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Hydrophobically-modified Chitosan Microspheres for Release of Diosgenin

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ABSTRACT

Chitosan microspheres (CS) prepared by water-in-oil emulsion/glutaraldehyde cross-linking-evaporation and simple coacervation/cross-linking with sodium tripolyphosphate were covalently linked to diosgenin hemiesters. The diosgenin content found using elemental analysis was ca. 6 to 42 wt-% and it showed dependence on the type of diosgenin hemiesters and on the method of preparation of the CS microspheres. Fourier transform infrared spectroscopy confirmed the hydrophobic functionalization of CS with the diosgenin hemiesters by amide bond formation. The effect of CS modification with diosgenin on the thermal properties was also studied using differential scanning calorimetry. Microsphere sizes determined using optical microscopy ranged from 60 to 700 μm , while scanning electron microscopy depicted morphology dependent on the selected method to obtain CS microspheres. In vitro release studies performed in aqueous medium indicated a drug release dependence on the diosgenin hemiester linkers, the steroid content and the acidity of the solution. Sustained diosgenin release in acidic aqueous solution (pH 6.0) reached from 34 to 81% after 48 h.

Keywords: chitosan microspheres; diosgenin; controlled release

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Introduction

Chitosan (CS), an heteropolysaccharide composed of $\beta(1\rightarrow4)$ linked glucosamine units, with some proportion of N-acetylglucosamine units, is obtained by extensive deacetylation of chitin [1]. CS itself is a biocompatible, biodegradable, nontoxic and mucoadhesive polysaccharide, widely used as delivery matrix for the release of drugs in humans and animals, and non-steroidal agrochemicals in agriculture [2,3]. The antifungal and antibacterial activity of chitosan and the reported ability to induce metabolic changes in plants allows it to increase the yield of crops, improving the germination of seeds and resistance against plagues [4,5].

Diosgenin, the most used substrate in synthesis of corticosteroids and some brassinosteroids, exhibits good anticancer activity [6]. On the other hand, synthetic brassinosteroids demonstrated antiproliferative, anticancer and anti-HIV activity in animals and humans upon in vitro bioassays [7,8]. Brassinosteroids are steroid plant hormones found in reproductive and vegetative plant tissues [9]. Different synthetic analogues of brassinosteroids are synthesized as agrochemicals [10]. Thus, the use of CS for controlled release of brassinosteroids allows potentiating the benefits of exogenous brassinosteroids application to plants as vegetal growth enhancers and protectors against plagues with the microbiocidal and stimulating effect of CS in crops [11,12].

This article reports on linking diosgenin hemiesters, as drug models of diosgenin-derived agrochemicals and potential anticancer drugs, to CS microspheres for controlled release. These delivery systems for diosgenin and diosgenin derivatives base their action in hydrolysis of the steroid-CS ester bond and diffusion from the CS matrix.

Materials and Methods

Chitosan (deacetylation degree, DD = 85.2% determined by $^1\text{H-NMR}$, $M_w = 2.55 \times 10^5$) 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 hemiesters were synthesized by base-catalyzed esterification of diosgenin with succinic, itaconic and maleic anhydrides in pyridine [13]. The solvents and chemicals employed were purchased from Sigma-Aldrich and used as received.

The structures of chitosan and diosgenin hemiesters (diosgenin hemisuccinate (MSD), diosgenin hemiitaconate (MID), diosgenin hemimaleate (MMD)) are shown in **Fig. 1**.

CS microspheres preparation

Chitosan microspheres (CS1) were obtained by simple coacervation-precipitation and cross-linking with sodium tripolyphosphate [14]. Briefly, a 2% (wt-%) CS solution in 2% (v/v) aqueous acetic acid was added dropwise to a 10% (w/v) NaOH in methanol. Dropping was performed manually using a syringe with a 22-gauge needle. The microspheres were rinsed with bi-distilled water to $\text{pH} < 8$ and then cross-linked in 1% (w/v) sodium tripolyphosphate aqueous solution at room temperature for 1 h. After cross-linking, microspheres were rinsed with bi-distilled water and stored at 4-8 °C for further use.

