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N,O6-partially acetylated chitosan nanoparticles hydrophobically-modified for controlled release of steroids and vitamin E

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ABSTRACT

Diosgenin, two synthetic analogs of brassinosteroids, testosterone and DL-α-tocopherol were covalently linked to synthetic water-soluble N,O6-partially acetylated chitosan, for their controlled release. Drug linking was confirmed by FTIR spectroscopy and proton NMR. Conjugates were also characterized by differential scanning calorimetry and wide-angle X-ray diffraction. These conjugates formed self-assembled nanoparticles in aqueous solution with particle sizes ranging from 197 to 358 nm and drug contents between 11.8 and 56.4% (w/w). Spherical 30–60 nm nanoparticles were observed by scanning electron microscopy and transmission electron microscopy upon drying. In vitro release studies performed at acid pH indicated a drug release dependence on substitution degree and particle sizes. Almost constant release rates were observed during the first 6–8 h. Brassinosteroids-modified nanoparticles showed good agrochemical activity in radish seeds bioassay at 10^{-1} to 10^{-4} mg mL^{-1}. Tocopheryl-modified nanoparticles exhibited radical scavenging activity in DPPH test.

1. Introduction

Chitosan, a natural polymer made of β(1 → 4) linked glucosamine units partially acetylated, is a biomaterial with mucoadhesive, microbicidal, antifungal, antioxidant and plant stimulatory properties (Kean & Thanou, 2010; Muzzarelli, Boudrant, Meyer, Manno, De Marchis, & Paoletti, 2012; Muzzarelli, 2009; Raafat & Sahl, 2009; Ravi Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). These biological properties have led to the development of several drug release formulations containing chitosan. However, many chitosan-based self-aggregates precipitate within a few days in biological solution (pH 7.4) (Jing-Mou, Li-Yan, Yi, & Yong-Jie, 2008). On the other hand, N,O6-partially acetylated chitosan, a derivative prepared by acetylation of chitosan, possess a good water solubility at pH 7.4 and acid conditions while retains microbicidal properties and biodegradability (Sashiwa, Kawasaki, Nakayama, Muraki, Yamamoto, & Aiba, 2002).

We have shown that introducing the appropriate changes in the structure of diosgenin and linking to chitosan it would be possible to prepare pH dependent delivery systems for the controlled release of potential diosgenin-based anticancer drugs and agrochemicals (Quiñones, Szopko, Schmidt, & Covas, 2011). However, these conjugates were not suitable for practical application because they exhibited very low aqueous solubility at physiological conditions (37 °C, pH 7.4).

The present work is devoted to the preparation of novel self-assembled nanoparticles by conjugating different bioactive compounds (diosgenin, a steroidal sapogenin with anticancer properties (Damodaran, Koduru, Kumar, Kyprianou, Srinivasan, & Venguswamy, 2009), two synthetic analogs of brassinosteroids (DI31 and S7) used as agrochemicals (Alonso, Cabrera, Coll, Jomarrón, & Robaina, 1995; Alonso-Becerra, Bernardo-Otero, Coll-Manchado, Guerra-Martínez, Martínez-Massanet, & Pérez-Martínez, 2007), testosterone and DL-a-tocopherol) to N,O6-partially acetylated chitosan, for achieving controlled release of the linked drugs.

Conjugation of the active compounds to the chitosan derivative was assessed by Fourier transform infrared spectroscopy (FTIR) and proton NMR, differential scanning calorimetry and wide-angle X-ray diffraction techniques. The hydrodynamic diameter and zeta potential of particles was determined in aqueous solution, and the shape and size of dried particles were inspected by scanning

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electron microscopy (SEM) and transmission electron microscopy (TEM). In addition, the release profiles of the covalently linked bioactive drugs from N,O6-partially acetylated chitosan nanoparticles at pH 6.0 were obtained. Preliminary agrochemical activity as plant growth stimulator of the brassinosteroids-modified N,O6-partially acetylated chitosan nanoparticles and the radical scavenging capacity of tocopheryl-modified N,O6-partially acetylated chitosan nanoparticles were also investigated.

2. Experimental

2.1. Materials

Steroidal and DL-α-tocopheryl hemisuccinates were synthesized by base-catalyzed traditional esterification in pyridine of commercial diosgenin, synthetic analogs of brassinosteroids DI31 and S7 (kindly provided by the Center of Natural Products, University of Havana), testosterone and DL-α-tocopherol with succinic anhydride, respectively (Abe, Hasunuma, & Kurokawa, 1976). Water soluble N,O6-partially acetylated chitosan (degree of acetylation 53.0 mol%, determined by elemental analysis) was prepared by acetylation of commercial chitosan (acetylation degree, DA = 20%, determined by ^{1}H NMR, $M_w = 4.3 \times 10^5$), dissolved in methanesulfonic acid with 10 equivalents of acetyl chloride (Sashiwa et al., 2002). As reported by Sashiwa et al., by this reaction procedure only partial N and O6 acetylation is obtained. Diosgenin, testosterone, DL-α-tocopherol, reagents and solvents employed were purchased from Sigma–Aldrich and used as received.

The structures of diosgenin hemisuccinyl N,O6-partially acetylated chitosan (I), DI-31 hemisuccinyl N,O6-partially acetylated chitosan (II), S7 hemisuccinyl N,O6-partially acetylated chitosan (III), testosterone hemisuccinyl N,O6-partially acetylated chitosan (IV), DL-α-tocopherol hemisuccinyl N,O6-partially acetylated chitosan (V) conjugates and N,O6-partially acetylated chitosan (VI) are shown in Fig. 1.

