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Novel drug delivery systems: Chitosan conjugates covalently attached to steroids with potential anticancer and agrochemical activity

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ABSTRACT

Chitosan (CS) was linked to diosgenin monoesters with potential anticancer and agrochemical activity employing several methods (i.e. solid phase microwave assisted synthesis with carbodiimides activation, acyl chloride approach and homogeneous reaction with carbodiimides activation in aqueous solution). The steroid contents found by elemental analysis were between 5 and 33% (w/w) and was dependent on the nature of the employed linker and the steroid–CS conjugates preparation method. Linking was confirmed by FTIR spectroscopy. Differential scanning calorimetry studies are presented. In vitro release studies performed in water at different pH indicated a drug release dependence on the dicarboxylic acid employed as linker, the steroid content and the acidity of the solution. The obtained conjugates can be potentially applied as films, microspheres or gels for delivery of plant growth regulators in agriculture and anticancer drugs.

1. Introduction

Chitosan (CS) is a cationic linear polysaccharide composed essentially of $\beta(1 \rightarrow 4)$ linked glucosamine units, with some proportion of N-acetylglucosamine units. CS is mainly obtained by extensive deacetylation of chitin present in the shells of crustaceans and molluscs, the cell walls of fungi and the cuticle of insects (Muzzarelli, 1997). Chitosan is a biocompatible, biodegradable, nontoxic and mucoadhesive polymer widely used as delivery matrix for controlled release of drugs in humans and animals, and non-steroidal agrochemicals in agriculture (Acosta, Heras, & Paños, 2008; Aminabhavi, Patil, Rokhade, & Shelke, 2007; Dunn et al., 1990; Keshavayya, Kulkarni, & Kulkarni, 2007; Minami & Shigemasa, 1995; Rinaudo, 2006; Shtilman et al., 2006; Shtilman & Tsatsakis, 1993). The antifungal activity of chitosan (Hirano & Nagao, 1989) and the reported ability to induce metabolic changes in plants allows it to increase the yield of crops, increasing the germination of seeds and resistance against plagues (Fristenski, Hadwiger, & Riggleman, 1984; Struszczyk, Pospieszny, & Kotlinski, 1989). Its employment has motivated numerous studies about the influence of CS acetylation degree on its fungicide activity, chemical properties and applications (Chatelet, Damour, & Domard, 2001; Einarsson et al., 2007; Gümüsderelioglu, Karakecili, & Seda, 2007). CS has been cross-linked with several substances for improving control in drug delivery (Acosta et al., 2008; Bumgardner et al., 2007; Keshavayya et al., 2007). The linkage of active compounds to chitosan (i.e. chitosan-peptides, CS-anti-inflammatory drugs) has been attracting more interest the last years as a useful way to create new formulations with better properties, biocompatibility and applications (Cheng et al., 2008; Ho et al., 2005; Jang and Nah, 2003).

Diosgenin (Liu, Ju, Wang, Wong, & Wu, 2005), dioscin (a natural saponin obtained from diosgenin) (Tao and Yu, 2002) and some synthetic saponins obtained from it exhibit antitumor, antibiotic and antifungal activity (Ando et al., 2000; Bhattarai et al., 2006; Chadwell, Ding, Orsak, Savage, & Taotofa, 2004; Dong, Hu, Iwasaki, Kobayashi, & Yao, 1996). Brassinosteroids are plant growth enhancers used as agrochemicals (Agüero, Alonso, Bernardo, Coll, & Pérez, 2006; Alonso, Cabrera, Coll, Jomarrón, & Robaina, 1995; Asami et al., 2009; Bhardwaj, Khurma, Ohri, & Sohal, 2007; Dinan, Voigt, & Whiting, 2001) with spirostanic or furostanic structures which are usually obtained from diosgenin by modification in the A and B steroidal nucleus (Agüero et al., 2006; Alonso, Cabrera, Coll, Jomarrón, & Robaina, 1995; Iglesias-Arteaga et al., 2005). Their benefits as agrochemicals and their anticancer activity are not fully expressed because they are quickly metabolised and the hydrophobic character limits their applications.

The preparation of CS–steroid conjugates will allow achieving the controlled delivery of these steroids combining the well documented effects of brassinosteroids on plants and animals with the above mentioned biological properties of chitosan

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(Hewajulige, Sivakumar, Sultanbawa, Wijesundera, & Wilson-Wijeratnam, 2007).

This article reports on the linking of dicarboxylic diosgenin monoesters (basic skeleton of commercial brassinosteroids with agrochemical activity) with chitosan to achieve their controlled release.

