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Self-assembled nanoparticles of glycol chitosan – Ergocalciferol succinate conjugate, for controlled release

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ABSTRACT

Glycol chitosan was linked to vitamin D2 hemisuccinate (ergocalciferol hemisuccinate) for controlled release through water-soluble carbodiimide activation. The resulting conjugate formed self-assembled nanoparticles in aqueous solution with particle size of 279 nm and ergocalciferol hemisuccinate content of 8.4% (w/w). Almost spherical 50–90 nm nanoparticles were observed by scanning and transmission electron microscopy upon drying. Drug linking to glycol chitosan was confirmed by FTIR spectroscopy and proton NMR. Particles were also characterized by differential scanning calorimetry and wide-angle X-ray diffraction studies. In vitro vitamin D2 release studies performed in water at acid pH indicated a drug release dependence on the solution acidity. Almost constant release rate was observed during the first 8 h. These results indicate that the obtained nanoparticles could be good candidates for vitamin D2 release to animals and humans.

1. Introduction

Self-assembled polymeric nanoparticles, engineered with controlled composition and defined properties, have received increasing interest recently for their potential applications in medicine, biotechnology and food chemistry, particularly as drug delivery systems and carriers (Chen et al., 2011). These amphiphilic nanoparticles consists of a hydrophobic core, usually a long hydrocarbon chain and/or aromatic groups, shielded by a hydrophilic shell in aqueous solution (Beheshti, Kjøniksen, Knudsen, Nyström, & Zhu, 2009; Jing-Mou, Li-Yan, Yi, & Yong-Jie, 2008). Chitosan, cellulose and other biopolymers are particularly promising for preparation of these nanoassemblies, due to their inherent biocompatibility (Babak & Desbriéres, 2010; Guthrie, Perrier, Roy, & Semsarilar, 2009). Chitosan is a natural polymer, consisting of β(1 → 4) linked glucosamine units, with some proportion of N-acetylglucosamine units. It is obtained by extensive deacetylation of chitin, a polysaccharide widely spread in nature and major component of the shells of crustaceans and molluscs, the cuticle of insects and cell walls of fungi (Boudran et al., 2012; Muzzarelli, 2011). Chitosan presents excellent biocompatible, biodegradable, mucoadhesive, microbicidal, antifungal and antioxidant properties (Domb, Muzzarelli, Muzzarelli, Ravi Kumar, & Sashiwa, 2004; Muzzarelli, 2009). Glycol chitosan possess a good water solubility at all pHs, biocompatibility and is widely used as carrier of hydrophobic drugs and genes (Cho et al., 2006). It has been hydrophobically functionalized with 5β-cholanic moieties; in order to prepare self-aggregated nanoparticles in aqueous solution for proteins and gene controlled release (Jing-Mou et al., 2008; Cho et al., 2006).

Vitamin D2 (ergocalciferol) is a lipophilic vitamin with important effects on adjusting the metabolism of calcium and phosphorus, regulating osteoblasts proliferation and skeleton development. Its deficit has been related to several diseases, such as multiple sclerosis, rheumatoid arthritis, type 1 diabetes and overall cancer mortality (Autier & Gandini, 2007; Holick, 2003). Some polymeric microcapsules/microspheres for controlled release of vitamin D2 have been prepared, i.e. chitosan/ethylcellulose complex microcapsules for intestinal release (Tian-Wei & Xin-Yuan, 2002).

The preparation of vitamin D2-modified glycol chitosan conjugate will allow accomplishing its controlled release, overcoming the low aqueous solubility of ergocalciferol, and combining its

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cancer protective effects on humans and animals with the microbicidal properties and biocompatibility of glycol chitosan (Amsden, Knight, & Shapka, 2007; Chen et al., 2008).

This article reports on the covalent linking of vitamin D2 hemisuccinate (ergocalciferol hemisuccinate) to glycol chitosan, forming self-assembled nanoparticles in aqueous solution, to achieve their controlled release.