2.1.1. Synthesis of steroid-modified N,O6-partially acetylated chitosan and tocopheryl-modified N,O6-partially acetylated chitosan conjugates

Steroidal hemisuccinates and tocopherol hemisuccinate (Fig. 1) were conjugated to synthetic water-soluble N,O6-partially acetylated chitosan by reaction with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide. Briefly, 200 mg (ca. 0.5 mmol) of N,O6-partially acetylated chitosan were dissolved in 8 mL of double-distilled water and diluted with 24 mL of anhydrous ethanol. Then 38 mg (0.20 mmol) of EDC and 23 mg (0.20 mmol) of N-hydroxisuccinimide were added and stirred until solution clearance. 75–90 mg (0.15 mmol) of steroidal hemisuccinates or 80 mg of tocopheryl hemisuccinate were dissolved in 32 mL of ethanol/water solution (85:15, v/v) and slowly added with stirring to the N,O6-partially acetylated chitosan solution. The reaction mixture was stirred 72 h at room temperature, dialyzed against ethanol/water mixture (90:10, 66:33, 50:50 and 0:100, v/v), each one for 2 days with 16 exchanges. The dialyzed solutions were lyophilized affording white, cotton wool-like products.

2.1.2. Preparation of the self-assembled nanoparticles

The synthesized steroid-modified and tocopheryl-modified N,O6-partially acetylated chitosans were able to form nanoparticles on aqueous solution after stirring overnight and probe tip sonication. To this end, the modified chitosan conjugates (ca. 0.5–2.0 mg mL^{-1}) were stirred overnight at 100 rpm on double-distilled water or phosphate-buffered saline solution (PBS) at pH 7.4, as required. The solutions were probe tip sonicated (Branson Sonifier W-250) at 20 W for 2 min on an ice bath. The sonication step was repeated five times. The pulse function was pulsed on 8.0 s and pulsed off 2.0 s (Jing-Mou et al., 2008).

2.2. Characterization

2.2.1. Characterization of steroid-modified and tocopheryl-modified N,O6-partially acetylated chitosan conjugates

The FTIR spectra of conjugates were obtained by the KBr pellet method using a Perkin-Elmer 1720 FTIR spectrophotometer with 32 scans and 4 cm^{-1} resolution. The ^{1}H NMR spectra were recorded with a VARIAN Oxford NMR AS400 spectrometer operating at 400.46 MHz for ^{1}H at 25 °C with concentrations ca. 25–8 mg mL^{-1} in d2-water and d4-methanol/d2-water (66%, v/v) and analyzed with the VNMRJ software, version 2.2 (Jing-Mou et al., 2008). Elemental analyses were performed on a Vario MicroCube Analyzer with burning temperature of 1150 °C. Wide-angle X-ray diffraction of the powered samples were performed using a Rigaku SmartLab X-Ray diffractometer with Cu K_α radiation (40 kV, 180 mA, $\lambda = 0.15418$ nm), data collected at a scan rate of 5° 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). Differential scanning calorimetry studies were conducted using sample weights of approximately 5 mg, under nitrogen dynamic flow of 20.0 mL min^{-1} and a heating-cooling rate of 10 °C min^{-1} (Quiñones et al., 2011). Samples were deposited in aluminum 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.

2.2.2. Characterization of nanoparticles

Dynamic light scattering (DLS) studies on the prepared nanoparticles were performed using Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at 25 °C to obtain the particle size and zeta potential. For zeta potential measurements nanoparticles were prepared in double-distilled water (ca. 1–2 mg mL^{-1}), while particle size measurements were conducted on PBS solution (ca. 0.5–1 mg mL^{-1}). The size and morphology of dried nanoparticles were studied by transmission electron microscopy (TEM) with a Philips CM20 operating at 200 kV and scanning electron microscopy (SEM) with a Nova NanoSEM 600 electron microscope. Each sample was stirred 48 h in double-distilled water (ca. 1 mg mL^{-1}), probe tip sonicated as already described and a drop of it was deposited on carbon plates. The excess solution was removed with filter paper and air-dried. The SEM samples were sputter-coated with gold. The TEM samples were negative stained with a drop of uranyl acetate solution (1%).

2.3. In vitro drug release studies

In vitro release of steroids and vitamin E from steroid-modified and tocopherol-modified N,O6-partially acetylated chitosan nanoparticles was studied by UV detection (Genesys 10 UV–Vis Spectrophotometer, Thermo Spectronic, Rochester, NY, USA) at 280 nm (I), 243 nm (II), 250 nm (III), 243 nm (IV) and 292 nm (V), respectively at pH 6.0. 10–15 milligrams of hydrophobically-modified N,O6-partially acetylated chitosan nanoparticles dissolved in 5 mL of PBS solution (pH 6.0) were placed in dialysis bags and dialyzed against the release media (PBS, pH 6.0, 40 mL) at 30 °C or 37 °C with constant agitation at 100 rpm. The entire media were removed at determined time intervals, and replaced with the same volume of fresh media. The amount

