruker DRX-500 at 20 °C. The beads were dissolved in HCl 0.1 M and freeze–dried. The acidified sample was dissolved in D_2O for NMR analysis.

2.8. Swelling kinetics

The swelling behavior of the CH/CMCG beads was evaluated by analyzing the bead volume change over time using an optical microscope. Swelling kinetics were performed in distilled water and phosphate buffer (pH 7.4). The bead diameter was measured for a sample of six beads, at 10 min time intervals during the first hour, and then every 20 min until a swelling equilibrium was reached.

2.9. In vitro BSA release

BSA-loaded microspheres (0.15 g) were immersed in 20 ml of phosphate buffer (pH 7.4) or distilled water at 37 °C with stirring. Aliquots of 3 ml were withdrawn at certain time intervals and replaced with an equal volume of fresh buffer solution or water. The amount of drug released was determined by measuring the absorbance of each aliquot at 280 nm.

3. Results and discussion

3.1. Characterization of CH/CMCG microspheres

The polyelectrolyte complex formed using carboxymethyl cashew gum (CMCG) with different DS values and similar molar mass and CH with different molar mass but similar DD values (see Section 2) were investigated. Spherical particles were obtained for all chitosan molar mass and CMCG DS values used (Fig. 1). The effect of the charge density of CMCG and CH molar mass on the average sphere diameter can be observed in Table 1. Dried microspheres with high DS of CMCG had large average particle diameter regardless of the CH molar mass. This may be explained by the high amount of CMCG incorporated when high DS of CMCG sample was used in the preparation of beads (Table 1). For microspheres formed with the same DS of CMCG, the beads formed with HCH sample had a larger sphere diameter. Since the DD of the two CH samples were similar, the increase in sphere diameter is likely to be mainly due to the increase in the CH chain length and also due to high CMCG incorporation.

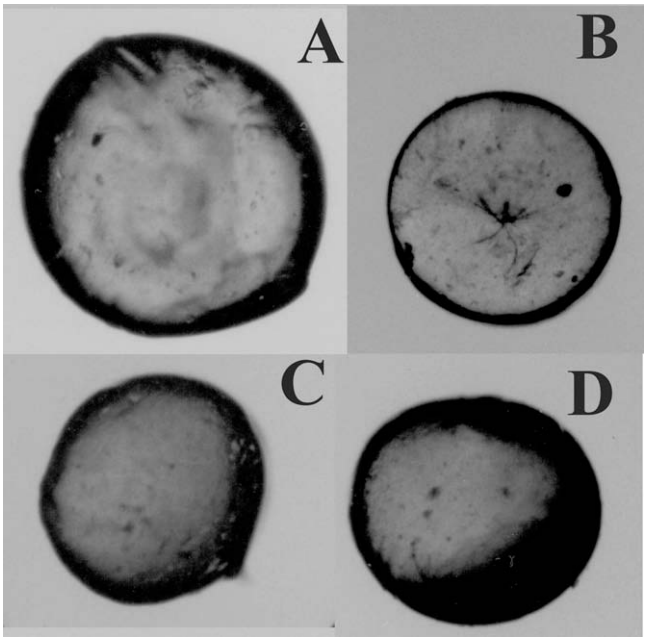

Fig. 1. Optical microscopy of microsphere: (A) HCH/CMCG 0.16; (B) HCH/CMCG 0.44; (C) LCH/CMCG 0.16 and (D) HCH/CMCG 0.44.

The average bead diameters obtained in this study were shorter than those obtained by Vasiliu et al. (2005), for the complexation of hyaluronan and chitosan (590–1550 μm) using different experimental conditions. For particles formed from carboxymethyl Konjac gum with chitosan, the reported particle diameter was in the range of 1200–1600 μm (Du et al., 2006). Microparticles produced by complexation of chitosan and alginate also had spherical forms and particle diameters ranging from 820 to 2100 μm, depending on the air flow rate of the bead formation device (Tan et al., 2003). The author shows that as high the air flow rate smaller is the bead. As in this work was used an air flow system with high flow rate (10 L/h), it is explained the small size of CMCH/CH beads produced.