2. Experimental

2.1. Materials

Glycol chitosan (acetylation degree, DA = 19.1% determined by ^{1}H NMR, $M_w = 4.1 \times 10^5$) was purchased from Sigma–Aldrich. Vitamin D2 hemisuccinate was synthesized by basic-catalyzed traditional esterification in pyridine of ergocalciferol with succinic anhydride (Abe, Hasunuma, & Kurokawa, 1976). The vitamin D2 (ergocalciferol), solvents and reagents employed were purchased from Sigma–Aldrich and used without further purification.

The structure of glycol chitosan–vitamin D2 conjugate is presented in Fig. 1.

2.2. Glycol chitosan–vitamin D2 conjugate preparation

2.2.1. Reaction in aqueous solution of glycol chitosan with vitamin D2 hemisuccinate activated with 1-ethyl-3-(3′-dimethylamino)carbodiimide hydrochloride (EDC)

130 mg (0.61 mmol) of glycol chitosan were dissolved in 8 mL of bi-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 mg (ca. 0.15 mmol) of ergocalciferol hemisuccinate were dissolved in 32 mL of ethanol/water solution (85:15, v/v) and slowly added with stirring to the glycol chitosan solution. The reaction mixture was stirred 72 h at room temperature, dialyzed (MWCO: 6000–8000) 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 solution was freeze-dried affording a white, cotton wool-like product.

2.3. Characterization

The glycol chitosan–vitamin D2 conjugate was characterized by FTIR spectroscopy using a Perkin-Elmer FTIR spectrophotometer with 32 scans and 4 cm^{-1} resolution. Sample was 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 was recorded with an OXFORD NMR AS400 (VARIAN) 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). Wide-angle X-ray diffraction (WAXD) analysis of the powered sample was performed using a Rigaku SmartLab X-ray diffractometer 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 5 mg, under a nitrogen dynamic flow of 20.0 mL min^{-1} and a heating–cooling rate of 10 °C min^{-1} (Basaran, Yazan, & Yenilmez, 2010). 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. Dynamic light scattering (DLS) study was performed using Zetasizer Nano (Malvern Instruments, UK) at 25 °C to obtain the particle size and Zeta potential. The size and morphology of dried nanoparticles was studied by transmission electron microscopy (TEM) with a Philips CM20 operating at 200 kV and scanning electron microscopy (SEM) with a FEI Nova NanoSEM 600. The sample was stirred 48 h in bi-distilled water (ca. 1 mg mL^{-1}) and a drop of it was deposited on carbon plates. The excess solution was removed with filter paper and air-dried. The SEM sample was coated with gold. The TEM sample was negative stained with a drop of uranyl acetate solution (1 wt.%).

2.4. In vitro drug release studies

In vitro drug release of vitamin D2 hemisuccinate linked to glycol chitosan nanoparticles was studied by UV detection at 265 nm of the delivered ergocalciferol at various pH. The glycol chitosan–vitamin D2 conjugate (10 mg) was placed in a dialysis bag (MWCO: 14,000) containing buffer solutions (5 mL) at different acidic pH and dialyzed against the release media (40 mL of PBS solutions at the same pH as the corresponding conjugate solution) at 37 °C with constant agitation at 100 rpm. The entire media was removed at determined time intervals, and replaced with the same volume of fresh media. The amount of ergocalciferol released was determined by UV spectrophotometry and calculated from a previously obtained calibration curve. These studies were conducted in triplicate.

3. Results and discussion

The employed method afforded ergocalciferol content of 3.9 mol% in the obtained glycol chitosan–vitamin D2 conjugate, equivalent to 8.4 wt.% of ergocalciferol hemisuccinate.

Glycol chitosan–vitamin D2 conjugate formed self-assembled nanoparticles in aqueous solution with 279 ± 7 nm average diameter, due to its hydrophilic/hydrophobic moieties. The particles structure is affected by the polarity of the media, as observed in proton NMR peak differences in d2-water compared to d4-methanol/d2-water (66%, v/v).