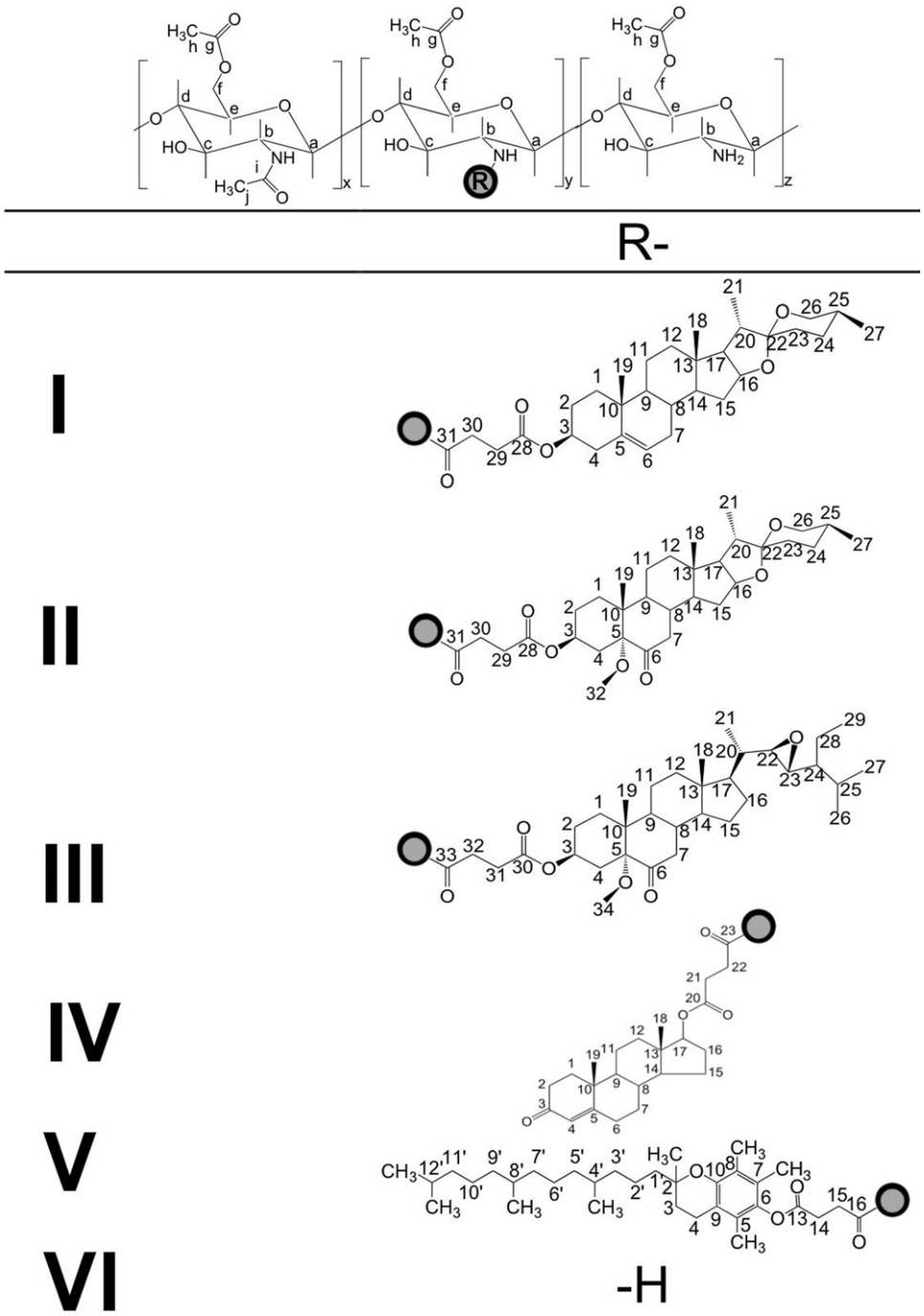

Fig. 1. Structure of diosgenin hemisuccinyl N,O6-partially acetylated chitosan (I), DI-31 hemisuccinyl N,O6-partially acetylated chitosan (II), S7 hemisuccinyl N,O6-partially acetylated chitosan (III), testosterone hemisuccinyl N,O6-partially acetylated chitosan (IV), DL-α-tocopherol hemisuccinyl N,O6-partially acetylated chitosan (V) conjugates and N,O6-partially acetylated chitosan (VI).

of steroids or DL-α-tocopherol released was determined by UV spectrophotometry and calculated from a previously obtained calibration curve. These studies were conducted in triplicate for each sample.

2.4. Biological activity

2.4.1. Radish cotyledon test

The radish (*Raphanus sativus*) test was employed in order to detect plant growth activity. This bioassay is based on the increased weight of the treated radish's cotyledons (auxin type activity). To this end, radish seeds previously sterilized by sodium hypochlorite treatment were germinated over wet filter paper in the dark at 25 °C, for 72 h (Alonso-Becerra et al., 2007). Cotyledons were separated of hypocotyls, weighted and treated with 5 mL of II or III nanoparticles in water (10^{-1} to 10^{-7} mg mL^{-1}); DI31 or S7 solutions (10^{-1} mg mL^{-1} in ethanol/water solution 50% (v/v) and diluted up to 10^{-2} to 10^{-7} mg mL^{-1}); N,O6-partially acetylated chitosan aqueous solution (10^{-1} to 10^{-7} mg mL^{-1}) or pure water (control). After 72 h, cotyledons weights were measured. These studies were conducted in triplicate for each sample and concentration (10 cotyledons each run).

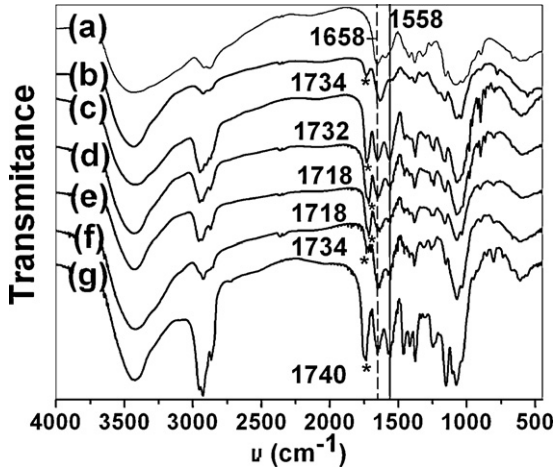

Fig. 2. Infrared spectra of: (a) CS, (b) VI, (c) I, (d) II, (e) III, (f) IV and (g) V (see Fig. 1 for structures).

2.4.2. Antioxidant activity test measured as DPPH radical scavenging activity

The antioxidant activity of the obtained tocopheryl-modified N,O6-partially acetylated chitosan nanoparticles was evaluated trough the in vitro test of DPPH (1,1′-diphenyl-2-picrylhydrazyl) (Hu, Tang, Yang, Zhang, & Zou, 2008) and compared with same molar concentrations of Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid). Briefly, 0.2 mL of ethanol and 0.3 mL of the release media (or same molar concentration of Trolox) at different time intervals were mixed in a 10 mL eppendorf tube and 2.5 mL of DPPH 75 μM in ethanol was added. The resulting solution was shaken vigorously, left to stand for 30 min in the dark and the absorbance at λ = 517 nm (A_{517}) was measured. The radical scavenging effect was calculated as:

$$\%\,\text{Radical scavenging effect} = \left[\frac{A_0 - (A_{517})}{A_0}\right] \times 100$$

where A_0 is the absorbance of blank DPPH at 517 nm. No corrections were needed to A_{517} because the samples were transparent at this wavelength.

