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Synthesis of cross-linked *N*-(2-carboxybenzyl)chitosan pH sensitive polyelectrolyte and its use for drug controlled delivery

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ABSTRACT

N-(2-Carboxybenzyl)chitosan (CBCS) polyelectrolyte was synthesized via a Schiff reaction of chitosan with 2-carboxybenzaldehyde, followed by reduction of the imine derivative with sodium borohydride. The polyampholyte chitosan derivative was cross-linked with glutaraldehyde leading to the formation of a pH-sensitive hydrogel. All prepared materials were characterized by FTIR, UV and ^{1}H NMR spectroscopies. The swelling behavior of the cross-linked *N*-(2-carboxybenzyl)chitosan hydrogel (CBCSG) was studied in aqueous solutions of varied pH (1, 5, 7 and 9). It was found that CBCSG swelled more in acidic solutions than in alkaline ones. Drug loading of fluconazole (a sparingly water-soluble drug) in CBCSG was prepared using the solvent evaporation method. WAXD data revealed that the drug was amorphous in the prepared formulations with 20% (w/w) drug content or less and crystalline at higher loading. Release of fluconazole from CBCSG was effectively sustained, indicating the suitability of the CBCSG to be used as a controlled-release system of fluconazole. The release rate was found to depend on drug loading and the pH of the release medium, which controlled the swelling capacity of the gel during drug release.

1. Introduction

Chitosan (CS), a copolymer of β-(1→4)-D-glucosamine and β-(1→4)-*N*-acetyl-D-glucosamine, is the (partially) deacetylated product of chitin [poly-β-(1→4)-*N*-acetyl-D-glucosamine], the second most abundant natural occurring polysaccharide after cellulose (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). Whereas chitin is insoluble in water due to the extensive hydrogen bonding of the acetyl group, chitosan is soluble in dilute acid solutions due to the ionization of the amino group and insoluble in solutions with $pH > 4.5$. Chitin, chitosan and their derivatives have been widely used in pharmaceutical systems, among numerous other applications (Kumar, 2000; Rinaudo, 2006; Shimono et al., 2002), on account of their lack of toxicity and excellent biocompatibility.

Amongst the chitosan derivatives utilized in pharmaceutical applications, of major importance is the *N*-alkylated chitosans. *N*-alkylation can be achieved via the reaction of chitosan with either an aldehyde or ketone followed by reduction, or via the reaction of chitosan with alkyl halides. However, the reaction of chitosan with alkyl halides usually leads to *N,O*-alkylated products (Kurita, 2001; Mourya & Inamdar, 2008). Carboxymethyl-chitosan (CMCS) is a water-soluble chitosan derivative, soluble at both acidic and basic physiologic conditions, which is obtained from the reaction of chloroacetic acid and chitosan in alkaline medium under relative mild conditions (50–60 °C). This conversion not only provides carboxymethyl-chitosan with aqueous solubility, but also brings some useful chemical, physical and biological properties to carboxymethyl-chitosan, such as high viscosity and large hydrodynamic volume, low toxicity, biocompatibility and antibacterial activity. These properties make carboxymethyl-chitosan an attractive material for applications in food products, cosmetics and pharmaceuticals (Muzzarelli, 1988). The degree of substitution and molecular weight of carboxymethyl-chitosan plays an important role in drug delivery. Thus, nanoparticles based on carboxymethyl-chitosan with higher molecular weight and degree of substitution exhibited enhanced drug loading and lower rate of drug release (Shi, Du, Yang, Zhang, & Sun, 2006).

A pH-sensitive chitosan derivative, *N*-(2-carboxybenzyl) chitosan (CBCS), has been synthesized from the reaction of chitosan with carboxybenzaldehyde (CBBA) and subsequent reduction of the imine derivative (CBCSS) (Muzzarelli, Tanfani, Marriotti, & Emanuelli, 1982; Lin, Chen, & Luo, 2007). In these studies, however, the imine derivative was not isolated and was reduced in situ (in the presence of unreacted carboxybenzaldehyde), rendering unclear exact chemical structure of the final product. A mixture of mono- and di-substituted products was probably synthesized. As the physicochemical and biological properties of the polymer depend on its structure, in order *N*-(2-carboxybenzyl)chitosan to

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be useful for biomedical applications its exact structure should be known. Thus in the present study, *N*-(2-carboxybenzyl)chitosan was synthesized from the reduction of isolated (purified) imine derivative. This made possible to investigate the effect of experimental conditions, such as reaction time and temperature, on the solubility and degree of substitution of the imine intermediate (CBCSS) and the final product (CBCS). The synthesized *N*-(2-carboxybenzyl)chitosan was then cross-linked with glutaraldehyde, leading to the formation of a pH-sensitive hydrogel (CBCSG). The swelling behavior of the CBCSG hydrogel in aqueous solutions of varied pH was studied. The hydrogel was loaded with the sparingly water-soluble drug fluconazole and the drug release properties at different pHs were also studied. Due to the mucoadhesive properties of chitosan derivatives (Salamat-Miller, Chittchang, & Johnston, 2005) fluconazole/CBCSG hydrogels may be useful as buccal controlled-release systems for the treatment of oropharyngeal candidiasis (Kuipers et al., 2002). Furthermore it is well known that chitosan and its derivatives can enhance the absorption of poorly water-soluble drugs as fluconazole (Schipper et al., 1997).

2. Experimental

2.1. Materials

High molecular weight ($M = 3.55 \times 10^5$ g/mol) chitosan (CS) from crab shells with a degree of deacetylation of 85% was obtained from Sigma–Aldrich. 2-Carboxybenzaldehyde (CBBA) of 99% purity was obtained from Acros Organics. Sodium borohydride (SB) (“BAKER” Grade) was purchased from J.T. Baker. The glutaraldehyde (GA) aqueous solution 50% (v/v) used was from Sigma–Aldrich. Fluconazole was purchased from Dr Reddy’s (Andra Pradesh, INDIA) as a white crystalline powder with assay 99.8% (limits 99.0–101.0%), slightly soluble in water and freely soluble in methanol.