Dynamic light scattering studies conducted in triplicate afforded average particles diameters of 279 ± 7 nm with a polydispersity index of 0.50 ± 0.03. These small nanoparticles were accompanied by ca. 5–8 mol% aggregates of about 4.7–4.8 μm size. The Zeta potential of 7.7 ± 0.1 mV is indicative of some instability or tendency to aggregation in aqueous solution.

Fig. 2 shows the SEM and TEM images of dried vitamin D2-modified glycol chitosan nanoparticles. Almost spherical shaped nanoparticles with ca. 50–80 nm mean diameters are observed at SEM micrographs. TEM image showed spherical particles with 70–90 nm mean diameters.

The FTIR spectrum of glycol chitosan–vitamin D2 conjugate is shown in Fig. 3. The spectrum of glycol chitosan is also included for comparison.

The IR spectrum of glycol chitosan (Fig. 3(a)), presented absorption peaks at 3432 cm^{-1} (O—H stretching overlapped with N—H stretching), 2926 cm^{-1} and 2870 cm^{-1} (aliphatic C—H stretching band), 1650 cm^{-1} (amide 1 band, C=O stretching of acetyl group), 1616 cm^{-1} (N—H bending) and 1450–1380 cm^{-1} (C—H bending). The absorption peaks at 1160 cm^{-1} (antisymmetric stretching of the C—O—C bridge), 1120 and 1066 cm^{-1} (skeletal vibrations involving the C—O stretching) related to its saccharide structure are observed (Coll, Curiel, Peniche, & Pérez, 2010; Jing-Mou et al., 2008).

The spectrum of glycol chitosan–vitamin D2 conjugate is dominated by the broad glycol chitosan peaks; however the intense C=O peak of the ester linkage is present at 1726 cm^{-1} (Fig. 3(b)). Intense

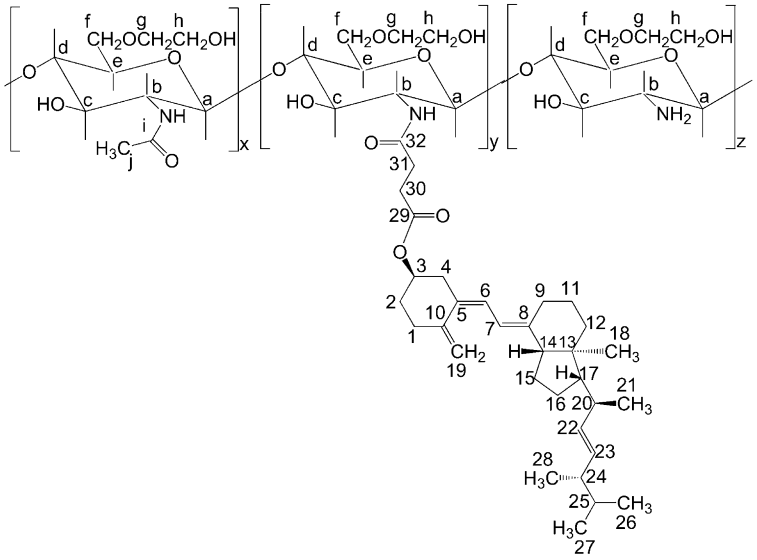

Fig. 1. Structure of glycol chitosan–vitamin D2 conjugate.