3. Results and discussion

The employed method afforded steroid-modified and tocopheryl-modified N,O6-partially acetylated chitosan conjugates with substitutions of up to 24.5 mol% (diosgenin), 22.6 mol% (DI31), 23.4 mol% (S7), 8.9 mol% (testosterone) and 51.5 mol% (DL-α-tocopherol); equivalent to weight contents of 33.2% (w/w) (diosgenin), 33.0% (w/w) (DI31), 34.7% (w/w) (S7), 11.8% (w/w) (testosterone) and 56.4% (w/w) (DL-α-tocopherol), respectively.

The FTIR spectra of the hydrophobically-modified conjugates are shown in Fig. 2. The spectra of chitosan and N,O6-partially acetylated chitosan are also included for comparison.

The IR spectrum of chitosan (Fig. 2(a)) presented characteristic absorption peaks at 2942–2784 cm^{-1} (aliphatic C—H stretching band), 1658 cm^{-1} (Amide I), 1597 cm^{-1} (NH_2 bending) and 1321 cm^{-1} (Amide III). Absorption peaks at 1154 cm^{-1} (antisymmetric 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 (Quiñones et al., 2011). N,O6-partially acetylated chitosan presents a quite similar IR spectrum (Fig. 2(b)), but a peak absorption at 1734 cm^{-1} (Acetate, C=O of ester linkage) and increased absorption at 1558 cm^{-1} (Amide II band, N—H bending of acetamide) are observed.

The IR spectrum of diosgenin-modified N,O6-partially acetylated chitosan conjugate (Fig. 2(c)) shows intense absorption peaks at 1732 cm^{-1} (C=O of ester linkage) and 1564 cm^{-1} (Amide II band, N—H bending of amide linkage). The IR spectra of brassinosteroid-modified N,O6-partially acetylated chitosan conjugates showed a quite intense C=O absorption at 1718 cm^{-1} (ketone group of DI31 and S7) overlapping the absorption peak at 1734 cm^{-1} (C=O of ester linkage) (Fig. 2(d) and (e)). The intense peaks at 1558 cm^{-1} (Amide II band, N—H bending), confirm the amide linkage between the N,O6-partially acetylated chitosan and the brassinosteroid hemisuccinates.

The IR spectrum of testosterone-modified N,O6-partially acetylated chitosan conjugate (Fig. 2(f)) shows intense peaks at 1734 cm^{-1} (C=O of ester linkage) and 1718 cm^{-1} (C=O of ketone group at C3). The intense absorption at 1558 cm^{-1} (Amide II band, N—H bending) confirms the amide linkage between the N,O6-partially acetylated chitosan and testosterone hemisuccinate.

Tocopheryl-modified N,O6-partially acetylated chitosan conjugate IR spectra (Fig. 2(g)) presents, in addition to chitosan peaks, C=O absorptions at 1740 cm^{-1} (C=O of ester linkage) and an intense peak at 1557 cm^{-1} (Amide II band, N—H bending); indicative of amide linkage between tocopheryl hemisuccinate and N,O6-partially acetylated chitosan.

The proton NMR spectrum of steroid-modified and tocopheryl-modified N,O6-partially acetylated chitosan conjugates are shown in Fig. 3. The placebo chitosan and N,O6-partially acetylated chitosan NMR spectra are shown in Fig. 1 of supplementary materials.

Proton NMR spectrum of chitosan (Fig. 1(I) of supplementary materials) showed characteristic peaks at 2.10 ppm (s, CH_3 of CH_3CO—, protons at C_j), 3.19 ppm (s, 1H, H_b), 3.75 ppm (s, 1H, H_e), 3.80 ppm (s, 1H, H_f') and 3.91 ppm (s, 3H, $H_c + H_d + H_f$) as reported for crab chitosans (Berrada et al., 2003). The ^{1}H NMR of N,O6-partially acetylated chitosan was dominated by chitosan peaks; but intense peaks at 1.94 ppm (s, CH_3 of CH_3CO—, protons at C_h), 2.83–2.93 ppm (s, H_b, of acetylated and free glucosamine units, respectively), 4.27/4.68 ppm (CH, C_a anomeric sugar proton of free and acetyl-substituted glucosamine units, respectively) are observed (Fig. 1(II) of supplementary materials).

The ^{1}H NMR spectra of diosgenin-modified N,O6-partially acetylated chitosan (Fig. 3(I)) presented several peaks at 1.20–1.35 ppm ($4CH_3$, H27 + H21 + H19 + H18), 2.59 ppm (CH_2, H29 + H30 of succinyl moiety), 4.18/4.20/4.58 ppm (CH, C_a anomeric sugar proton of free glucosamine, diosgenin-substituted glucosamine and acetyl-substituted glucosamine units respectively). The brassinosteroid-modified chitosans (Fig. 3(II) and (III)) showed intense peaks at 0.88–1.40/0.74–1.40 ppm (CH_3, H18 + H19 + H26 + H27 + H21 at II and III), 2.54–2.62/2.55–2.69 ppm (CH_2, H29 + H30 and H31 + H32 of succinyl moiety at II and III, respectively), 4.10/4.11 ppm (CH, C_a anomeric sugar proton of free glucosamine units at II and III, respectively), 4.23/4.21 (CH, C_a anomeric sugar proton of steroid-substituted glucosamine units at II and III, respectively) and 4.62 ppm (CH, C_a anomeric sugar proton of acetyl-substituted glucosamine units at II).