Fig. 2 shows the ^{1}H NMR spectrum of CH/CMCG spheres. The spectrum is complex and shows anomeric signals of acetyl glucosamine and glucosamine, respectively, at δ 4.5 (H-1 A) and δ 4.90 (H-1 D), along with the H-1 of galactose, rhamnose and glucose units of carboxymethyl cashew gum, respectively, at δ 4.44 (H-1 Gal), 4.80 and 4.94. Signals of the acetyl protons of chitosan and methyl group of rhamnose od CMCG at δ 2.1 (H- Ac) and δ 1.25 (Me-Rham), respectively, were also observed. The NMR spectrum thus confirms the presence of both polysaccharides in the spheres.

3.2. Swelling behavior of CH/CMCG beads

The swelling behavior of CH/CMCG dried beads in water and pH 7.4 buffer was evaluated by analyzing bead volume changes over time, using an optical microscope. Polyelectrolyte complexes of chitosan tend to dissolve under gastric pH conditions. The effects of the DS of CMCG and CH molar mass on the swelling of the beads in distilled water are shown in Fig. 3. For PEC formed using HCH and low DS of CMCG, swelling of the spheres was not observed (Fig. 3A). On increasing the DS to 0.44, an increase of 6% in the bead diameter was observed. Using LCH (Fig. 3B), swelling was observed for both CMCG samples and CMCG sample with higher DS showed greater swelling. The charge ratio (n^+/n^-).of polysaccharides present in the beads seems to be important on the swelling. When an excess of n^+ charge is present, a smaller swelling is observed. As the charge ratio tend to 1 the swelling ratio increases.

The swelling behavior of samples in pH 7.4 buffer is shown in Fig. 4. In this medium all samples absorbed water. At this pH, the COO^- groups of carboxymethyl cashew gum are ionized leading to polyelectrolyte complex repulsion between the ionized groups and a consequent expansion of the network. Using HCH (Fig. 4A), less swelling was observed, which may be due to the formation of a more compact network with longer chitosan chains. At pH 7.4, samples with a lower DS of CMCG showed less swelling, probably due to a smaller number of ionized groups in the samples, leading to small repulsion of carboxylate groups in the CMCG chain and thus a less extended network is formed.

The diffusion coefficient (D_v) in pure water and in buffer pH 7.4 was calculated using the methodology described by Harogoppad and Aminabhavi (1992):

$$\frac{\Delta V_t}{V_0} = 4 \cdot \left(\frac{\Delta V_\infty}{V_0 \cdot D_0}\right) \cdot \left(\frac{D_v}{\Pi}\right)^{1/2} \cdot t^{1/2} \quad (2)$$

D_v is calculated from the slope of the initial linear plot of $\frac{\Delta V_t}{V_0}$ *versus* $t^{1/2}$ by:

$$D_v = \left((1.773 \cdot \text{slope})\frac{V_0 D_0}{4\Delta V_\infty}\right)^2 \quad (3)$$

The D_v data for CH/CMCG beads in water and in phosphate buffer pH 7.4 are shown in Table 2. Higher D_v values were obtained for the highest CMCG charge density in both media. For the highest DS of CMCG, and consequently charge density, this was more pronounced for LCH, with values of 212% (in distilled water) and

Table 1
Effect of DS of CMCG and CH molar mass on average sphere diameter.

CMCG DS	HCH[a]			LCH[b]		
	d (μm)[c]	CMCG (%)[d]	n^+/n^-ratio[e]	d (μm)[c]	CMCG (%)[d]	n^+/n^-ratio[e]
0.16	530 ± 4.0	72.9	3.3	500 ± 4.0	71.4	3.4
0.44	580 ± 6.0	79.3	1.1	550 ± 5.0	77.4	1.1

[a] HCH MM = 2.35×10^5 g/mol.
[b] LCH MM = 7.84×10^4 g/mol.
[c] d = Dried sphere diameter.
[d] % of CMCG incorporated.
[e] Charge molar ratio of spheres.