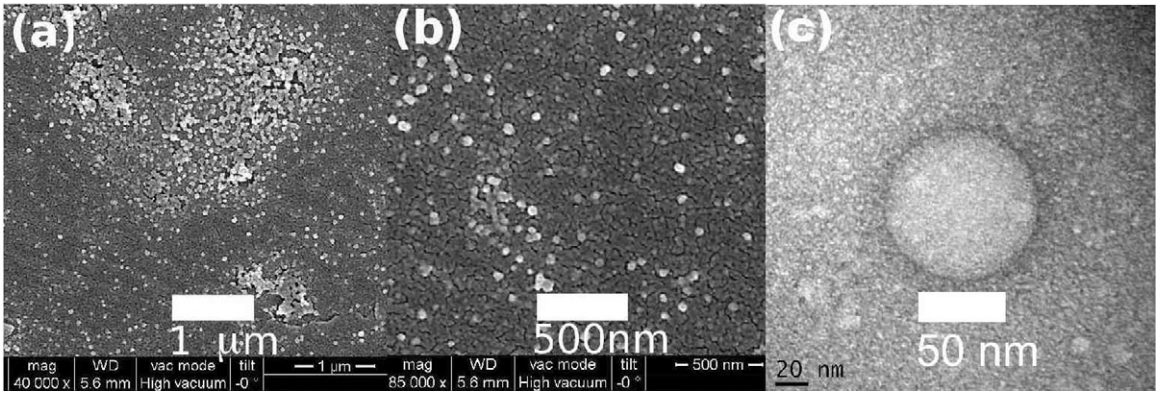

Fig. 2. Scanning electron micrographs of glycol chitosan–vitamin D2 conjugate nanoparticles at (a) 40,000×, (b) 85,000× magnifications, (c) transmission electron micrographs at 100,000× magnifications (see Fig. 1 for structure).

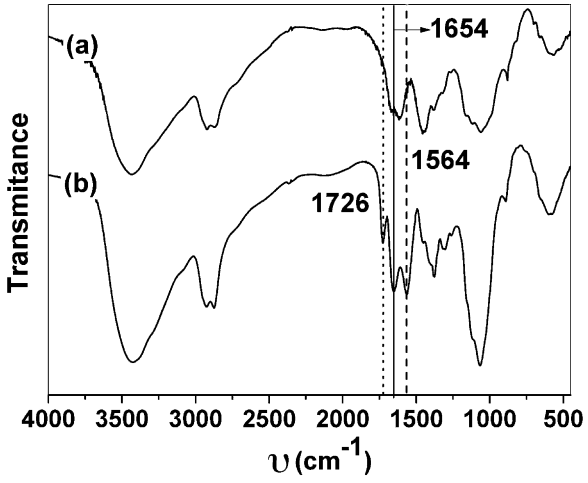

Fig. 3. Infrared spectra of: (a) glycol chitosan and (b) glycol chitosan–vitamin D2 conjugate (see Fig. 1 for structure).

peak at 1564 cm^{-1} (amide II band, N—H bending) and the increase in the amide I band at 1654 cm^{-1} confirm the amide linkage between the glycol chitosan and ergocalciferol hemisuccinate.

The proton NMR spectrum of glycol chitosan–vitamin D2 conjugate is shown in Fig. 4. The placebo glycol chitosan is also showed for comparison.

The ^{1}H NMR spectrum of glycol chitosan (Fig. 4(I)(a)) showed the characteristic signals of the saccharide protons at 2.09 ppm (s, CH_3 of CH_3CO—), 2.74 ppm (s, 2H, C_b sugar protons of N-unsubstituted glucosamine units), 3.2–4.0 ppm (C_c to C_h sugar protons) and 4.49 ppm (s, C_a anomeric sugar proton) (Gray et al., 2001). Compared with glycol chitosan, in the proton NMR spectrum of glycol chitosan–vitamin D2 (Fig. 4(I)(b), and (II)), the peaks at 0.90 ppm (CH_3, H18 + H26 + H27), 0.98 ppm (CH_3, H21 + H28), 1.29 ppm/1.34 ppm (CH2, H12 + H16), 1.35 ppm/1.38 ppm (CH2, H11 + H15), 2.53–2.61 ppm (CH_2, H30 + H31 of succinyl moiety), 4.20 ppm and 4.60 ppm (CH, C_a anomeric sugar proton of ergocalciferol substituted and free glycol chitosan, respectively; CH_2, methylene protons of glycol residues of glycol chitosan polymer chain) (Jing-Mou et al., 2008) are observed. Addition of d4-methanol to previously prepared d2-water solution of glycol chitosan–vitamin D2, until a composition of d4-methanol/d2-water (66%, v/v), enhance the peak intensity in the proton NMR