Testosterone-modified N,O6-partially acetylated chitosan (Fig. 3(IV)) presented peaks at 0.96 ppm (CH_3, H18), 1.31–1.40 ppm ($CH + CH_2 + CH_3$, H6 + H7 + H11 + H12 + H15 + H19), 2.39–2.63 ppm (CH_2, H21 + H22 of succinyl moiety) and 4.13/4.24 ppm (CH, C_a anomeric sugar proton of free glucosamine and testosterone-substituted glucosamine units, respectively).

The proton NMR spectrum of tocopheryl-modified N,O6-partially acetylated chitosan (Fig. 3(V)) showed, in addition to chitosan peaks, signals at 1.08–1.11 ppm (CH_3, 4′-CH_3 + 8′-CH_3 + 12′-CH_3), 1.26–1.43 ppm ($CH_3 + CH_2$, 2-CH_3 + H1′ to H12′), 2.61 ppm (CH_2, H14 + H15 of succinyl moiety), 4.13/4.23 ppm (CH, C_a anomeric sugar proton of free glucosamine and tocopheryl-substituted glucosamine units, respectively).

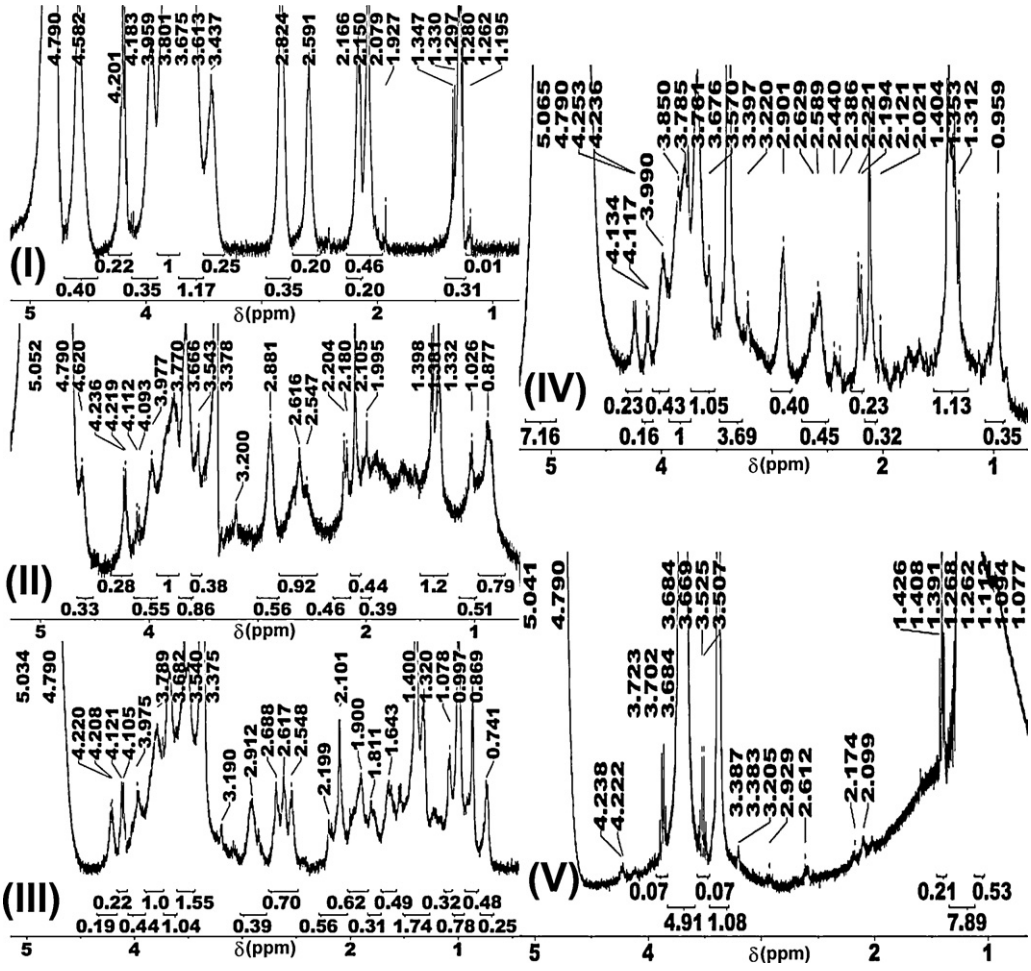

Fig. 3. Proton NMR spectra of: (a) I, (b) II, (b) III, (c) IV and (d) V at 8 mg mL^{-1} in CD_3OD/D_2O (2:1) at 25 °C (see Fig. 1 for structures).

The wide-angle X-ray diffraction patterns of chitosan, N,O6-partially acetylated chitosan, steroids-modified and tocopheryl-modified N,O6-partially acetylated chitosan conjugates are shown in Fig. 4.

Chitosan presents the characteristic peaks at 2θ 10.5° and 19.8° (Fig. 4(a)), attributed to the $(0\,2\,0)_h$ planes of the hydrated crystalline structure and the reflections of the hydrated polymorph respectively (David, Domard, Lucas, Osorio-Madrazo, Peniche-Covas, & Trombotto, 2010). N,O6-partially acetylated chitosan shows a broad and intense peak at 20.1° (Fig. 4(b)), showing a destruction of the native chitosan packing structure.

The hydrophobically-modified N,O6-partially acetylated chitosan conjugates showed several intense peaks at 5.6°, 15.5° and 19.5° (I); 12.3°, 14.8° and 17.4° (II); 11.7°, 14.7° and 17.8° (III); 23.3° (IV) and 19.4° (V) (Fig. 4(c)–(g)). The absence of a broad and intense peak at 20.1° attributed to N,O6-partially acetylated chitosan is indicative of its absence as crystalline phase. The

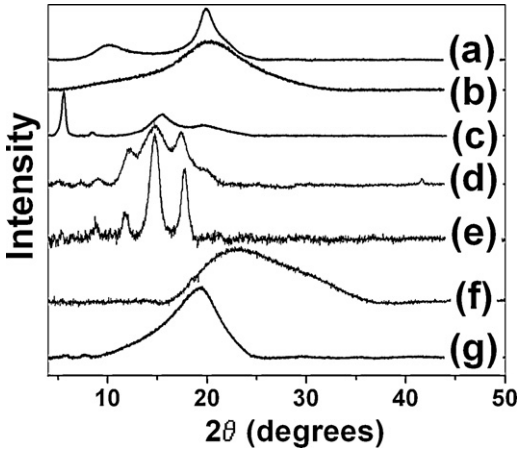

Fig. 4. Wide-angle X-ray diffraction patterns of: (a) CS, (b) VI, (c) I, (d) II, (e) III, (f) IV and (g) V (see Fig. 1 for structures).