2.2. Synthesis of N-(2-carboxybenzyl)chitosan (CBCS)

Chitosan powder (4.0 g) was gradually added, under constant mechanical stirring, into 500 mL aqueous acetic acid 0.7% (v/v). After complete dissolution, 30 mL of 2-carboxybenzaldehyde 17% (w/v) solution in ethanol were added dropwise. The amount of 2-carboxybenzaldehyde used corresponded to a 2:1 mole ratio of reactive groups CHO/NH_2. After stirring in a water bath at specified temperature and time period (Table 1) the mixture was cooled down to room temperature and either poured into an excess of acetone to isolate the Schiff base derivative (syntheses 1–3, Table 1) or 20 mL of SB aqueous solution 8% (w/v) were added dropwise and under constant stirring to bring about direct reduction of the Schiff base. In the latter case, stirring was continued for 2 h (synthesis 4, Table 1). In the case of syntheses 1–3, 2.0 g of the purified Schiff base was dissolved in 250 mL aqueous acetic acid 0.7% (v/v) where 10 mL of sodium borohydride aqueous solution 8% (w/v) was added dropwise, followed by a 2 h stirring period. The *N*-(2-carboxybenzyl)chitosan derivatives produced were precipitated by adding an adequate amount of acetone. Schiff bases and *N*-alkyl chitosan derivatives were purified by soaking in acetone for 24 h and dialyzed against deionized water for 3 days. The purpose of using a nitrogen purge in the case of synthesis 3 (synthesis at higher temperature) was the protection from oxygen, since reactions such as depolymerization and oxidation are favored at temperatures higher than 55 °C.

2.3. Preparation of cross-linked N-(2-carboxybenzyl)chitosan derivatives (CBCSG) and cross-linked chitosan (CSG)

N-(2-Carboxybenzyl)chitosan derivatives in solution 0.4% (w/v) were prepared in 500 mL aqueous acetic acid 0.7% (v/v). Under intense stirring, 6 mL of glutaraldehyde aqueous solution 1% (v/v) was added dropwise. The amount of glutaraldehyde used was such that it could have reacted with 15% of the amino groups of a zero degree-substituted polysaccharide. After a 5 min stirring period, the bubble-free solution was poured into a glass beaker and left there for 12 h allowing formation of the gel network. The hydrogel was then frozen at −18 °C and dried in a freeze-dryer to yield the xerogel. For comparison purposes, chitosan xerogel was also prepared with the same method.

2.4. Fluconazole loading into cross-linked N-(2-carboxybenzyl)chitosan (CBCSG) xerogel

Fluconazole was loaded in CBCSG using the solvent evaporation method. CBCSG xerogel was dispersed in ethanol at room temperature and allowed to swell for 48 h. Fluconazole dissolved in ethanol was added dropwise and under stirring in the CBCSG gel. The mixture was left at room temperature under constant stirring in order to evaporate the solvent and to yield the drug loaded CBCSG. CBCSG/fluconazole formulations were prepared with drug loading 10%, 20%, 30% and 40% (w/w).

2.5. Fourier transformed-infrared spectroscopy (FTIR)

FTIR spectra were obtained using a PerkinElmer FTIR spectrometer, model Spectrum One. A small amount of each material was mixed with KBr and compressed to tablets. The IR spectra of these tablets were obtained in absorbance mode and in the spectral region of 450–4000 cm^{-1} using a resolution of 4 cm^{-1} and 64 co-added scans.

2.6. Ultraviolet spectroscopy (UV)

UV spectra were recorded in the region 200–300 nm using a Shimadzu UV spectrometer, model UV-1601. UV spectra of CBCS were obtained from 0.005% (w/v) solutions in 0.01 M HCl (0.01 g in 200 mL). UV spectra of CBBA solutions in HCl were also obtained. CBBA concentration in these solutions varied so as to correspond to the amount of CBBA that would have reacted with 0.01 g CS to produce a derivative with a 10%, 25%, 50%, 75% or 100% DS. Calculations were performed using equations given in acid–base titration.

Table 1
Experimental conditions for preparing *N*-(2-carboxybenzyl)chitosan.

Synthesis	Intermediate imine isolation	Imine formation reaction time [h]	Temp. [°C]	N_2 purge	Coding	
					Imine derivative	Alkylated derivative
1	Yes	1	50	No	CBCSS1	CBCS1
2	Yes	5	50	No	CBCSS2	CBCS2
3	Yes	5	80	Yes	CBCSS3	CBCS3
4	No	5	50	No	–	CBCS4

2.7. Nuclear magnetic resonance spectroscopy (NMR)

^{1}H NMR spectra were obtained using a Bruker spectrometer, model AMX 400, operating at a frequency of 400 MHz for protons. Dimethyl sulfoxide (DMSO) was used as a solvent. The number of scans was 10 and the sweep width was 6 kHz.

2.8. Wide-angle X-ray diffractometry (WAXD)

WAXD study of the samples was performed over the range 2θ from 5° to 60°, at steps of 0.05°. A Philips PW1710 powder diffractometer with Cu Kα nickel-filtered radiation was used.

2.9. Acid–base titration

A 0.1% (w/v) *N*-(2-carboxybenzyl)chitosan solution was prepared into a conical flask by dissolving appropriate amount of *N*-(2-carboxybenzyl)chitosan in 50 mL of distilled water. Two drops of 0.1% (w/v) phenol red solution in ethanol were added into the flask as an indicator and 0.01M NaOH standard solution was added titrimetrically from a burette until the yellow color of the indicator (acid pH) changed into light red (neutral pH). As a blank titration, 50 mL of distilled water was used in place of the *N*-(2-carboxybenzyl)chitosan solution.