2. Experimental

2.1. Materials

Chitosan (deacetylation degree, DD = 85.2% determined by ^{1}H-NMR, $M_v = 2.55 \times 10^5$) was obtained by extensive deacetylation of chitin isolated from shells of common lobster (*Panulirus argus*) at the Center of Biomaterials of the University of Havana, Cuba. Diosgenin monoesters were synthesized by acid-catalyzed microwave assisted reaction of diosgenin with succinic, maleic and itaconic anhydrides, respectively (Martínez-García and Martínez, 2008; Coll, Curiel, Peniche, & Pérez, 2010). The solvents and reagents employed were purchased from Sigma–Aldrich and used without further purification.

The structures of diosgenin monoesters: diosgenin monosuccinate (MSD), diosgenin monoitaconate (MID), diosgenin monomaleate (MMD) and chitosan are shown in Fig. 1.

2.2. CS–steroid conjugates preparation

2.2.1. Solid phase microwave assisted synthesis of chitosan–steroid conjugates activated by carbodiimides

100 mg (0.51 mmol) of chitosan were mixed in a mortar with 150 mg (c.a. 0.3 mmol) of diosgenin monoesters (MSD, MID and MMD, respectively) and then 80 mg (0.39 mmol) of N,N′-dicyclohexilcarbodiimide (DCC) or 0.1 ml (0.64 mmol) of N,N′-diisopropylcarbodiimide (DIC) were added and grounded (CS/Monoester/DCC or CS/Monoester/DIC molar ratios were 5/3/4 and 8/5/10, respectively). Some drops of N,N-dimethylformamide (DMF) were added with mixing for ensuring efficient transference of microwave radiation into the reaction mixture. The resulting paste was irradiated for 3–6 min with a potency of 150 W and irradiation periods of 30 s. The reaction was stopped when TLC chromatography showed steroid exhaustion. The resulting solids were washed several times with methanol, ethyl acetate, diethyl ether in this sequence and dried in vacuum overnight at 50 °C.

2.2.2. Acyl chloride synthesis

The corresponding acyl chlorides of diosgenin monoesters were obtained by refluxing these compounds for half of hour in excess of thionyl chloride (Chu et al., 2005; Pearson and Roush, 1999). Afterwards thionyl chloride was distilled out and the resulting black powder was stored in vacuum.

200 mg (c.a. 0.4 mmol) of MSD, MID or MMD chlorides were dissolved in dry DMF and 100 mg (0.51 mmol) of CS were added. The obtained suspension was stirred for 48 h, filtered out and washed successively with methanol, ethyl acetate and diethyl ether. The powder was left overnight at 50 °C in vacuum.

The coupling of the obtained diosgenin acyl chlorides with CS was also performed in toluene using zinc as catalyst.

200 mg (c.a. 0.4 mmol) of the corresponding diosgenin acyl chloride were dissolved in absolute toluene and 100 mg (0.51 mmol) CS and 8 mg (less than 10% vs. acyl chloride) of zinc were added in this order. The resulting suspension was left under magnetic stirring at room temperature for 48 h. The powder was filtered out, washed and dried under vacuum as previously described.