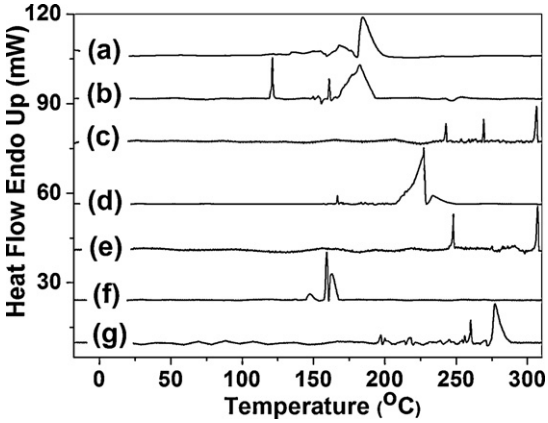

Fig. 5. DSC curves of (I): (a) CS, (b) VI, (c) I, (d) II, (e) III, (f) IV and (g) V (see Fig. 1 for structures).

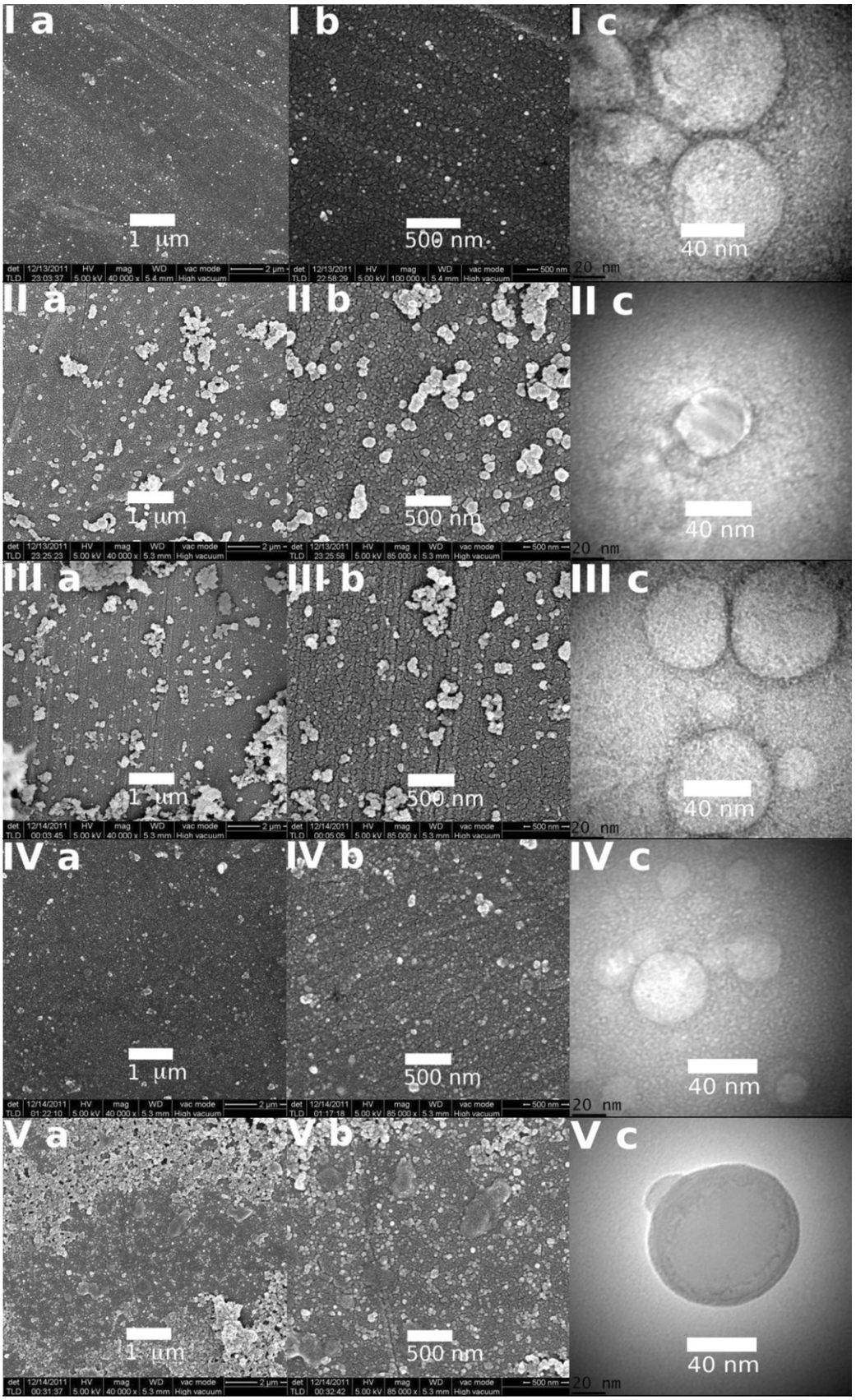

Fig. 6. Scanning electron micrographs of: (I) I, (II) II, (III) III, (IV) IV and (V) V nanoparticles at (a) 40,000×, (b) 85,000× magnifications, (c) transmission electron micrograph at 100,000× magnifications with negative staining (see Fig. 1 for structures).