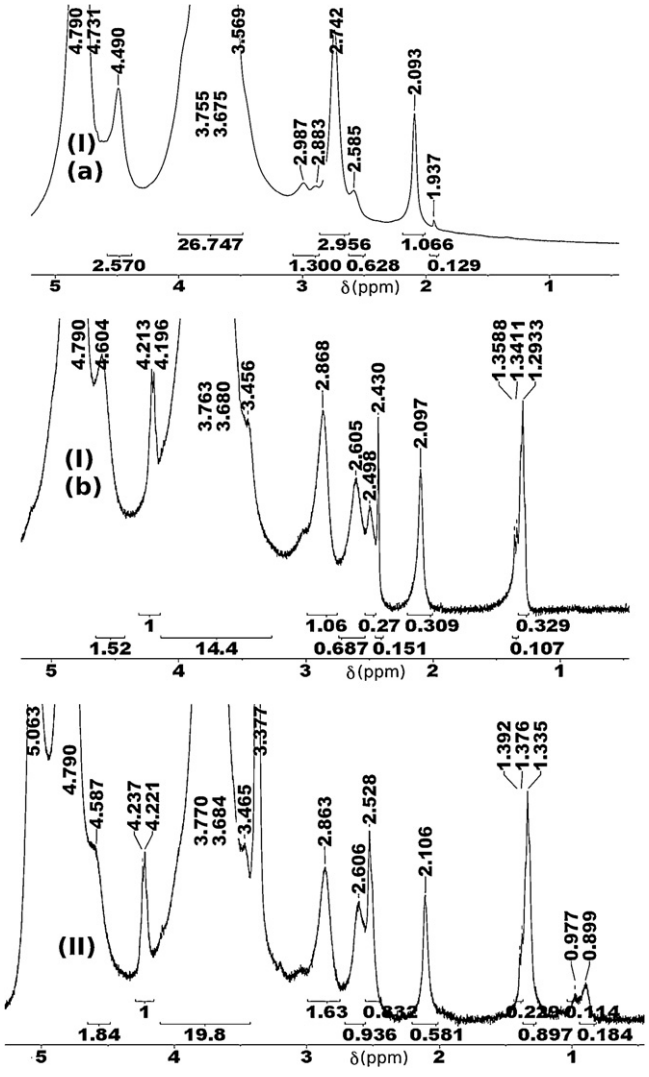

Fig. 4. Proton NMR spectra of (I): (a) glycol chitosan and (b) glycol chitosan–vitamin D2 conjugate at 25 mg mL^{-1} in D_2O; (II): glycol chitosan–vitamin D2 conjugate at 8 mg mL^{-1} in CD_3OD/D_2O (2:1) at 25 °C (see Fig. 1 for structure).

spectrum and revealed the less intense methyl peaks of ergocalciferol hemisuccinyl moiety (at 0.90 ppm and 0.98 ppm, Fig. 4(II)), due to a better solubility and amphiphilic behavior of ergocalciferol hemisuccinate glycol chitosan conjugate (Fig. 4(II)).

The wide-angle X-ray diffraction patterns of ergocalciferol hemisuccinate, glycol chitosan and glycol chitosan–vitamin D2 conjugate are shown in Fig. 5.

Glycol chitosan presents two intense peaks at 2θ 7.7° and 20.1°.

The glycol chitosan–vitamin D2 showed a broad and intense peak at 20.9°. The absence of intense peak at 20.1° attributed to glycol chitosan is indicatives of glycol chitosan lack as crystalline phase. The characteristic peaks of pure ergocalciferol hemisuccinate were also absent (see Table 1).