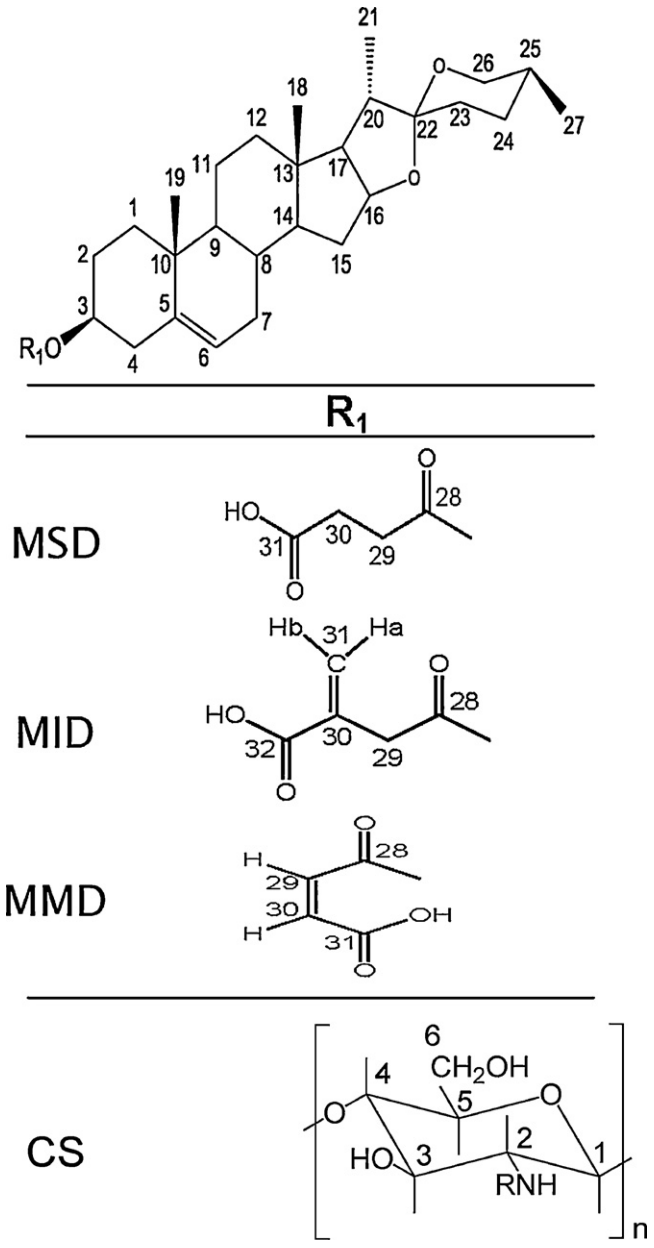

Fig. 1. Structure of diosgenin monoesters (MSD, MID, MMD) and chitosan.

2.2.3. Reaction in aqueous solution activated with 1-ethyl-3-(3′-dimethylamino)carbodiimide hydrochloride (EDC)

100 mg (c.a. 0.2 mmol) of diosgenin monoesters were dissolved in DMF and cooled down to −10 °C. Then 60 mg (0.24 mmol) of EDC were added and stirred 10 min at −5 to −10 °C for activate the free carboxylic group of these monoesters. This solution was dropped into 10 ml of 2% (w/w) chitosan solution in 2% (v/v) aqueous acetic acid and stirred for 24 h at room temperature. The solvents were evaporated, washed and dried as described.

2.3. Characterization

The CS–steroid conjugates were characterized by FTIR spectroscopy using a Perkin-Elmer FTIR spectrophotometer with 32 scans and 4 cm^{-1} resolution. Samples were prepared by the KBr pellet method. Elemental analysis was performed on a Varian MicroCube Analyzer with burning temperature of 1150 °C. The ^{1}H NMR spectra were recorded with a Bruker BioSpin GmbH

Table 1
Steroid content (wt-%) by elemental analysis.

Samples	%		%		%
MSD-CS1	<5	MSD-CS2	<5	MSD-CS3	11
MID-CS1	13	MID-CS2	20	MID-CS3	23
MMD-CS1	7	MMD-CS2	9	MMD-CS3	18
MSD-CS4	8	MSD-CS5	24		
MID-CS4	14	MID-CS5	33		
MMD-CS4	11	MMD-CS5	27		

CS1: diosgenin–chitosan conjugates obtained by microwave reaction with DCC.
CS2: diosgenin–chitosan conjugates obtained by microwave reaction with DIC.
CS3: diosgenin–chitosan conjugates obtained by acyl chloride/DMF.
CS4: diosgenin–chitosan conjugates obtained by acyl chloride/toluene/Zn.
CS5: diosgenin–chitosan conjugates obtained by EDC in water.

spectrometer operating at 500.13 MHz for proton at 70 °C with concentrations c.a. 30 mg mL^{-1} in d4-acetic acid/d2-water (25% v/v) and analyzed with the TopSpin 2.1 software (Berrada et al., 2003; Hirai, Nakajima, & Odani, 1991). Wide-angle X-ray diffraction (WAXD) analysis of the powered samples was performed using a Rigaku III diffractometer (Rigaku Corp., Japan) with Cu K_α radiation (40 kV, 30 mA, $\lambda = 0.15418$ nm), data collected at a scan rate of 1° min^{-1} with a scan angle from 4 to 50°. Calorimetric curves were obtained with a Perkin-Elmer Differential Scanning Calorimeter Pyris 1 and analyzed with the Pyris 1 software (version 6.0.0.033). DSC studies were conducted using sample weights of approximately 8 mg, under a nitrogen dynamic flow of 20.0 mL min^{-1} and heating-cooling speed of 10 °C min^{-1} (Dockal, Santos, & Soares, 2003). Samples were deposited in aluminium capsules and hermetically sealed. Indium was used to calibrate the instrument. Enthalpy (ΔH in J/g dry weight) and peak temperature were computed automatically. Samples were heated and cooled from −30 to 300 °C. Analytical thin layer chromatography (TLC) was performed using Merck silica gel 60F254 aluminium sheets.