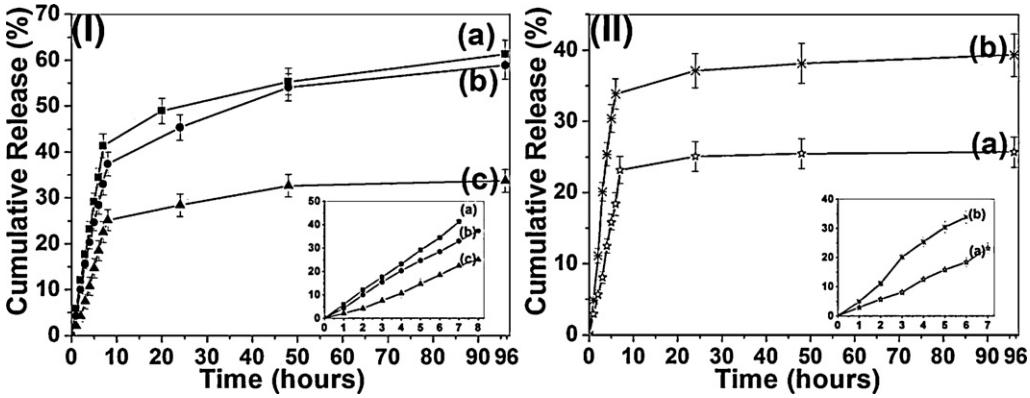

Fig. 7. In vitro release profile of (I): (a) I, (b) II, (c) III in phosphate buffered solution (pH = 6.0) at 30 °C; (II) (a) IV and (b) V in phosphate buffered solution (pH = 6.0) at 37 °C. Data are the mean ± standard deviation ($n = 3$) (see Fig. 1 for structures).

characteristic peaks of pure steroid hemisuccinates and tocopheryl hemisuccinate were also absent (see Fig. 2 and Table 1 of supplementary materials). Instead, some peaks associated to new crystalline phases of the obtained conjugates were observed.

The thermal behavior of chitosan samples has been shown to be dependent on the natural source, the purity of samples and the preparation conditions (Mau, Yang, & Yen, 2009). However, the main thermal effects presented by a chitosan from a particular source can be interpreted on the basis of the behavior of chitosan from other sources.

Chitosan (Fig. 5(a)) showed two endothermic peaks at 170.4 °C and 187.0 °C, with associated peak enthalpy (ΔH) of 3.5 J/g and 120.6 J/g, respectively. These endothermic effects must result mainly from the melting and dissociation of chitosan crystals, by comparison with reports for crab chitosans (Quiñones et al., 2011). N,O6-partially acetylated chitosan presents three endothermic peaks at 120.3 °C, 161.3 °C and 183.1 °C, with associated ΔH of 45.9 J/g, 26.5 J/g and 987.2 J/g, respectively (Fig. 5(b)).

The steroids-modified N,O6-partially acetylated chitosan exhibited several endothermic peaks (Fig. 5(c)–(f)) at 242.4 °C 269.2 °C and 306.6 °C (I), with related ΔH of 9.7 J/g, 8.6 J/g and 22.7 J/g, respectively; 166.9 °C, 227.9 °C and 234.4 °C (II), with related ΔH of 17.8 J/g, 1333.3 J/g and 199.3 J/g, respectively; 248.5 °C and 306.7 °C (III), with related ΔH of 33.3 J/g and 36.5 J/g, respectively; 148.2 °C, 160.1 °C and 162.8 °C (IV), with associated ΔH of 32.4 J/g, 70.4 J/g and 84.0 J/g, respectively. Tocopherol-modified N,O6-partially acetylated chitosan showed two endothermic peaks at 259.6 °C and 276.4 °C, with associated ΔH of 60.3 J/g and 504.3 J/g (Fig. 5(g)). These peaks can result from the melting of steroids-modified or tocopherol-modified N,O6-partially acetylated chitosan conjugates, dissociation and decomposition of chitosan chains.

Dynamic light scattering studies conducted in triplicate afforded average particles diameters in PBS solution of 325 ± 2 nm with a polydispersity index (PDI) of 0.17 ± 0.01 (I), 358 ± 6 nm PDI: 0.42 ± 0.08 (II), 197 ± 5 nm PDI: 0.54 ± 0.07 (III), 206.0 ± 0.9 nm PDI: 0.39 ± 0.03 (IV) and 275 ± 5 nm PDI: 0.58 ± 0.08 (V). These nanoparticles were accompanied by ca. 3–8 mol% aggregates of about 3.6–5.3 μm size. Zeta potential values were 22.7 ± 0.4 mV (I), 15.0 ± 0.1 mV (II), 19 ± 3 mV (III), 7 ± 1 mV (IV) and 14.9 ± 0.7 mV (V).

In order to obtain morphological information on the individual particles, scanning electron microscopy and transmission electron microscopy techniques were employed. Fig. 6 shows the scanning electron microscopy and transmission electron microscopy images of dried steroid-modified and tocopheryl-modified N,O6-partially acetylated chitosan nanoparticles. The SEM micrographs showed almost spherical individual nanoparticles and some aggregates. TEM images showed spherical particles with 30–60 nm mean diameters.

The size measured by the DLS technique was the hydrodynamic diameters of aggregated particles and/or hydrated individual particles, while scanning and transmission electron micrographs depicted the size in the dried state of the particles. Therefore, particles size is smaller in electron microscopy techniques.

The release profiles of steroids-modified N,O6-partially acetylated chitosan nanoparticles (I, II and III) at 30 °C in PBS (pH 6.0), expressed as percent cumulative release against time, are shown in Fig. 7(I). Fig. 7(II) shows the release profiles of testosterone-modified and tocopheryl-modified N,O6-partially acetylated chitosan nanoparticles (IV and V) at 37 °C in PBS (pH 6.0). These studies were performed at pH 6 since acidic conditions are needed to achieve the hydrolysis of the ester linkage and the release of the steroids and vitamin E.

The steroids-modified N,O6-partially acetylated chitosan nanoparticles presented sustained release with almost constant release rates during the first 8 h (Fig. 7(I)(a)–(c)). Releases were highly dependent on the particle sizes in PBS, reaching 61% (I) and 58% (II) after 96 h for the conjugates with hydrodynamic diameters between 330 and 360 nm; while the smaller III nanoparticles released a 34% of S7 after 96 h.