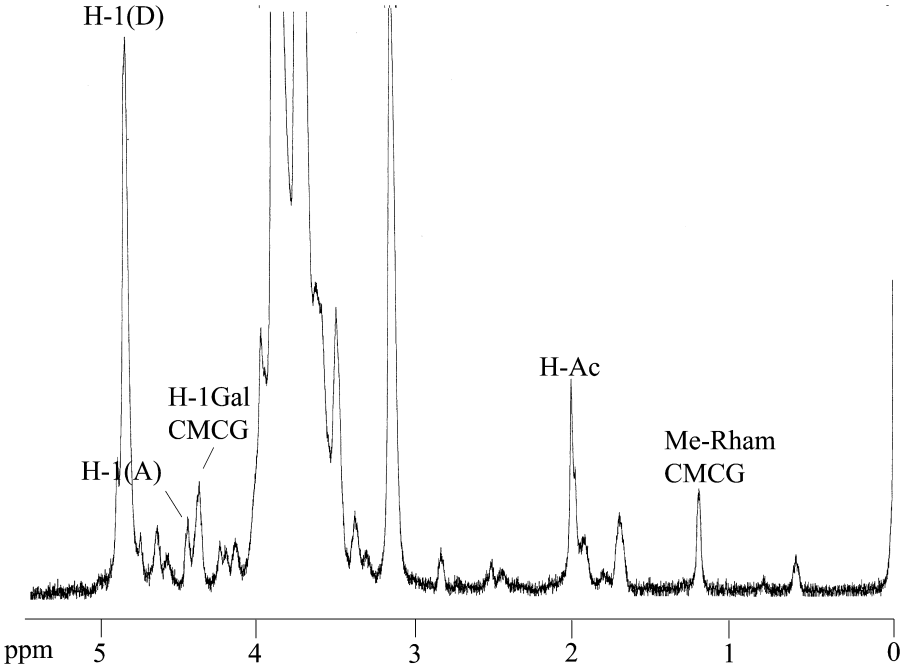

Fig. 2. ^{1}H NMR in D_2O at 70 °C of HCH/CMCG 0.44.

194% (in buffer pH 7.4). The diffusion coefficient values were found to be higher in pH buffer than in water. In a previous study, the swelling degree of chitosan/cashew gum reacetylated gel was found to be lower in phosphate buffer (pH 7.4) in comparison with that in distilled water (Maciel et al., 2006; Paula, Gomes, & de Paula, 2002). The difference in the behavior observed for CH/CMCG microspheres may be due to an increase in the charge density when the cashew gum was carboxymethylated. At pH 7.4 the carboxyl groups of CMCG are ionized, and so an increase in the charge repulsion occurs, leading to a network expansion, resulting in an increase in the diffusion coefficient. At a given DS of CMCG, the higher the CH molar mass the lower is the D_v value. The% decrease in D_v is more pronounced in pH 7.4 (134%) than in distilled water (12.7%) for DS = 0.44. Although the swelling degree of CH/CMCG beads is not comparatively high, the diffusion coefficient values obtained in this study are similar to those obtained by Lin et al.(2005) for chitosan/dextran sulfate beads crosslinked with tripolyphosphate (D_v ranging from 2.8×10^{-7} to 8.1×10^{-7} cm^2/s, at pH 6.8).

3.3. Effect of CH molar mass and CMCG DS on the release of BSA from CH/CMCG microspheres

Our data revealed that neither the CH molar mass nor the CMCG DS affected the loading efficiency (LE%) of CH/CMCG beads, as almost the same LE values (10.0 ± 0.2%) were obtained for all beads. For microcapsules of alginate/chitosan, an increase in chitosan molar mass was found to lead to increased Ketoprofen loading (Tan et al., 2003).

The effect of chitosan molar mass and CMCG DS on the release of BSA was investigated in phosphate buffer (pH 7.4). Our experiments revealed that at pH 1.2, beads were dissolved in a few minutes. In Fig. 5 it can be seen that the beads were not efficient at sustaining the BSA release for a time larger than 80 min, may be because the beads were not crosslinked. A general trend seems to be evident, whereby microspheres with same CMCG DS exhibit similar release profiles up to 80 min, regardless of CH molar mass. The release rate of BSA was lower for LCH than for HCH. A similar result was found by Tan et al. (2003) for alginate/chitosan beads with different CH molar mass for the release of Ketoprofen. However, Ko, Park, Hwang, Park, and Lee (2002) found that an increase in chitosan molar mass decreased the release of felodipine significantly and Polk, Amsden, Yao, Peng, and Goosen (1994) showed that on decreasing CH molar mass, an increase in albumin release rate was observed. On the other hand, the effect of DS of CMCG has a strong influence on release rate, although the maximum% of BSA released was not affect by either DS of CMCG or chitosan molar mass. The higher DS of CMCG showed a faster release rate, and this may be explained by the high diffusion coefficient observed for samples with high DS, for which an increase in drug release is expected. Microspheres formed with lower charge density CMCG (DS = 0.16) took twice the time to reach similar release profiles.