The calculations were performed with the assumption that the acidity of the solutions was attributed to the total amount of carboxyl groups and that the amino groups in the pH range of the titration (from slightly acidic to neutral) remained unionized. The volume (mL) of NaOH solution consumed for each titration was converted to moles of carboxyl groups per sample gram. The conversion of the previous value to degree of substitution (DS) (%) was performed using Eq. (1). The degree of polymerization of CBCS assumed to be the same with the degree of polymerization of chitosan, which was calculated using Eq. (2). Molecular weight of *N*-(2-carboxybenzyl)chitosan was calculated using Eq. (3).

$$1\ \text{g of CBCS corresponds to } \frac{\text{DP}\left(\text{DD}/100\right)\left(\text{DS}/100\right)}{M_{\text{CBCS}}} \times \text{mole of carboxyl groups} \tag{1}$$

$$M_{\text{CS}} = \left[\frac{1-\text{DD}}{100} M_{N-\text{acetylglucosamine}} + \frac{\text{DD}}{100} M_{\text{glucosamine}}\right] \text{DP} \tag{2}$$

$$M_{\text{CBCS}} = \left[\frac{1-\text{DD}}{100} M_{N-\text{acetylglucosamine}} + \frac{\text{DD}(1-\text{DS})}{100} M_{\text{glucosamine}} + \frac{\text{DD DS}}{100} M_{N-(2-\text{carboxybenzyl})\text{glucosamine}}\right] \text{DP} \tag{3}$$

where: DP is the degree of polymerization, DD is the % degree of deacetylation of CS and CBCS, DS is the % degree of substitution, *M* is the molecular weight.

2.10. Swelling of the xerogels

Cross-linked chitosan and cross-linked *N*-(2-carboxybenzyl)chitosan samples (0.01 g) were placed into plastic nets which were immersed into aqueous solutions with different pHs. At appropriate time intervals, samples were taken off from the swelling medium and weighed after the removal of the excess water. The swelling ratio (SR) was expressed by the following formula, $\text{SR} = (W_t - W_0)/W_0$, where W_t was the sample's weight at time t and W_0 was the initial xerogel's weight. Swelling studies were carried out in HCl aqueous solutions with pHs 1 and 5, phosphate-buffered solution with pH 7 and NaOH aqueous solutions with pH 9.

2.11. Release studies

A dissolution apparatus II (paddle method) type Distek 2100C was used. CBCSG/fluconazole samples were placed into cellulose dialysis membrane bags with a molecular weight cut-off 12.400, tied up and placed into the dissolution vessels. Dissolution testing was performed at 37 ± 0.5 °C in one stage. Dissolution medium consisted of 500 mL of either phosphate buffer (pH = 7) or citrate buffer (pH = 5). At predetermined time intervals 4 mL of aqueous solution were withdrawn from the release medium using an automatic sampler type Distek Evolution 4300. Samples were filtered by nylon filters (Wattman 0.45 μm) and analyzed by HPLC. The HPLC system (Prominence system of Shimadzu) consisted of degasser DGU-20A5, liquid chromatograph LC-20 AD, auto sampler SIL-20AC, UV/vis detector SPD-20A and column oven CTO-20AC. The column type performed was a NC-04 (250 mm × 4.0 mm), NUCLEOSIL 100-5-CN 5.0 μm. The mobile phase was citrate buffer (pH = 5):acetonitrile (75:25, v/v), and the analyte was detected at 210 nm. The flow rate of the mobile phase was 1 mL/min and the column temperature was 30 °C.

3. Results and discussion

3.1. Synthesis of N-(2-carboxybenzyl)chitosan (CBCS)

N-(2-Carboxybenzyl)chitosan was first synthesized by Muzzarelli et al. (1982) who studied its chelating properties. More recently Lin et al. (2007) synthesized *N*-(2-carboxybenzyl)chitosan which was used to prepare an orally administrated colon-specific drug delivery system. In the present study, in order to evaluate the effect of experimental conditions, such as reaction time and temperature, on the solubility and degree of substitution of the final product, imine derivatives (CBCSS) were prepared under different experimental conditions (Table 1). This is because the production of the imine derivative is the most critical stage in the synthesis of the final product.

The introduction of the carboxyl groups, due to the reaction of chitosan amine groups ($-\text{NH}_2$) with aldehyde groups of 2-carboxybenzaldehyde, leads initially to the formation of an amphoteric polyelectrolyte containing both cationic and anionic charges (Fig. 1). This polyelectrolyte is a Schiff base (imine derivative). In the second stage the purified or unpurified Schiff base was reduced with sodium borohydride to the *N*-(2-carboxybenzyl)chitosan derivatives. At the finally stage, cross-linking of the produced *N*-(2-carboxybenzyl)chitosan derivative with glutaraldehyde leads to the formation of a hydrogel, which could be highly useful as drug delivery system (Gupta, Vermani, & Garg, 2002; Hoare & Kohane, 2008).

Interestingly, while *N*-(2-carboxybenzyl)chitosan derivatives from syntheses 2 and 3 (Table 1) were insoluble over the whole pH range, *N*-(2-carboxybenzyl)chitosan derivatives from syntheses 1 and 4 (Table 1) were soluble in water. Moreover, while we managed to dissolve the imine derivatives from syntheses 2 and 3 (CBCSS2&3) in DMSO, this was impossible to accomplish for the alkylated derivatives (CBCS2&3). The reduced solubility of CBCSS2&3 and CBCS2&3 can be explained by considering the probable inter- and/or intra-molecular hydrogen bonding or ammonium salt formation between amino and carboxyl groups (Muzzarelli, Ilari, & Petrarulo, 1994). The latter assumption is supported by the fact that the derivatives from synthesis 1, which had been carried out in reduced reaction time resulting to smaller degree of substitution, had relatively increased solubility. The reason that solubility of the derivatives from synthesis 2 is reduced while solubility of the derivative from synthesis 4 is not, may be that in the latter case bi-substituted derivatives were formed due to