Chitosan microspheres (CS2) were also prepared in a water-in-oil emulsion using glutaraldehyde as cross-linker. To this end, a 2% (wt-%) CS solution in 1% (v/v) aqueous acetic acid was vigorously stirred at 1200 rpm. 50 mL of bi-distilled water was added and stirred for an additional 1 h. Span 40 (2 g), Span 80 (1 g) and 1L of cyclohexane were added. The emulsion was heated to 40 °C and stirred for another 1 h. 4 mL of aqueous glutaraldehyde solution (50 %, w/v) was slowly added dropwise for 15 min. The reaction was continued overnight. The CS microspheres were filtered out, washed twice with an excess of bi-distilled water, methanol, diethyl ether and dried at 50 °C in vacuum [15].

Eclipse E400 optical microscope (Nikon, Japan). The morphology of CS microspheres was studied with a JEOL JSM 6300F scanning electron microscope (Jeol Ltd., Japan) operating at 1 kV. Dried CS microspheres were sputtered on a graphite plate and analyzed without coating.

***In-vitro* drug release studies**

25 mg of diosgenin-modified CS microspheres were placed in a volumetric flask containing phosphate buffered saline (PBS) solution at total volume of 25 mL and incubated at 30 °C with constant agitation at 100 rpm. 1 mL of solution was periodically withdrawn of the flask and replaced with equal volume of fresh

solution to maintain sink conditions. The amount of released diosgenin was determined using UV detection (Genesys 10 UV–Vis Spectrophotometer, Thermo Spectronic, Rochester, NY, USA) 280 nm (diosgenin).

Results and discussion

The method employed to prepare the diosgenin-modified CS microspheres involved mild esterification (stirring at room temperature) of primary –OH at C6 position in CS microspheres with the diosgenin hemiesters and efficiently afforded steroid contents between 6 and 42 wt-% (see Table 1). Achieved diosgenin content was higher employing CS1 microspheres.

Table 1. Degree of substitution (DS) and steroid content (wt-%) by elemental analysis; dried particles sizes (d, um) and frequency distribution (FQ, %) of diosgenin-modified CS microspheres.

Sample	DS	wt-%	d (um)	FQ (%)
MSD-CS1	0.13	23.3	300 ± 50	100
MID-CS1	0.36	42.1	590 ± 80	100
MMD-CS1	0.06	12.0	700 ± 90	100
MSD-CS2	0.08	15.8	130 ± 30	100
MID-CS2	0.29	37.9	60 ± 20	57
			140 ± 20	43
MMD-CS2	0.03	6.3	250 ± 30	40
			380 ± 30	60

*CS1: chitosan microspheres obtained by simple coacervation-precipitation and cross-linking with sodium tripolyphosphate. **CS2: chitosan microspheres obtained by water-in-oil emulsion and glutaraldehyde cross-linking.

Characterization of diosgenin-modified CS microspheres

The blank and diosgenin-modified CS microspheres had spherical shape. After drying in vacuum at 60 °C, their size was significantly reduced and some of them showed irregular shapes when inspected with the optical microscope. The size distribution of the CS microspheres was evaluated after measuring the diameter of 85–90 particles. The size of CS microspheres showed dependence on the

selected preparation method. The mean values for CS1 microspheres varied from 250 to 790 um, while for CS2 microspheres they ranged from 40 to 280 um. These microsphere sizes are consistent with those reported in other studies [16], but smaller than the observed in diosgenin- and brassinosteroid-loaded CS microspheres [14]. CS2 microspheres containing MID and MMD showed bimodal size distributions. This was probably due to an aggregation of CS microspheres.