2.4. In vitro drug release studies of steroids

In vitro drug release of diosgenin monoesters linked to CS powder was studied by UV detection of the delivered steroid at various pH. 25 mg of diosgenin–CS linked conjugates were placed in volumetric flask containing buffer solutions (25 mL) at different acidic pH and incubated at 30 °C with constant agitation at 100 rpm. 1 mL of solution was withdrawn of the flask, 30 min after preparation of the suspension and compared with the spectrum of 1.5×10^{-4} mol L^{-1} pure diosgenin monoester. The amount of released steroids was calculated from a previously obtained calibration curve.

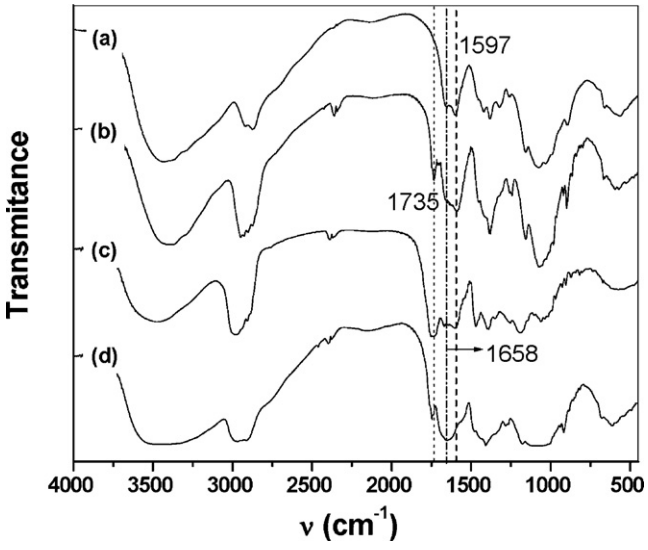

Fig. 2. Infrared spectra of (a) CS, (b) MSD-CS, (c) MID-CS and (d) MMD-CS (see Fig. 1 for names).