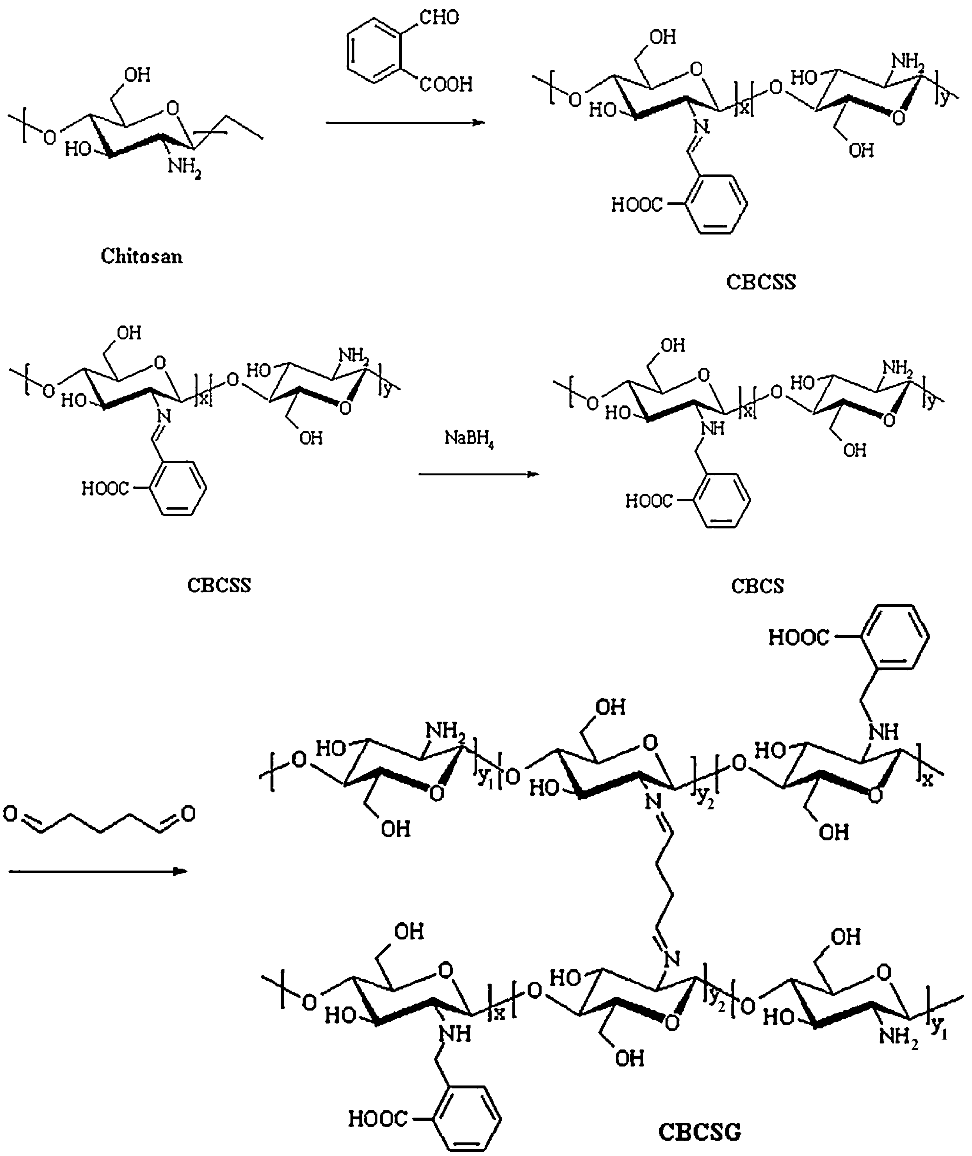

Fig. 1. Synthetic scheme for *N*-(2-carboxybenzyl)chitosan (CBCS) and cross-linked *N*-(2-carboxybenzyl)chitosan (CBCSG).

the longer contact period of chitosan with CBBA. In this case, CBCS's secondary nucleophile nitrogen can attack another CBBA molecule and lead to the formation of a bi-substituted product. Bi-substitution of chitosan after in situ reduction has been confirmed in the past with ^{1}H NMR studies on chitosan's reaction products with formaldehyde (Muzzarelli & Tanfani, 1985) and glyoxylic acid (Muzzarelli et al., 1994). This was the reason we decided to isolate and purify the imine intermediate before reducing it.

From the above study it is concluded that soluble derivatives can be prepared only using the experimental conditions of syntheses 1 and 4 (Table 1). The intermediate product of synthesis 1 was isolated while in synthesis 4 was not. The temperature in both the cases was the same (50 °C) but the reaction time was different. Since the produced materials in both synthetic ways seem to be similar it was decided to use the synthesis 4 avoiding the isolation of the intermediate product and its treatment. It seems that using higher reaction temperatures or reaction times non-soluble products can be prepared, which is undesirable for further handling.