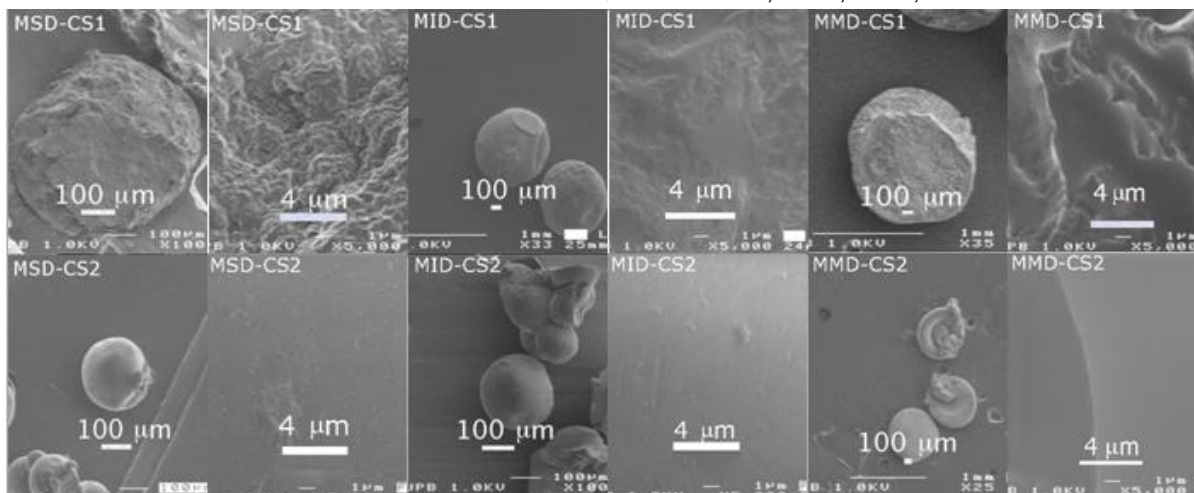


Fig. 2. Scanning electron micrographs of hydrophobically-modified CS microspheres at 100X and 5,000X magnifications (scale bars 100 μm and 4 μm, respectively) (to see structures in **Fig. 1**).

Fig. 2 shows the SEM images of CS1 and CS2 microspheres after diosgenin immobilization.

The diosgenin-modified CS1 microspheres presented some wrinkles on the surface and a porous surface at higher magnifications. On the other hand, diosgenin-modified CS2 microspheres showed smoother surface morphologies exhibiting a more compact structure at higher magnifications. These differences in surface morphologies may be attributed mainly at the preparation method of

the CS microspheres. The rougher surface of CS1 microspheres is probable related to a lower cross-linking degree of CS chains caused by sodium tripolyphosphate, which make CS more suitable to chemical modification with the diosgenin hemiesters. This might explain the higher degree of substitution observed in CS1 microspheres (**Table 1**).

The FTIR spectra of blank CS and diosgenin-modified CS microspheres are shown in **Fig. 3**.

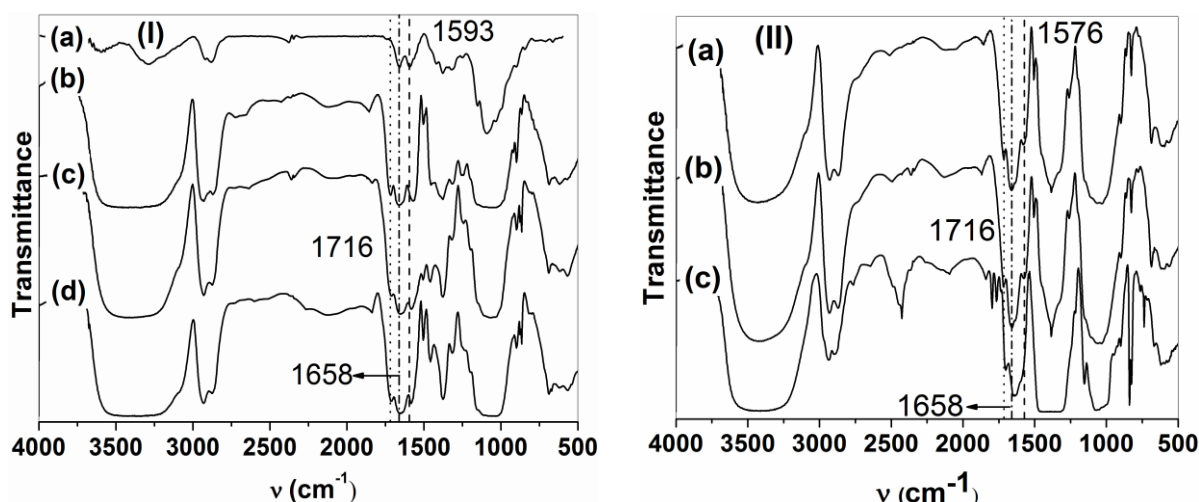


Fig. 3. FTIR spectra of (I) (a) CS, (b) MSD-CS1, (c) MID-CS1, (d) MMD-CS1; (II) (a) MSD-CS2, (b) MID-CS2, (c) MMD-CS2 (see structures in **Fig. 1**).