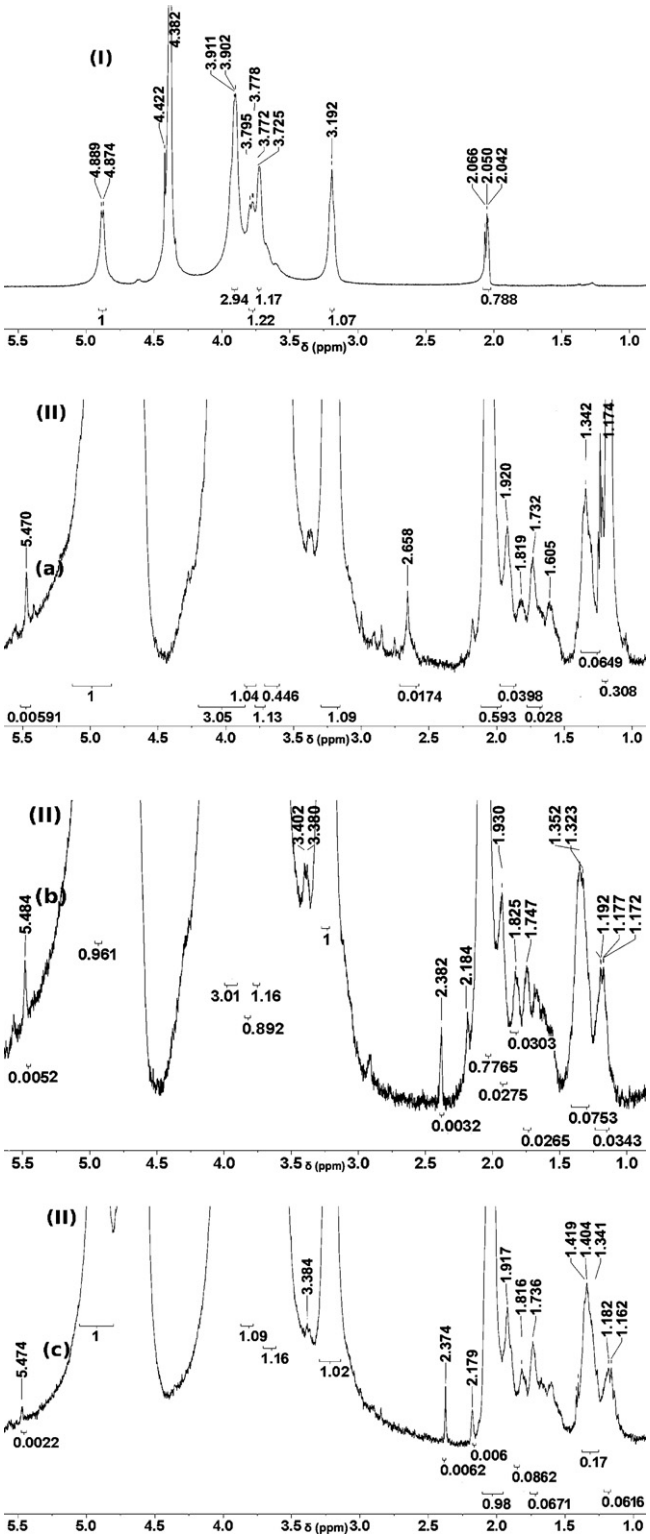

Fig. 3. Proton NMR spectra of (I): CS; (II) (a) MSD-CS, (b) MID-CS and (c) MMD-CS (see Fig. 1 for names).

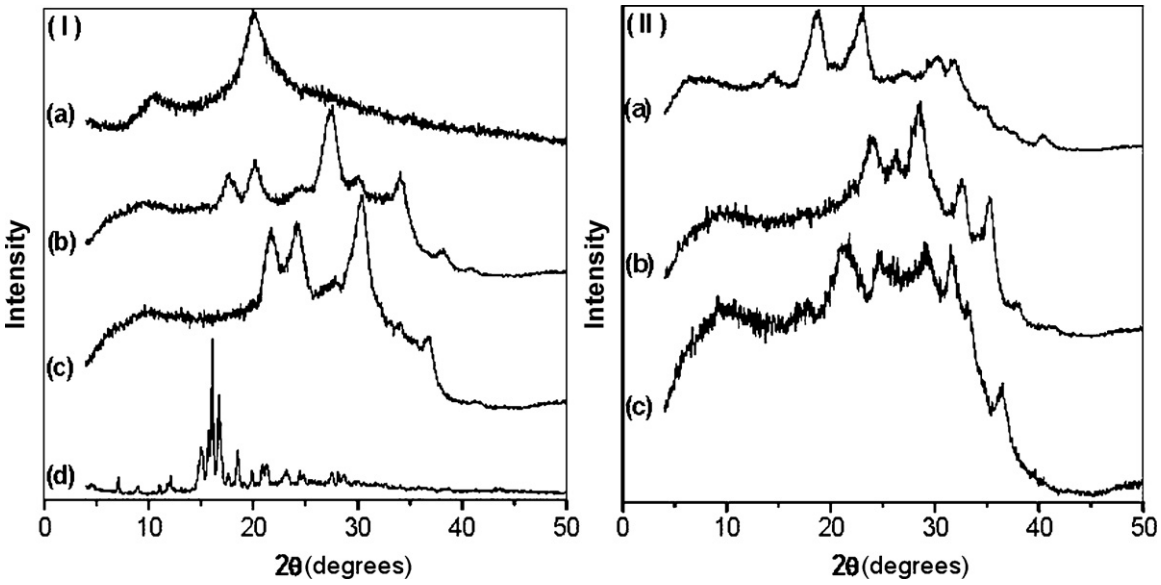

Fig. 4. Wide-angle X-ray diffraction patterns of chitosan, diosgenin monoesters and diosgenin-linked chitosan conjugates. (I): (a) CS, (b) MSD, (c) MID and (d) MMD; (II): (a) MSD-CS, (b) MID-CS and (c) MMD-CS (see Fig. 1 for names).

The release profile of diosgenin monosuccinate was studied by periodically recording the UV absorbance of aliquots at 280 nm and the concentration of the solution was determined as above. These studies were conducted in triplicate for each sample.

3. Results and discussion

3.1. Preparation of CS–steroid conjugates

The different methods employed to prepare the CS–steroid conjugates, afforded steroid contents ranging from 5 to 33 1wt-% (see Table 1). The higher steroids linking were obtained when the reaction was conducted in aqueous solution activated with EDC (CS5). This may be attributed to the homogeneous conditions of the reaction, ensuring a better contact between reactants. In contrast, linking performed in solid phase (CS1: monoester/CS/DCC) afforded the lower steroid content, as expected for an heterogeneous reaction. The general trend of observed diosgenin monoesters reactivity with CS was: MID > MMD > MSD.