The DSC curves of glycol chitosan placebo and the vitamin D2-modified glycol chitosan, under nitrogen flow in the temperature range from −30 to 300 °C are shown in Fig. 6. DSC curve of glycol chitosan placebo (Fig. 6(a)) showed three endothermic peaks at 123.6 °C, 175.1 °C and 180.9 °C. Their onset and completion temperatures are listed in Table 2, together with their associated peak enthalpy (ΔH). These endothermic effects must result mainly from the melting and dissociation of glycol chitosan crystals, by comparison with reports for chitosans (Coll et al., 2010). The DSC of linked glycol chitosan-ergocalciferol conjugate (Fig. 6(b)) presents two

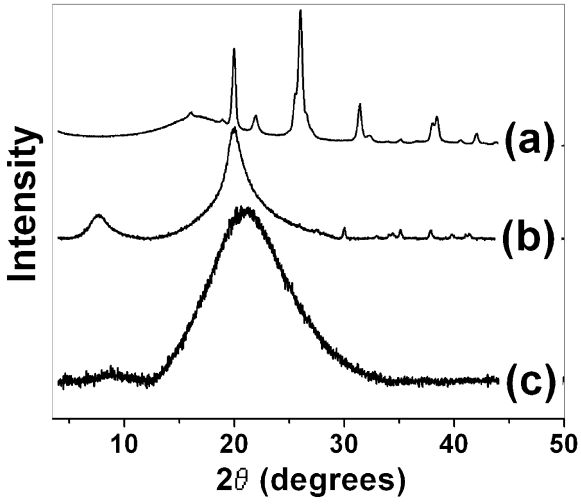

Fig. 5. Wide-angle X-ray diffraction patterns of: (a) vitamin D2 hemisuccinate, (b) glycol chitosan and (c) glycol chitosan–vitamin D2 conjugate (see Fig. 1 for structure).

intense endothermic peaks at 125.5 °C and 152.2 °C (see Table 2) with associated ΔH of 229.0 J/g and 225.2 J/g, respectively. These peaks can result from the melting of glycol chitosan with linked ergocalciferol hemisuccinate, dissociation and decomposition of modified glycol chitosan chains.

The vitamin D2 released (in weight per cent) after 30 min at different pH was 2% at pH 6.0, 5% at pH 5.0, 11% at pH 4.0 and 18% at pH 2.0. The ergocalciferol released increases as the solution acidity increases.

The in vitro release is mainly pH dependent and controlled by the hydrolysis of the ester linkage between the vitamin D2 and the succinyl group on the glycol chitosan–vitamin D2 nanoparticles.

The release profile of ergocalciferol at 37 °C in buffer solution (pH = 6.0), expressed as per cent cumulative release against time for ergocalciferol hemisuccinate-modified glycol chitosan is presented in Fig. 7. This study was performed at pH 6 to simulate as much as possible physiological conditions (pH 7.4), but acidic conditions are needed to achieve the hydrolysis of the ester linkage and the release of the vitamin D2.

Ergocalciferol hemisuccinate presented sustained release with almost constant release rate (zero order kinetics) during the first 8 h. Release was not quantitative, reaching a cumulative release of

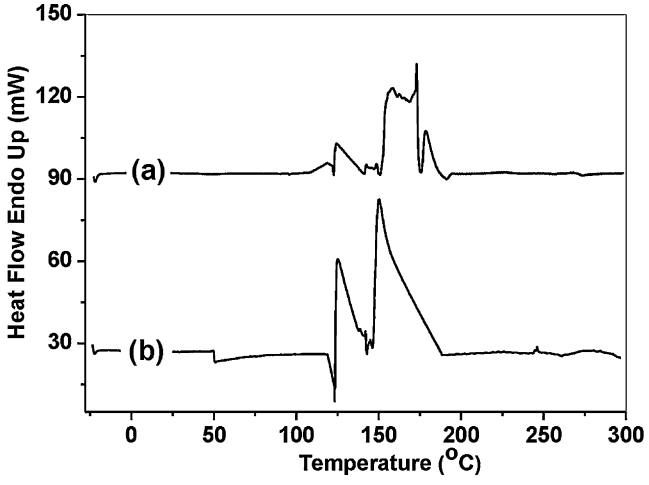

Fig. 6. DSC Curves of: (a) glycol chitosan and (b) glycol chitosan–vitamin D2 conjugate (see Fig. 1 for structure).