The IR spectrum of CS presented characteristic absorption peaks at 2942-2784 cm^{-1} (aliphatic C-H stretching band), 1658 cm^{-1} (Amide I) and 1593 cm^{-1} ($-\text{NH}_2$ bending) and 1321 cm^{-1}

(Amide III). The 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 [14].

The FTIR spectra of diosgenin-modified CS microspheres are dominated by the intense and broad CS absorption bands; however, the distinctive C=O absorption bands of formed diosgenin-CS ester bond are observed at 1713-1717 cm⁻¹ (**Fig. 3**). The Amide I and -NH₂ bands of CS at 1658 and 1593 cm⁻¹ overlapped the C=O absorption bands, producing a broad band ranging from 1700 to 1500 cm⁻¹.

The thermal behavior of CS samples has been shown to be strongly dependent on the natural source, the purity of samples and the preparation conditions [14,17]. However, the main thermal effects observed in a chitosan from a particular source can be interpreted on the basis of the thermal behavior of the CS obtained from other sources.

The thermogram of blank CS1 microspheres exhibited three endothermic peaks at 105.5, 167.4, and 181.1 °C; while the CS2 microspheres showed two endothermic peaks at 170.4 and 187.0 °C, respectively. Their onset and completion temperatures are listed in **Table 2**, together with their associated peak enthalpies (ΔH). The total ΔH of these effects is 115.1 J g⁻¹ and 124.1 J g⁻¹ for CS1 and CS2 microspheres, respectively. These endothermic effects must result mainly from the melting and dissociation of chitosan crystals, by comparison with reports for crab chitosans [14,17,18]. The DSC curves of diosgenin-modified CS microspheres present an intense endothermic peak between 165–209 °C (**Table 2**) associated with ΔH of 83–146 J g⁻¹. These peaks may be explained by the melting of CS crystals modified with the diosgenin hemiesters and dissociation of CS chains.

Table 2. Thermal properties and main thermal effects of parent and diosgenin-modified CS microspheres (see **Fig. 1** for structures).

Sample	Endotherm (°C)			ΔH (J g ⁻¹)
	Onset	Peak	Completion	
CS1	127.9	150.5	155.7	10.2
	155.7	167.4	177.2	26.2
	177.2	181.1	195.3	78.9
CS2	161.7	174.2	186.4	35.3
	188.1	192.3	199.5	91.5
MSD-CS1	177.2	196.3	229.4	145.1
MID-CS1	199.1	208.4	214.6	123.9
	155.1	165.0	180.4	82.9
MMD-CS1	154.9	160.7	171.4	29.9
	178.1	183.7	192.6	108.7
MSD-CS2	183.4	190.7	197.6	106.2
	152.4	166.5	176.2	57.3
MID-CS2	218.2	224.8	232.9	146.0
MMD-CS2	160.5	197.4	250.0	122.4

In vitro release studies

Fig. 4 (I) presents the UV absorption spectra of MSD-CS1 microspheres (25 mg) both in diosgenin solution (3.9×10⁻⁴ mol L⁻¹) and in buffer solutions (25 mL) at different acidic pH measured 30 min after preparation of the

suspension, whereas **Fig. 4 (II)** shows the diosgenin release profiles from diosgenin-modified CS1 microspheres in PBS solution (pH 6.0).

Diosgenin release is mainly pH dependent and is controlled by the ester hydrolysis of the

diosgenin hemiester in the diosgenin-modified CS microspheres (**Fig. 4** (I)). This permits to control the kinetics of the release by adjusting the pH.

The *in vitro* drug release profiles were governed by the dicarboxylic linker between diosgenin and CS. In all cases a sustained release of diosgenin is observed, which is characterized by an almost constant release rate (zero order

kinetics) during the first 12 h. The different release patterns might be due to different diosgenin ester hydrolysis rates. The observed general trend of diosgenin release rate was MSD-CS1>MID-CS1>MMD-CS1. This trend was different that the observed for diffusion controlled release of diosgenin hemiesters from diosgenin-loaded CS microspheres [14].