3.2. Polymer characterization

The FTIR spectra of pure CS, CBBA, CBCSS, *N*-(2-carboxybenzyl)chitosan (CBCS) and cross-linked CBCS from synthesis 4 (CBCSG) are presented in Fig. 2. In the chitosan spectrum, the peaks at 3418 and 3271 cm^{-1} are attributed to the –OH and –NH$_2$ groups, respectively, whereas other characteristic bands are that of >N–H group (in-plane bending) which are recorded at 1562 cm^{-1}. The absorption band at 1153 cm^{-1} was the asymmetric stretching of the C–O–C bridge, while the bands at 1075 and 1033 cm^{-1} assigned to the skeletal vibration of C–O stretching. 2-Carboxybenzaldehyde (CBBA) spectra show two peaks at 1760 and 1740 cm^{-1} due to the two carbonyls of its aldehyde and carboxyl groups respectively. From the prepared CS derivatives in the imine derivative CBCSS spectra showed clearly the peak at 1750 cm^{-1} can be assigned to carbonyl vibrations due to the incorporation of 2-carboxybenzaldehyde into chitosan backbone. Furthermore, new peaks appeared at 1586, 1447, 757 and 705 cm^{-1}. The first two peaks are framework vibrations of the aromatic ring, while the last two are attributed to the aromatic C–H

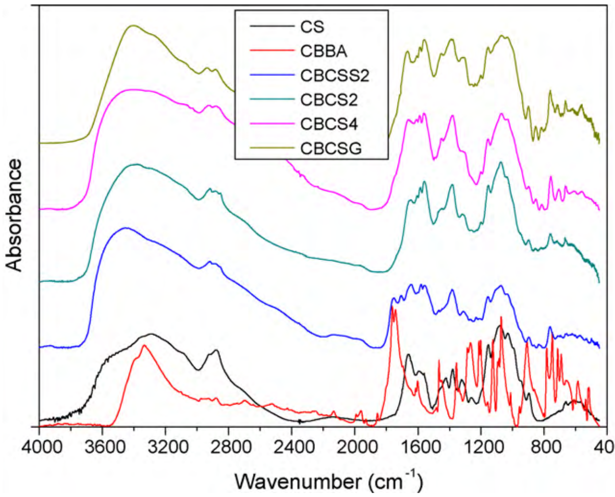

Fig. 2. FTIR spectra of CS, CBBA, CBCSS, CBCS and CBCSG.

bending vibration. The spectra of *N*-(2-carboxybenzyl)chitosan are in all cases identical and thus only the spectrum of synthesis 2 is presented. All *N*-(2-carboxybenzyl)chitosan spectra showed new peaks at 1604 cm^{-1} that can be assigned to $–CO_2^-$ asymmetrical stretching vibrations and a shoulder at 1707 cm^{-1} due to the carboxyl group (Muzzarelli et al., 1982; Lin et al., 2007). Moreover, an increase in absorption at 1660 cm^{-1} is observed in CBCSG and all CBCSS spectra. This is due to imine bond (C=N) vibrations that overlap chitosan's amide I (C=O) absorption band and confirms the cross-linking reaction and the successful formation of the Schiff bases (imines). Finally, peak intensity at 1074 and 1026 cm^{-1} was measured and the A_{1026}/A_{1074} ratio was calculated for CS and for all derivatives. These peaks are attributed to bending vibrations of the C–O bond in secondary and primary hydroxyl groups of the polysaccharide, respectively. The fact that the A_{1026}/A_{1074} ratio remained virtually unaffected (0.92 ± 0.02) means that CS is alkylated at the amino group and not at the primary hydroxyl group (Chen et al., 2004).

The imide (–N=CH–) group of the Schiff base could not be detected in the IR spectra (Fig. 2). In order to characterize the CBCSS1,2&3 derivatives ^{1}H NMR spectroscopy was also employed. The ^{1}H NMR spectra for CBCSS1,2&3 were identical. Thus, the spectrum of CBCSS2 is only presented in Fig. 3 and assignments of the ^{1}H NMR spectra are presented in Table 2. The appearance of the characteristic multiple peak of aromatic protons (7.6–7.8 ppm) confirmed the successful introduction of the 2-carboxybenzyl group to CS backbone. It is also interesting that where the peak for the protons of the acetyl group was expected (~2 ppm) three peaks emerged at 1.9, 2.0 and 2.1 ppm. This suggests that three different groups of acetyl protons are present, each one of them with a different chemical microenvironment, probably due to participation of these groups in the formation of different hydrogen bonds. Finally, the imine proton (H_7) gives a peak at 10.2 ppm (dos Santos, Dockal, & Cavalheiro, 2005) which confirms the formation of the Schiff base intermediate during CBCS synthesis.

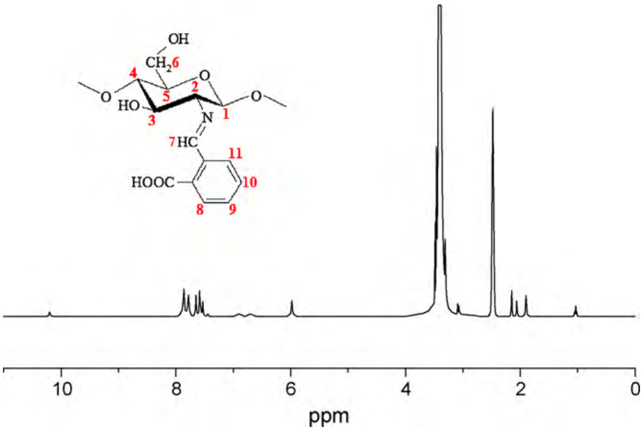

Fig. 3. ^{1}H NMR spectra of CBCSS2 in DMSO and proton numbering.

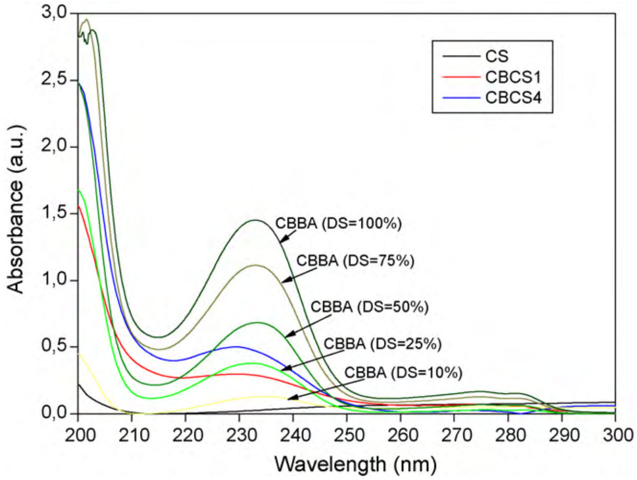

Fig. 4. UV spectra of CS, CBCS and CBBA in concentrations corresponding to various DS.