Table 1
X-ray peaks of ergocalciferol hemisuccinate (MSVitD2), glycol chitosan (GC) and glycol chitosan–vitamin D2 conjugate (GC–MSVitD2) by wide angle X-ray diffraction (see Fig. 1 for structure).

Samples	2θ (°)								
MSVitD2	16.1[a]	20.0	22.0[a]	26.0	31.4	38.1	38.4	42.0[a]	–
GC	7.7	20.1	30.0[a]	34.4[a]	35.1[a]	37.9[a]	39.8[a]	41.0[a]	41.3[a]
GC–MSVitD2	8.9[a]	20.9	–	–	–	–	–	–	–

[a] Stand for peaks of low intensity.

Table 2
Thermal properties and main endothermal effects of glycol chitosan (GC) and glycol chitosan–vitamin D2 conjugate (GC–MSVitD2) (see Fig. 1 for structure).

Samples	Endotherm (°C)			
	Onset	Peak	Completion	ΔH (J/g)
GC	121.7	123.6	141.7	81.9
	151.7	175.1	177.6	510.5
	177.6	180.9	195.2	72.1
GC–MSVitD2	123.7	125.5	139.1	229.0
	148.2	152.2	163.6	225.2

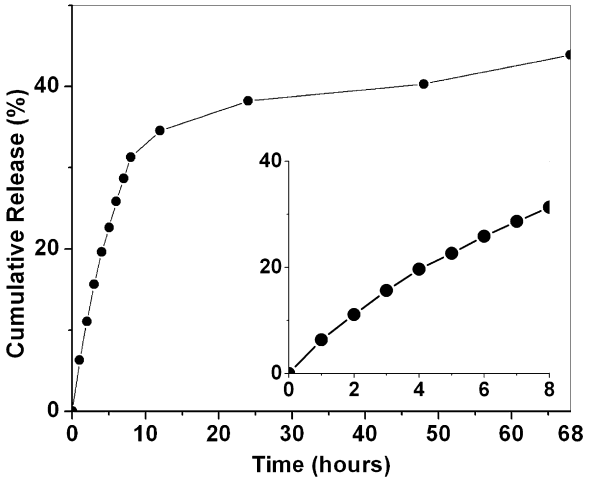

Fig. 7. In vitro release profile at 37 ± 2 °C of vitamin D2 from glycol chitosan–vitamin D2 conjugate in buffer solution (pH = 6.0) (see Fig. 1 for structure).

44% after 68 h. However the unreleased vitamin D2 linked to glycol chitosan should be available after enzymatic degradation.

4. Conclusions

Glycol chitosan was linked to ergocalciferol hemisuccinate with a functionalization of up to ca. 4 mol% as confirmed by elemental analysis and proton NMR. This conjugates were able to form self-assembled nanoparticles in aqueous solution, as result of partial hydrophobic modification of glycol chitosan with the vitamin D2 hemisuccinate. The vitamin D2 release percent in water at different acid pH indicated a drug release dependence on the acidity of the solution. In vitro sustained release was observed up to 68 h, reaching a 44 wt.% of the ergocalciferol linked to the glycol chitosan. These results indicate that the obtained nanoparticles could be good candidates for vitamin D2 release to animals and humans. Besides, the proposed methodology could be extended to other lipophilic vitamins or bioactive compounds, in order to achieve their controlled release.