3.2. FTIR spectroscopy

The FTIR spectra of CS–diosgenin conjugates obtained by reaction in aqueous solution activated by EDC (CS5) are shown in Fig. 2. The spectrum of CS is also included for comparison.

The IR spectrum of chitosan presented characteristics absorption peaks at 2942–2784 cm^{-1} (aliphatic C–H stretching band), 1658 cm^{-1} (Amide 1) and 1597 cm^{-1} ($-NH_2$) bending and 1321 cm^{-1} (Amide III). The absorption peaks at 1154 cm^{-1} (anti-symmetric stretching of the C–O–C bridge), 1082 and 1032 cm^{-1} (skeletal vibrations involving the C–O stretching) are due to its saccharide structure (Coll et al., 2010).

The spectra of diosgenin–chitosan conjugates are dominated by the intense and broad CS peaks; however the distinctive

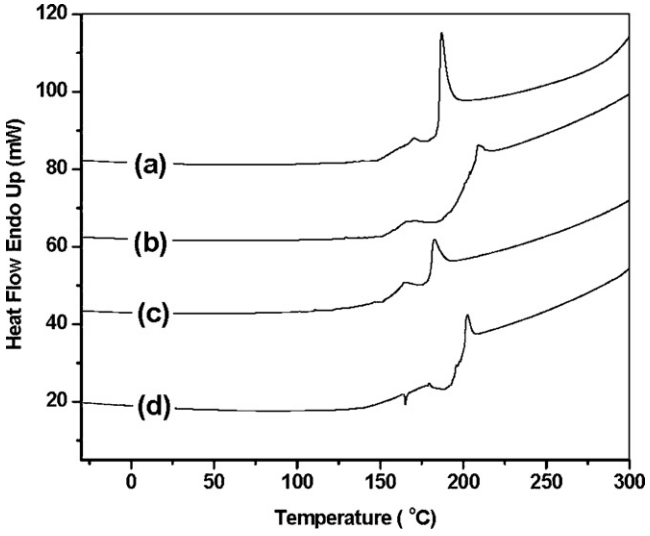

Fig. 5. DSC Curves of chitosan and diosgenin-linked chitosan conjugates: (a) CS, (b) MSD-CS, (c) MID-CS and (d) MMD-CS (see Fig. 1 for names).

Table 2
X-ray peaks of CS, diosgenin monoesters and diosgenin linked chitosan conjugates by WAXD.

Samples	2 θ (degrees)								
CS	10.7	20.0	–	–	–	–	–	–	–
MSD	17.8	20.2	24.7*	27.6	30.1*	34.1	38.2*	40.9*	–
MID	–	21.8	24.2	–	30.5	34.1*	36.7*	–	–
MMD	7.2*	9.0*	11.0*	12.2*	15.0	15.8	16.1	16.8	17.6*
	18.6	19.9*	20.9*	21.3*	23.2*	24.5*	27.5*	28.1*	28.7*
MSD-CS	14.5*	18.9	23.1	30.3	32.0	40.4*	–	–	–
MID-CS	23.7	24.1	26.3	28.5	32.7	35.3	–	–	–
MMD-CS	21.8	24.6	29.0	31.5	33.1*	36.5	–	–	–

* Stand for peaks of low intensity.

C=O peaks of ester linkage are present at 1740–1715 cm^{-1} (see Fig. 2).

These peaks are overlapping the Amide I and $-NH_2$ peaks at 1658 and 1597 cm^{-1}, producing a broad band ranging from 1700 to1500 cm^{-1}.

3.3. NMR results

The proton NMR spectra of CS–diosgenin conjugates obtained by reaction in aqueous solution activated by EDC (CS5) are shown in Fig. 3. The placebo CS is also showed for comparison.

The ^1H NMR spectrum of CS showed the characteristics signals of saccharide protons at 2.10 ppm (s, CH_3 of CH_3CO-), 3.18 ppm (s, 1H, H-2), 3.75 ppm (s, 1H, H-5), 3.80 ppm (s, 1H, H-6′) and 3.95 ppm (s, 3H, H-6 + H-4 + H-3) as reported for crab chitosans (Berrada et al., 2003; Hirai et al., 1991).

The proton NMR spectra of chitosan–diosgenin conjugates are dominated by the intense and broad CS signals, but the less intense steroid peaks are visible at great magnifications. These steroid peaks in the conjugates are shifted in comparison with proton NMR of the pure steroid, recorded at room temperature in $CDCl_3$ or DMSO, as result of solvent and temperature effect on chemical shift. However can be attributed the peaks at 1.17–1.18 ppm (d, 3H, J = 10 Hz, H-27), 1.34–1.40 ppm (s + d, 6H, H-21 + H-19), 2.37–2.38 ppm (s, 1H, H-29, H-26 ax) and 5.47–5.48 ppm (s, 1H, H-6).