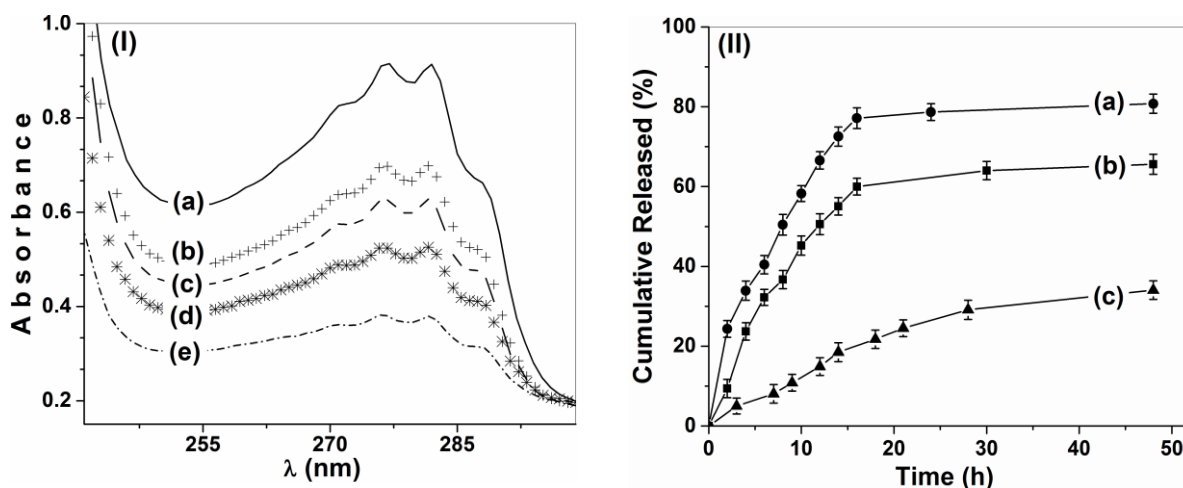


Fig. 4. (I) UV spectra of MSD-CS1 microspheres in (a) diosgenin at $3.9 \times 10^{-4} \text{ mol L}^{-1}$, (b) PBS at pH 2.0, (c) PBS at pH 4.0, (d) PBS at pH 5.0, (e) PBS at pH 6.0; (II) diosgenin release profiles from diosgenin-modified CS1 microspheres (a) MSD-CS1, (b) MID-CS1 and (c) MMD-CS1 in PBS solution (pH 6.0) at 30 °C (see **Fig. 1** for structures).

Conclusion

CS microspheres were successfully linked to diosgenin hemiesters as confirmed by ATR-FTIR spectroscopy and elemental analysis. The steroid contents found were between 6 to 42 wt-% and showed a dependence on the type of the employed diosgenin hemiester and the method of preparation of the CS microspheres. Particle sizes ranged from 40 to 790 μm, and the morphology was also dependent on the method of CS microsphere preparation. The esterification of CS was somewhat more efficient with the more coarse CS1 particles. The *in vitro* release studies performed in aqueous medium at different acidic pH indicated a drug release dependence on the dicarboxylic acid employed as linker, the degree of substitution of CS and the acidity of the solution. The period of diosgenin release

was extended to 48 h. These results indicate that by introducing the appropriate changes in the diosgenin-linker adduct, it would be possible to design efficient pH dependent delivery systems for sustained release of diosgenin and related agrochemicals as well as anticancer drugs.

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Abbreviations: attenuated total reflectance Fourier transform infrared (ATR-FTIR); chitosan (CS); N,N-dimethylacetamide (DMA); degree of deacetylation (DD); degree of substitution (DS); differential scanning calorimetry (DSC); frequency distribution (FQ); enthalpy (ΔH); human immunodeficiency virus (HIV); diosgenin hemisuccinate (MSD); diosgenin hemiitaconate (MID); diosgenin hemimaleate (MMD); phosphate buffered saline (PBS); scanning electron microscopy (SEM); ultraviolet (UV); weight percent (wt.%)

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