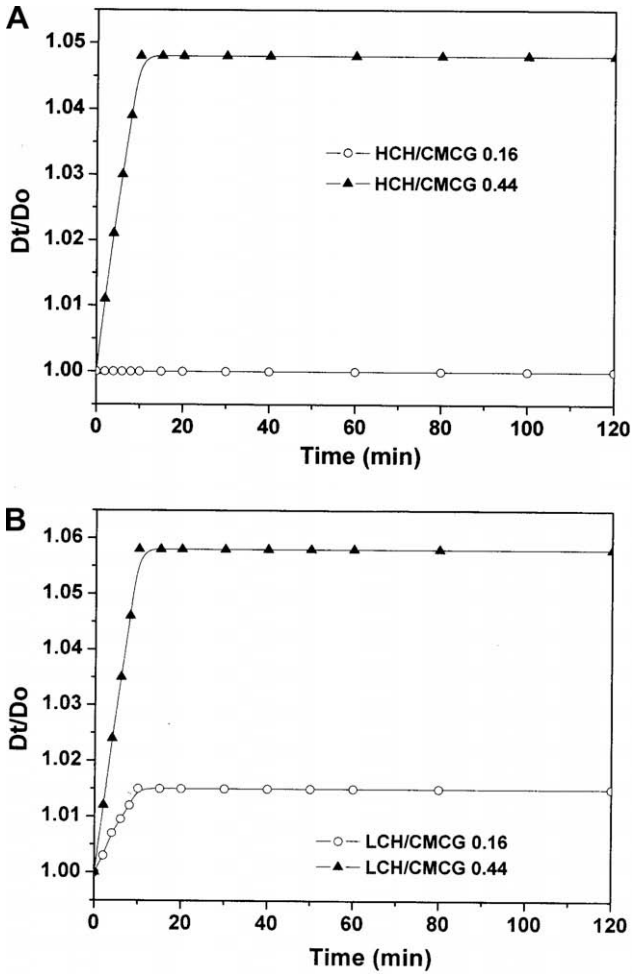

Fig. 3. Plot of D_t/D_0 vs. Swelling time in water for CH/CMCG: (A) HCH/CMCG 0.16 and 0.44; (B) LCH/CMCG 0.16 and 0.44.

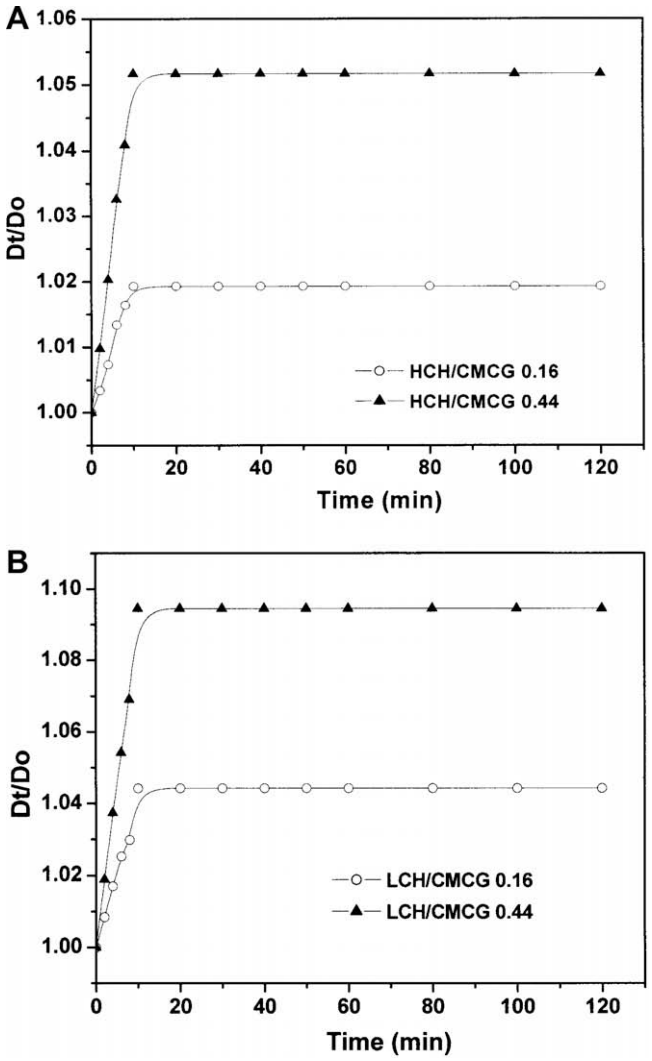

Fig. 4. Plot of D_t/D_0 vs. swelling time (t) at pH 7.4 for CH/CMCG: (A) HCH/CMCG 0.16 and 0.44; (B) LCH/CMCG 0.16 and 0.44.