3.3. Degree of substitution

The UV spectra of CS, CBCS1, CBCS4 and CBBA in 0.01 HCl solutions are presented in Fig. 4. UV spectra of alkyl derivatives from synthesis 2 and 3 were not recorded due to reduced solubility. Neat CS showed no absorption at the examined ultraviolet region 200–300 nm. On the contrary, CBBA presents three absorption maxima for λ_{max} = 233, 275 and 282 nm. The primary λ_{max} is at 233 nm and corresponds to a $\pi \rightarrow \pi^*$ transition of the conjugated π electrons of CBBA, while the absorption maxima at 275 and 282 nm corresponds to $n \rightarrow \pi^*$ transitions. The $\pi \rightarrow \pi^*$ transition is also visible in case of CBCS1 and CBCS4 at 230 nm, indicating the incorporation of 2-carboxybenzaldehyde into chitosan backbone in agreement with the FTIR spectra (Fig. 2). The observed blue shift (3 nm) is probably due to reduction of the conjugated electrons from 10 in the case of CBBA to 8 in the case of CBCS. From these spectra and the absorption at 230 nm the amount of incorporated 2-carboxybenzaldehyde into chitosan could be calculated.

Table 2
Assignments of the ^{1}H NMR spectra signals.

Sample	Chemical shift (δ) [ppm]						
	H_1	H_2	$H_{3–6}$	H_7	$H_{8–11}$	H_{Ac}	H_{DMSO}
CBCSS2&3	6	3.1	3.3–3.6	10.2	7.6–7.8	1.9, 2.0, 2.1	2.5

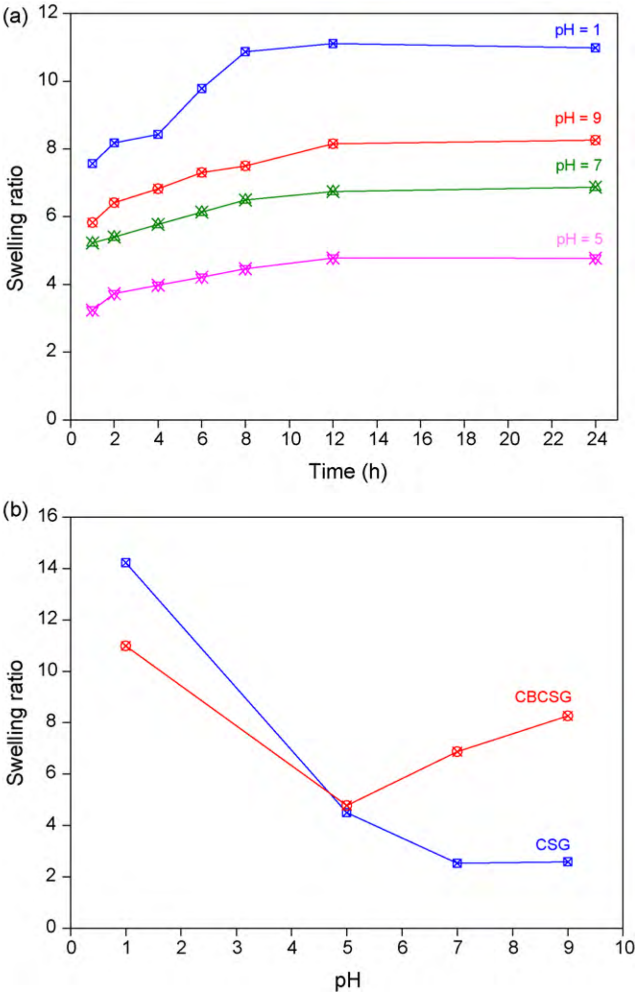

Fig. 5. (a) Swelling ratio (SR) of CBCSG1 at different pH versus time and (b) SR of CBCSG and CSG after 12 h in aqueous solutions of different pHs.

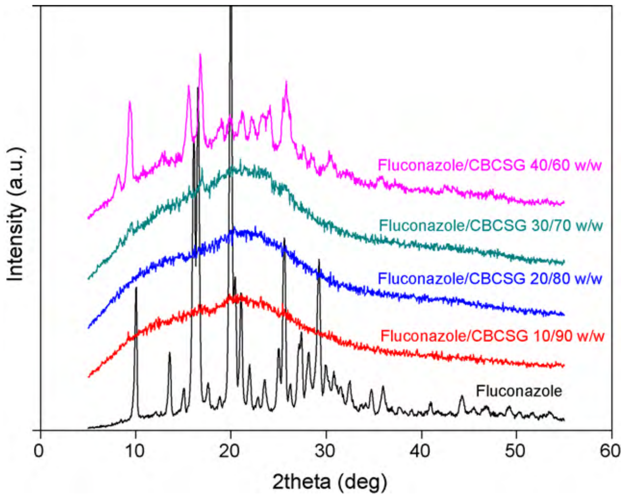

Fig. 6. WAXD patterns for fluconazole and CBCSG loaded with different concentrations of fluconazole.

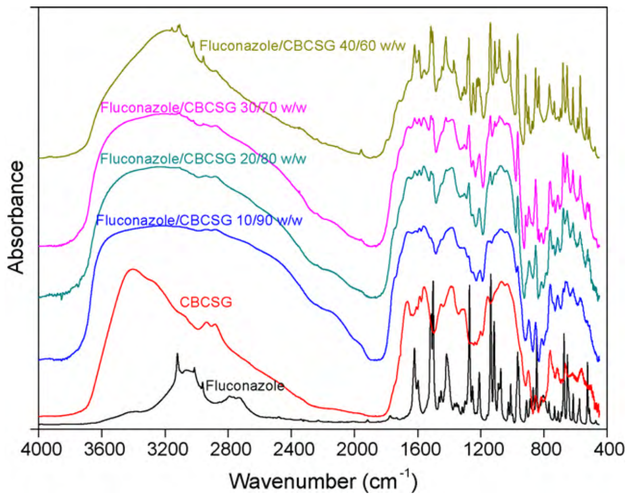

Fig. 7. FTIR spectra of fluconazole and CBCSG loaded with different amounts of fluconazole.