3.4. X-ray diffraction

The wide-angle X-ray diffraction patterns of CS, diosgenin monoesters and CS–diosgenin conjugates (CS5) are shown in Fig. 4.

CS presented lower crystallinity than the pure diosgenin monoesters, but defined peaks at 2θ 10.7 and 20.0° attributed to the $(0\,2\,0)_h$ planes of the hydrated crystalline structure and to the contributions of peaks around 20°, related to reflections of the hydrated polymorph, respectively (David et al., 2010). These defined peaks are also observed in chitosans from crab shells (Mau, Yang, & Yen, 2009).

The pure diosgenin monoesters presented several intense peaks ranging from 2θ 15.0 to 34.1° (see Table 2).

The linked CS–diosgenin conjugates present intense and broad peaks between 18.9 and 36.5°. The absence of intense peaks at 10–11° and 20° attributed to CS are indicatives of CS lack as crystalline phase. The characteristic peaks of pure steroids were also absent. Instead several new peaks were observed, associated to the new crystalline phases of the obtained CS–diosgenin conjugates.

3.5. Differential scanning calorimetry

The thermal behaviour of CS samples has been shown to be dependent on the natural source (Dockal et al., 2003), the purity of samples and the preparation conditions (Mau et al., 2009).However, the main thermal effects presented by a chitosan from a particular source can be interpreted on the basis of the behaviour of CS from other sources.

The DSC curves of CS placebo and the linked CS–diosgenin conjugates (CS5), under nitrogen in the temperature range from −30 to 300 °C are shown in Fig. 5. The DSC of CS placebo (Fig. 5(a)) showed two endothermic peaks at 170.4 and 187.0 °C, respectively. Their onset and completion temperatures are listed in Table 3, together with their associated peak enthalpy (ΔH). The total ΔH of these effects is 124.1 J/g. Similar endothermic effects have been reported for crab chitosans (Coll, Curiel, Peniche, & Pérez, 2010). In contrast, DSC of linked CS–diosgenin conjugates (CS5) (Fig. 5(b)–(d)) present intense endothermic peaks between 164 and 210 °C (see Table 3) with associated ΔH of 29–197 J/g.

Table 3
Thermal properties of chitosan and main endothermal effects linked CS-diosgenin conjugates.

Samples	Onset	Endotherm (°C)		
		Peak	Completion	ΔH (J/g)
CS	167.2	170.4	174.1	3.5
	181.5	187.0	197.8	120.6
CS-MSD	153.1	165.3	179.4	45.8
	192.3	208.9	219.8	156.3
CS-MID	154.1	164.3	171.1	29.0
	179.1	182.4	193.2	116.8
CS-MMD	193.4	202.4	209.7	196.8