The mechanism of drug release can be evaluated using the semi empirical equation proposed by Ritger and Peppas (1987):

$$M_t/M_\alpha = kt^n \quad (4)$$

where M_t/M_α denotes the fraction of drug released, t is the release time and k is the constant characteristic of the system. The diffusional exponent n indicates the mechanism of drug release. For a spherical, swellable polymeric matrix, a Fickian system is described by the diffusion phenomenon and assumes an n value of 0.43 (Ritger & Peppas, 1987). For a drug released by the diffusion and relaxation phenomenon (non-Fickian system), n values are in the range of $0.43 < n < 0.85$ (Ritger & Peppas, 1987). Table 3 shows the diffusional exponent for the CH/CMCG beads. All the bead systems investigated had a non-Fickian mechanism of BSA release. A non-Fickian mechanism has previously been found for several drug delivery devices based on chitosan (Lin, Yu, & Yang 2005; Peng, Zhang, & Kennedy, 2006). Release systems prepared with low molar mass chitosan have lower diffusion exponents than those employing HCH.

Table 2
Diffusion coefficient in CH/CMCG polyelectrolyte complex beads.

DS of CMCG	$D_v \times 10^7$(cm^2/s) in distilled water		$D_v \times 10^7$(cm^2/s) in pH 7.4 buffer	
	LCH[a]	HCH[b]	LCH[a]	HCH[b]
0.16	3.15	–	7.30	3.86
0.44	9.84	8.73	21.5	9.17

[a] LCH MM = 7.84×10^4 g/mol.
[b] HCH MM = 2.35×10^5 g/mol.

4. Conclusions

CH/CMCG microspheres were prepared and their diameter was found to vary proportionally to both charge density (DS of CMCG) and chitosan molar mass. Diffusion coefficients (D_v) increased with CMCG DS in both media (distilled water and pH buffer pH 7.4) and decreased with chitosan molar mass. For low chitosan molar mass (LCH) swelling behavior in water increased with CMCG charge density. For high chitosan molar mass (HCH), low DS samples, swelling was not observed in water. At pH 7.4 less swelling was observed for HCH sample. BSA release rates were fast for beads prepared with high DS CMCG, whereas those prepared with lower charge density (DS = 0.16) released the drug for twice the time. The data obtained showed good agreement with results reported in the literature for similar systems.

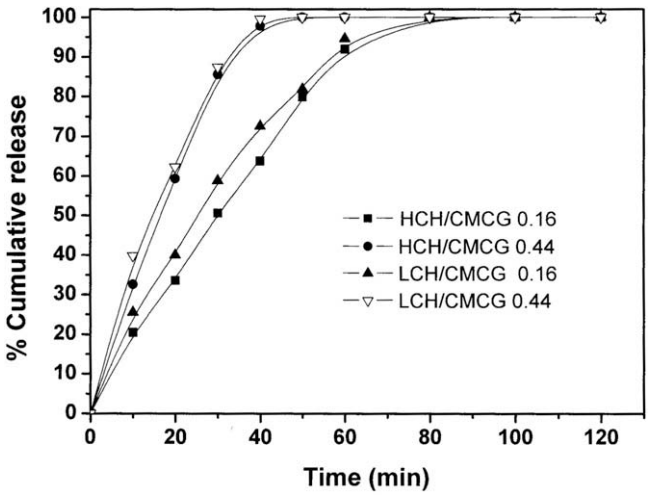

Fig. 5. Effect of CH molar mass and DS of CMCG on *in vitro* release profile of BSA.

Table 3
Kinetics data for the release of BSA from CH/CMCG microspheres.

DS of CMCG	*n* values	
	LCH[a]	HCH[b]
0.16	0.74 ± 0.03	0.81 ± 0.01
0.44	0.68 ± 0.03	0.81 ± 0.03

[a] LCH MM = 7.84×10^4 g/mol.
[b] HCH MM = 2.35×10^5 g/mol.

Acknowledgments

The authors gratefully acknowledge financial support from Rede Nanoglicobiotec/ CNPq and CAPES/PRODOC and CENAURMN for the NMR spectra.

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