From the UV spectra of CBBA (Fig. 4) the degree of substitution corresponding to each CBBA solution versus the solutions' absorbance at 233 nm was recorded. This dependence was found to be described by the equation: DS (%) = $69.962A_{233} + 0.9493$ ($R^2 = 0.9977$). By replacing CBCS's absorbance at 230 nm in the equation, and by making the assumption that the molar extinction coefficients, ε, of CBBA and CBCS are not significantly different, the DS (%) for CBCS1&4 were calculated and are presented in Table 3. As can be seen, the degree of substitution is higher for CBCS4 compared to CBCS1, since the reaction time during CBCSS4 (intermediate imine derivative) was higher (Table 1) and a higher number of chitosan amino groups could react with aldehyde groups of 2-carboxybenzaldehyde reagent.

Similar are the DS results obtained from the titration analysis (Table 3). In this case, the volume (mL) of NaOH solution consumed in acid–base titration was converted to moles of carboxyl groups per sample gram and to DS (%), using Eq. (1) (Section 2.9). With both derivatives, higher DS values were obtained with the UV spectroscopy than with the titration method. The observed differences in the DS (%) values obtained with the two techniques is probably attributed to partially amino group ionization during titration or/and to hyperchromic effect in UV spectra.

3.4. Swelling of the hydrogels

In the following studies CBCS1 which is better characterized and well purified, compared with CBCS4, and its cross-linked derivative CBCSG1 was used. The swelling behavior of CBCSG1, as shown in Fig. 5, demonstrates that the prepared material is pH sensitive. Furthermore, as can be seen CBCSG hydrogel practically reached swelling equilibrium after 12 h (Fig. 5a). CBCSG exhibited the highest swelling ratio (SR) at pH = 1. This means that amino groups,

Table 3
DS of CBCS as calculated from UV spectrometry and acid–base titration.

Sample	UV spectroscopy		Acid–base titration		
	A_{230}	DS (%)	NaOH (mL)	DS (mol/g)	DS (%)
Blank			0.3		
CBCS1	0.29	21	4.2	7.8×10^{-4}	17
CBCS4	0.49	35	5.95	1.13×10^{-3}	26

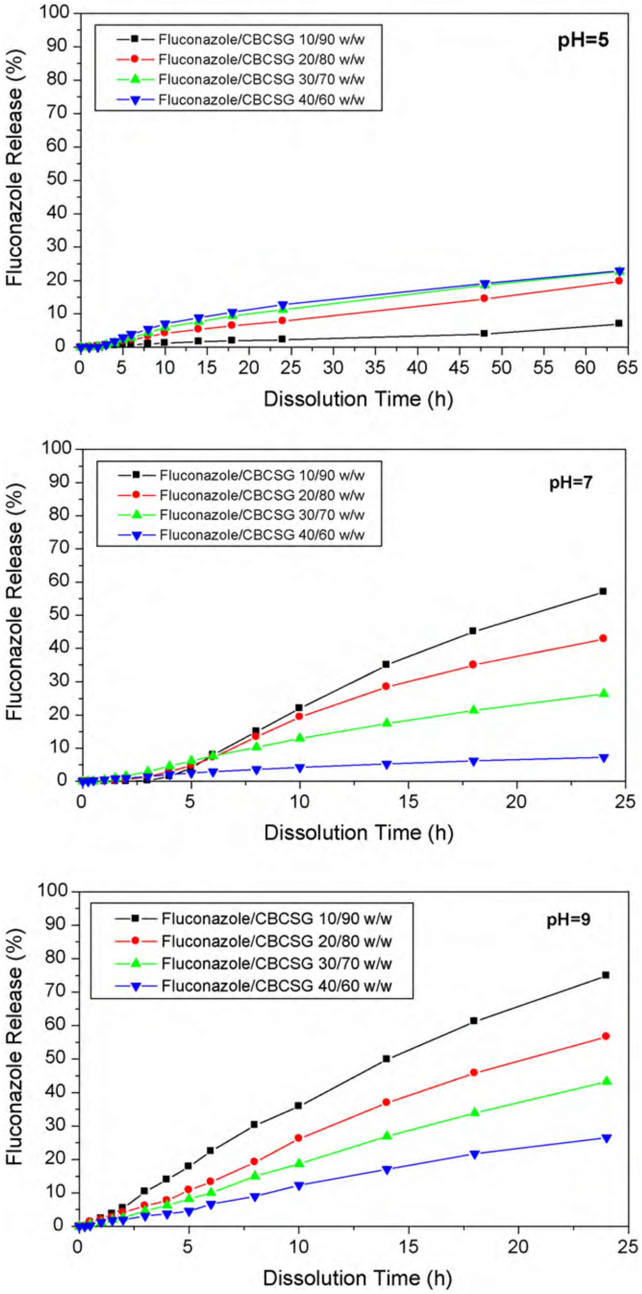

Fig. 8. Fluconazole release from CBCSG in pH = 5, pH = 7 and pH = 9 buffered solutions.