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References

Abe, T., Hasunuma, K., & Kurokawa, M. (1976). *Vitamin E orotate and a method of producing the same*. US: 3,944,550.
Amsden, B. G., Knight, D. K., & Shapka, S. N. (2007). Structure, depolymerization, and cytocompatibility evaluation of glycol chitosan. *Journal of Biomedical Materials Research Part A*, *83A*(3), 787–798.
Autier, P., & Gandini, S. (2007). Vitamin D supplementation and total mortality: A meta-analysis of randomized controlled trials. *Archive International Medicine*, *167*, 1730–1737.
Babak, V., & Desbriéres, J. (2010). Interfacial properties of chitin and chitosan based systems. *Soft Matter*, *6*, 2358–2363.
Basaran, E., Yazan, Y., & Yenilmez, E. (2010). Release characteristics of vitamin E incorporated chitosan microspheres and in vitro–in vivo evaluation for topical application. *Carbohydrate Polymers*, *84*(2), 807–811.
Beheshti, N., Kjøniksen, A.-L., Knudsen, K. D., Nyström, B., & Zhu, K. (2009). Rheological and structural aspects on association of hydrophobically modified polysaccharides. *Soft Matter*, *5*, 1328–1339.
Boudran, J., DeMarchis, M., Manno, N., Meyer, D., Muzzarelli, R. A. A., & Paoletti, M. G. (2012). Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial. *Carbohydrate Polymers*, *87*, 995–1012.
Chen, X., Choi, K., Choi, Y., Jeong, S. Y., Leary, J. F., Lee, D.-E., et al. (2011). Tumor-homing photosensitizer-conjugated glycol chitosan nanoparticles for synchronous photodynamic imaging and therapy based on cellular on/off system. *Biomaterials*, *32*(16), 4021–4029.
Chen, X. G., Kong, M., Liu, C. G., Liu, C. S., Meng, X. H., & Yu, L. J. (2008). Antibacterial mechanism of chitosan microspheres in a solid dispersing system against *E. coli*. *Colloids and Surfaces B: Biointerfaces*, *65*, 197–202.
Cho, Y. W., Chung, H., Jeong, S. Y., Kim, K., Park, J. H., & Son, Y. J. (2006). Preparation and characterization of self-assembled nanoparticles based on glycol chitosan bearing adriamycin. *Colloid Polymer Science*, *284*(7), 763–770.
Coll, Y., Curiel, H., Peniche, C., & Pérez, J. (2010). Microspheres of chitosan for controlled delivery of brassinosteroids with biological activity as agrochemicals. *Carbohydrate Polymers*, *80*, 915–921.
Domb, A. J., Muzzarelli, C., Muzzarelli, R. A. A., Ravi Kumar, M. N. V., & Sashiwa, H. (2004). Chitosan chemistry and pharmaceutical perspectives. *Chemical Reviews*, *104*, 6017–6084.
Gray, A. I., Mahmoud, A., Sadiq, L., Schätzleinä, A. G., Uchegbu, I. F., Waigh, R. D., et al. (2001). Quaternary ammonium palmitoyl glycol chitosan—A new polysoap for drug delivery. *International Journal of Pharmaceutics*, *224*, 185–199.
Guthrie, J. T., Perrier, S., Roy, D., & Semsarilar, M. (2009). Cellulose modification by polymer grafting: A review. *Chemical Society Reviews*, *38*, 2046–2064.
Holick, M. F. (2003). Vitamin D: A millenium perspective. *Journal Cell Biochemistry*, *88*, 296–307.
Jing-Mou, Y., Li-Yan, Q., Yi, J., & Yong-Jie, L. (2008). Self-aggregated nanoparticles of cholesterol-modified glycol chitosan conjugate: Preparation, characterization, and preliminary assessment as a new drug delivery carrier. *European Polymer Journal*, *44*, 555–565.
Muzzarelli, R. A. A. (2011). Chitin nanostructures in living organisms. In S. Gupta (Ed.), *Chitin: Formation and diagenesis* (pp. 1–34). Dordrecht: Springer.
Muzzarelli, R. A. A. (2009). Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone. *Carbohydrate Polymers*, *76*(2), 167–182.
Tian-Wei, T., & Xin-Yuan, S. (2002). Preparation of chitosan/ethylcellulose complex microcapsule and its application in controlled release of Vitamin D2. *Biomaterials*, *23*, 4469–4473.