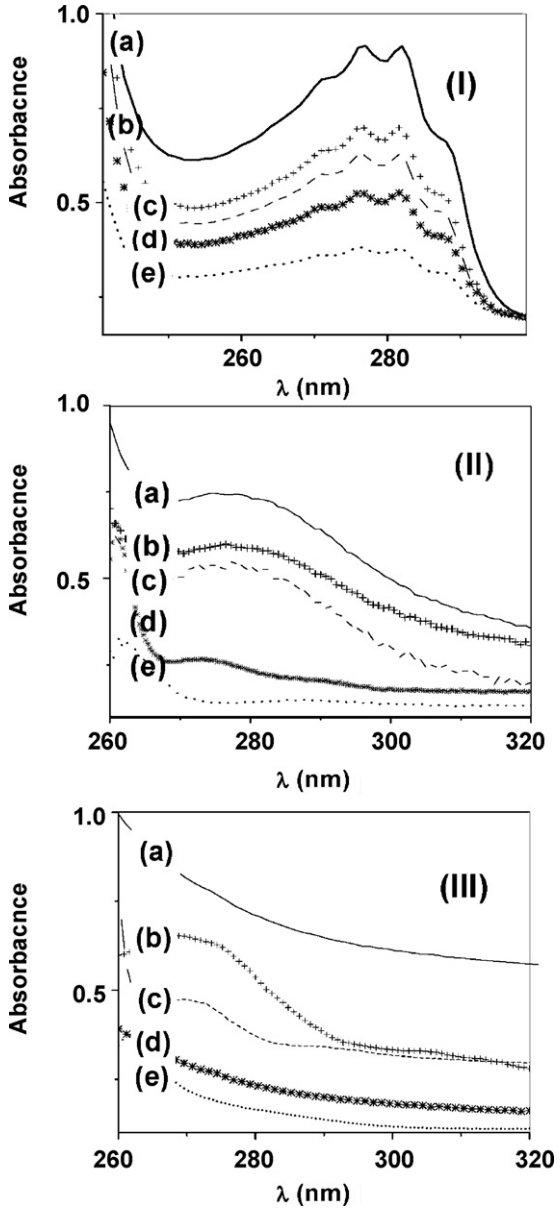

Fig. 6. In vitro UV spectra at 30 °C of (I) MSD-CS at (a) MSD, (b) pH 2, (c) pH 4, (d) pH 5, (e) pH 6.5; (II) MID-CS at (a) MID, (b) pH 2, (c) pH 4, (d) pH 5, (e) pH 6.5; (III) MMD-CS at (a) MMD, (b) pH 2, (c) pH 4, (d) pH 5, (e) pH 6.5 (see Fig. 1 for names).

3.6. Release studies at different pH

The UV absorption spectra of linked diosgenin–CS conjugates obtained by reaction in aqueous solution and activation with EDC (CS5) at different pH are presented in Fig. 6.

Table 4
% Steroid released at 30 °C in buffer solutions after 30 min.

Sample	% (pH 6.5)	% (pH 5)	% (pH 4)	% (pH 2)
MSD-CS	8	13	16	21
MID-CS	<5	6	10	14
MMD-CS	5	10	12	16

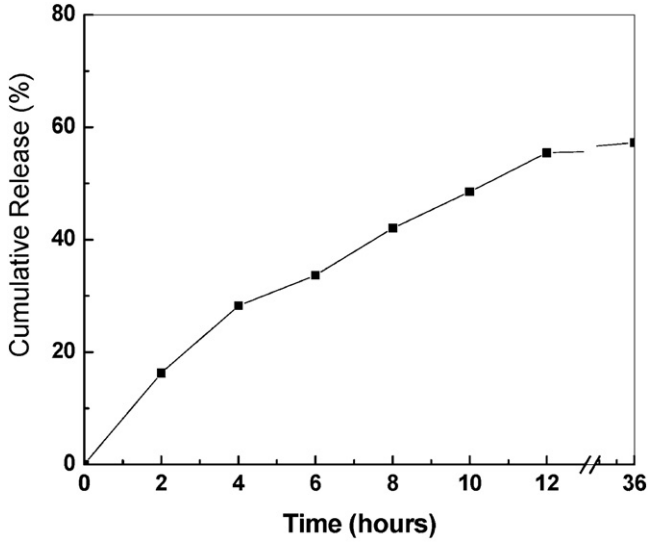

Fig. 7. In vitro release profile at 30 °C of diosgenin monosuccinate linked to chitosan in buffer solution (pH = 6.0) (see Fig. 1 for names).

The steroid releases (Fig. 6) are mainly pH dependent and controlled by the hydrolysis of the ester linkage between the steroid and chitosan.

The calculated steroid released (in weight per cent) after 30 min at different pH are shown in Table 4. The steroid released increases with the solution acidity for the studied compounds. Diosgenin monosuccinate was the fastest released, steroid while the diosgenin monoitaconate was delivery slowly.

The release profile of diosgenin monosuccinate at 30 °C in buffer solution (pH = 6.0), expressed as per cent cumulative release against time for linked CS–diosgenin monosuccinate is presented in Fig. 7.

Diosgenin monosuccinate presented a sustained release with almost constant release rate (zero order kinetics) during the first 12 h. The release was not quantitative, reaching roughly a cumulative release of 60% after 36 h. However the unreleased steroid linked to chitosan could be available after enzymatic degradation.

4. Conclusions

Chitosan was successfully linked to diosgenin monoesters as confirmed by different analytical techniques. Steroid contents were between 5 and 33 wt-% and showed dependence on the employed carboxylic linker and the linking method (homogeneous or heterogeneous conditions). The linking process was more efficient when reaction was conducted in homogeneous conditions (CS5). The UV spectra recorded in water at different acidic pH indicated a drug release dependence on the acidity of the solution, the dicarboxylic acid employed as linker and the steroid content. Sustained release profile was obtained for diosgenin monosuccinate linked to diosgenin. These results indicate that by introducing the appropriate changes in the steroid structure and linking to chitosan it would be possible to design efficient pH dependent delivery systems for the sustained release of steroid based agrochemicals and anticancer drugs.

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