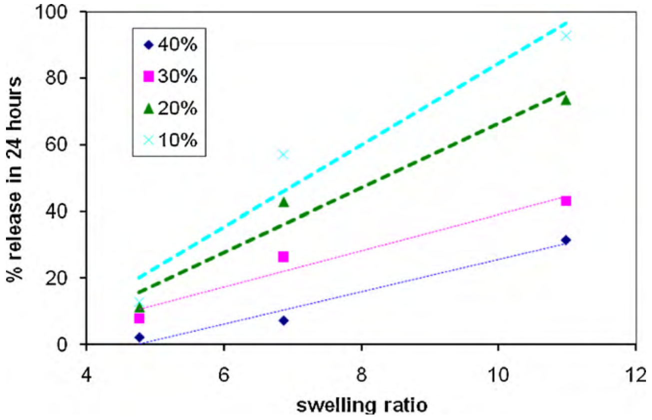

Fig. 9. Rate of fluconazole release (expressed as % release in 24 h) versus the swelling ratio of the gel at the respective pH of the release medium where the release rate was measured.

which are responsible for CBCSG swelling in this pH, still exist in significant numbers. SR of CBCSG reached its lower value at pH 5 while in neutral and alkaline solutions, is increased again due to the ionization of the carboxyl groups of CBCSG. Cross-linked chitosan (CSG) lacks carboxyl groups and its SR decreases continuously as the pH increases from 1 to 9 (Fig. 5b).

3.5. *Characterization of drug loaded formulations*

The solid state of fluconazole/CBCSG formulations (solid dispersions of fluconazole in CBCSG xerogel) was studied by WAXD.As can be seen in Fig. 6 the formulation with 40% (w/w) drug content showed some crystalline peaks indicating that an over-saturated dispersion of fluconazole was prepared. Furthermore, most of these peaks were located at different positions compared to those of the as-received fluconazole sample, indicating that a second polymorph of the drug has been formed (Papageorgiou et al., 2008). Responsible for the modification of fluconazole crystals should probably be the solvent (ethanol) used for the preparation of the solid dispersions. It is common for drugs to crystallize in different crystal forms depending on the conditions of crystallization (Papadimitriou & Bikiaris, 2009; Papadimitriou, Bikiaris, Avgoustakis, Karavas, & Georgarakis, 2008; Papageorgiou et al., 2006). On the other hand, solid dispersions with 20% (w/w) drug content or less showed no crystalline reflections indicating that the drug in these dispersions was amorphous. Amorphization of the drug was probably due to the extensive hydrogen bonding of the drug with the CBCSG (Karavas, Georgarakis, Docoslis, & Bikiaris, 2007). This is supported by the FTIR spectra of the solid dispersions (Fig. 7). The broad absorption band at ~3400 cm^{-1} in CBCSG's spectra is a result of the superimposition of the O–H stretching vibration, the N–H extension vibration and the hydrogen bond vibrations of the polysaccharide. The fact that the band changes both shape and wavenumber indicates the formation of hydrogen bonds between fluconazole and CBCSG.

3.6. *Drug release*

Hydrogels are three-dimensional, hydrophilic, polymeric networks capable of imbibing large amounts of water or biological fluids. The networks are composed of homopolymers or copolymers, and are insoluble due to the presence of chemical cross-links (tie-points, junctions), or physical cross-links, such as entanglements or crystallites (Berger et al., 2004; Langer & Peppas, 2003; Peppas, Bures, Leobandung, & Ichikawa, 2000). The swelling properties of hydrogels exhibiting pH-dependent swelling behavior relate to the formation of ionic networks. These ionic networks contain either acidic or basic pendant groups. In aqueous media of appropriate pH and ionic strength, the pendant groups can ionize, developing charges on the gel. As a result of the electrostatic repulsions, the uptake of solvent in the network is increased. There are many advantages of using ionic over neutral networks in drug delivery. Their characteristics can be exploited for applications in a wide variety of biomedical applications, such as dental adhesives and restorations, controlled-release devices, prodrugs and adjuvants, and biocompatible materials.

The rate of fluconazole release from the CBCSG gels is effectively sustained, indicating the suitability of the CBCSG to be used as a controlled-release system of fluconazole (Fig. 8).

At all three pHs tested, the release rate of fluconazole from the CBCSG gels decreases with increasing drug loading (drug weight proportion) (Fig. 8). This unexpected behavior can be attributed to the solid-state properties of the drug dispersion in the gel. As the WAXD spectra indicated (Fig. 6), at low drug loadings (10–20%, w/w drug) fluconazole is in the more rapidly dissolving amorphous state whereas at high drug loadings (30–40%, w/w drug) it is in the less rapidly dissolving crystalline state. Comparing the release profiles at the different pHs, one can easily see that for the same drug loading the release rate of fluconazole increases when the pH of the release medium increases from pH = 5 to pH = 9 (Fig. 8). It would appear that the rate of fluconazole release from the CBCSG gels depends on the swelling capacity of the gel at the particular release medium used. Indeed, an almost linear increase of release rate (expressed as % release at 24 h) is observed when the release rate is plotted against the SR of the gel at the respective pH where the release rate was measured (Fig. 9).

4. Conclusions

N-(2-Carboxybenzyl)chitosan (CBCS) polyelectrolyte was synthesized via a Schiff reaction of chitosan with 2-carboxybenzaldehyde (CBBA), followed by reduction of the imine derivative (CBCSS) with sodium borohydride (SB). Reaction time and temperatures were found to be critical for the solubility of *N*-(2-carboxybenzyl)chitosan polyelectrolyte. High reaction temperatures or reaction times lead to insoluble *N*-(2-carboxybenzyl)chitosan, while low temperature (50 °C) and reaction time 5 h without the isolation of the imine intermediate seems to be the most ideal conditions. Cross-linked *N*-(2-carboxybenzyl)chitosan with glutaraldehyde (GA) is a pH-sensitive hydrogel (CBCSG) which can be loaded with drug and applied as a pH-sensitive controlled drug delivery system. Fluconazole release from this hydrogel depends on drug content, since at higher loading the release rate decreases. Release rate is also affected by the pH of the release medium as it affects the swelling of the hydrogel. Thus, the lowest fluconazole release rate was observed at pH 5 where the swelling ratio of the hydrogel was the lowest and the highest release rate at pH 9 where the swelling ratio was the highest.

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