The testosterone-modified and tocopheryl-modified N,O6-partially acetylated chitosan nanoparticles also showed sustained release with almost constant release rates (zero order kinetics) during the first 6–7 h. Releases were not quantitative, reaching a cumulative release of 26% (IV) and 39% (V) after 96 h (Fig. 7(II)(a) and (b)). The V nanoparticles, with higher substitution degree and bigger hydrodynamic diameter in PBS, released faster and more quantity of the covalently linked drug than the smaller and less substituted testosterone-modified N,O6-partially acetylated chitosan nanoparticles.

Fig. 8 shows the plant growth biological activity of the synthetic brassinosteroids, N,O6-partially acetylated chitosan and brassinosteroids-modified N,O6-partially acetylated chitosan nanoparticles in the radish cotyledons bioassay.

Plant growth stimulator activities of the synthetic brassinosteroids DI31 and S7 are quite similar, showing best results at 10^{-3} and 10^{-4} mg mL^{-1} concentrations, but almost a doubled cotyledons weight was reached as compared to control with the lowest concentrations (10^{-6} and 10^{-7} mg mL^{-1}). N,O6-partially acetylated chitosan exerts a growth stimulator effect at higher (10^{-1} to 10^{-4} mg mL^{-1}) and medium concentrations (10^{-5} mg mL^{-1}); but no activity is observed at low concentration (10^{-6} to 10^{-7} mg mL^{-1}).

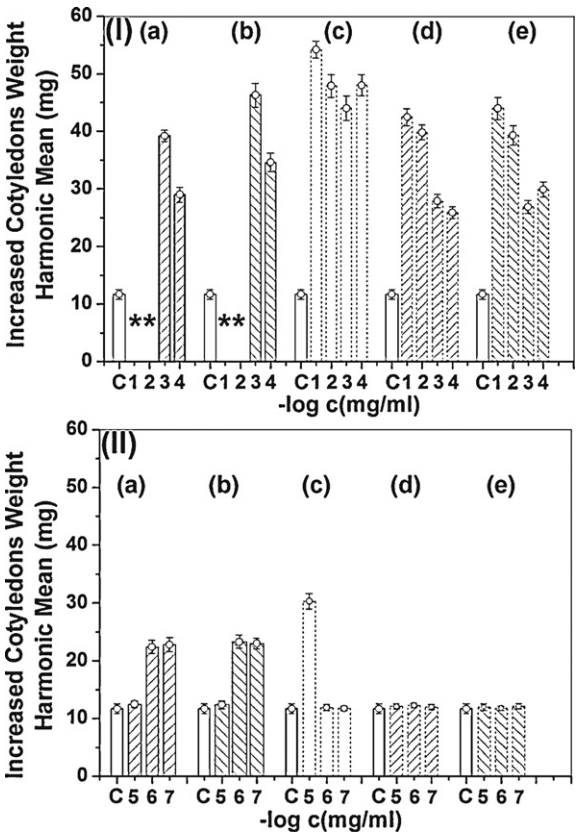

Fig. 8. Biological activity as plant growth regulator of (I): (a) DI31, (b) S7, (c) VI, (d) II and (e) III at control (C) and 10^{-1} to 10^{-4} mg mL^{-1} at 25 °C; (II): (a) DI31, (b) S7, (c) VI, (d) II and (e) III at control (C) and 10^{-5} to 10^{-7} mg mL^{-1} at 25 °C. (*) Not measured because cotyledons died as result of high ethanol content. Data are the mean ± standard deviation (n = 3) (see Fig. 1 for structures).

The studied II and III nanoparticles in aqueous solution presented stimulatory activities at high concentrations (10^{-1} to 10^{-4} mg mL^{-1}) (weight increased approximately three or four times compared to control). Not activity was observed at lowest concentrations (10^{-5} to 10^{-7} mg mL^{-1}).

The antioxidant activities at 2, 4, 6, 8 and 96 h of the tocopheryl-modified N,O6-partially acetylated chitosan nanoparticles in PBS (pH 6.0), expressed as % radical scavenging activity by DPPH test, were 35%, 44%, 61%, 75% and 81%, respectively. The antioxidant power in the release medium increased with time in the same way as tocopherol release. It was around 78–84% when compared to Trolox solutions with same molar concentrations. Based on these results it can be concluded that the processes carried out during the synthesis of tocopheryl-modified N,O6-partially acetylated chitosan conjugate, the preparation of the nanoparticles and the release experiments did not compromise the antioxidant activity of DL-α-tocopherol.

4. Conclusions

Diosgenin, two synthetic analogs of brassinosteroids, testosterone and DL-α-tocopherol were covalently linked to synthetic water-soluble N,O6-partially acetylated chitosan, for controlled release, as confirmed by FTIR spectroscopy and proton NMR. Differential scanning calorimetry and wide-angle X-ray diffraction studies showed structural changes of chitosan packing, as result of hydrophobic modification on chitosan chains due to the covalent linking of the bioactive molecules. Resulting conjugates formed self-assembled nanoparticles in PBS with hydrodynamic diameters ranging from 197 to 358 nm and drug contents between 11.8 and 56.4% (w/w). Spherical 30–60 nm nanoparticles were observed by scanning electron and transmission electron microscopy upon drying. In vitro release studies performed in PBS (pH 6.0) indicated a drug release dependence on substitution degree and hydrated particle sizes. Almost constant release rates were observed during the first 6–8 h. Brassinosteroids-modified nanoparticles showed good agrochemical activity in radish seeds bioassay at 10^{-1} to 10^{-4} mg mL^{-1}. The vitamin E released of tocopheryl-modified N,O6-partially acetylated chitosan nanoparticles retained good antioxidant activity. These results indicate that these hydrophobically-modified N,O6-partially acetylated chitosan nanoparticles are good candidates for the sustained release of steroids and vitamin E.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.07.080.

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