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Differential Scanning Calorimetry Evidence of the Enhancement of β-Sitosterol Absorption across Biological Membranes Mediated by β-Cyclodextrins

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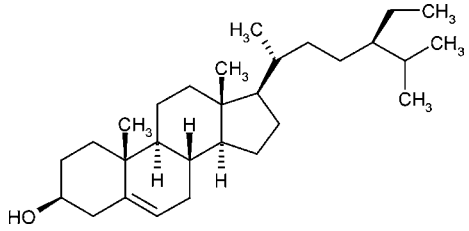
β-Sitosterol is a plant sterol that has received much attention because of its effectiveness in reducing the absorption of dietary cholesterol, as well as in offering protection from cardiovascular diseases and cancer development. Thus, the knowledge of the interaction of β-sitosterol with biological membranes can help in understanding its mechanism of action. In the present paper, the differential scanning calorimetry technique has been used to study the interaction of β-sitosterol with a biomembrane model constituted by dimyristoylphosphatidylcholine multilamellar vesicles. Furthermore, kinetic experiments have been carried out to follow the uptake of β-sitosterol by biomembranes and the effect of β-cyclodextrins on such a process. Our results indicate that opportune concentrations of β-cyclodextrins improve the uptake of β-sitosterol by phospholipid membranes.

KEYWORDS: β-Cyclodextrins; differential scanning calorimetry; dimyristoylphosphatidylcholine; model membranes; β-sitosterol

INTRODUCTION

Phytosterols, naturally occurring substances found in plants, are the counterparts of cholesterol in animal products. Phytosterols are present in a normal diet, consisting of β-sitosterol (**Scheme 1**), campesterol, and stigmasterol in the largest proportion in most sources, and are absorbed proportionally to cholesterol but to a much lesser extent. The interest in studying phytosterols has been initially due to their effectiveness in reducing the absorption of dietary cholesterol and thus offering protection from cardiovascular diseases (*1*). However, β-sitosterol has been shown to exhibit antiinflammatory, antineoplastic, antipyretic, and immunomodulating activities (*2*−*5*). It should be pointed out that in recent years a great deal of interest has been given to dietary factors, which could influence cancer development, and in particular to the types of dietary fats. Epidemiological and experimental studies suggest that dietary phytosterols may offer protection from the most common cancers in Western societies, such as colon, breast, and prostate cancers. In particular, β-sitosterol has been shown to inhibit cancer development by inhibiting tumor initiation and promotion and by inducing cell differentiation, although it is unclear if this effect is related to the cholesterol-lowering effects of phytosterols or to other yet unknown mechanisms (*6*). Other studies have suggested that phytosterols may have beneficial effects on prostate disorders.

Scheme 1 β-Sitosterol Structure



Oral exposure to phytosterols does not appear to cause adverse effects, but laboratory animals receiving phytosterols parenterally have demonstrated adverse effects including evidence of estrogenicity (*2*). In particular, very high plasma concentrations of sitosterol may have potentially cytotoxic effects and interfere with cellular functions. It seems evident that the adverse effects of phytosterols are dependent on bioavailability.

Together with fat-soluble vitamins and carotenoids, phytosterols are highly lipophilic vegetable food constituents (*7*); furthermore, while similar in structure to cholesterol, phytosterols possess substitutions at position C-24 that are responsible for their poor absorption. In fact, it has been suggested that in humans and other mammals only approximately 5% of ingested plant sterols are absorbed; furthermore, sitosterol accumulated in mucosal cells of sitosterol-fed animals, while plasma sitosterol has a high affinity for steroid-synthesizing tissues.

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Thus, several preparations have been formulated specifically to increase water solubility and thus the bioavailability of phytosterols. Water-soluble phytostanols have been formulated as lecithin-emulsified micelles with potential applications in nonlipid-based foods (*8*). However, micellar preparations of lipophilic pharmaceuticals are formulated specifically to increase their solubility and hence also their absorption from the gut. Phytosterols formulated with emulfiers into water-soluble micelles may be more soluble within the gut and have the potential for increased absorption when consumed orally (*9*).

Cyclodextrins (CDs), a family of cyclic oligosaccharides obtained from starch by enzymatic degradation, are composed of α-1,4-linked glucopyranose subunits (*10*). These macrocyclic carbohydrates with apolar internal cavities can form complexes with various biomolecules by means of intramolecular interactions of the type "host−guest" without the formation of covalent bonds; the binding of guest molecules with the CD host is not permanent but rather is a dynamic equilibrium.

Because of the inclusion complex forming properties, CDs are widely used in many industrial products, technologies, and analytical methods. In particular, hydrophilic CDs can solubilize and modify the release of poorly water-soluble drugs and, thus, may be used for the enhancement of drug absorption across biological membranes. Furthermore, CDs possess all characteristics (quality, cost performance, bioadaptability, and negligible toxic effects) required for drug carriers from the safety viewpoint.

β-CDs, which are composed of seven α-1,4-linked glycosyl units, are the most accessible, the lowest priced, and generally the most useful with respect to the other compounds of this class (*11*). In particular, complexes with β-CDs can significantly increase the water solubility and bioavailability of β-sitosterol. However, no systematic study has been carried out to characterize the efficiency of CDs complexes to improve β-sitosterol delivery and interaction with biological membranes. At this aim, we have investigated by means of differential scanning calorimetry (DSC) the interaction between β-sitosterol and dimyristoylphosphatidylcholine (DMPC) vesicles used as biomembrane models. DMPC vesicles are characterized by a sharp phase transition from an ordered gel-like structure to a disordered fluidlike structure upon heating. This phase change happens at a characteristic transition temperature (T_m), and it can be revealed by the DSC technique as an endothermic peak with a related enthalpy change (ΔH). The presence of foreign substances dissolved inside the lipid bilayer modifies the calorimetric curves depending on the kind of interaction between the foreign substance and the lipid (for instance, affecting the T_m and the ΔH), related to the amount of compound dissolved in the lipid matrix (*12*−*16*).

We have performed different series of experiments to detect the interaction between β-sitosterol and phospholipid vesicles and to follow the uptake of β-sitosterol by biomembranes in the presence or not of β-CDs, with the aim to demonstrate that the presence of CDs could improve the uptake process.

MATERIALS AND METHODS

Materials. β-Sitosterol (purity ≥95%) was purchased from Sigma (Germany). Synthetic l-α-DMPC was obtained from Genzyme (Switzerland). β-CD (purity ≥99%) was purchased from Fluka (Germany).

Lipids were chromatographically pure as assessed by two-dimensional thin-layer chromatography. Phospholipid concentrations were determined by phosphorus analysis following the procedure described by Rouser et al. (*17*). The buffer solution [50 mM Tris(hydroxymethyl)-aminomethane, Tris] was adjusted to pH 7.4 with hydrochloric acid.

Liposomes Preparation. Multilamellar vesicles (MLVs) were prepared in the absence and presence of increasing molar fractions of β-sitosterol. Two stock solutions of DMPC and β-sitosterol in chloroform/methanol (1:1; v:v) were prepared. Aliquots of the DMPC solution were delivered in glass tubes in order to have the same amount (0.01032 mmol) of lipid, and aliquots of the β-sitosterol solution were added in order to have the following molar fractions: 0.00, 0.015, 0.03, 0.045, 0.06, 0.09, 0.12, and 0.15 of compound with respect to the lipid. The solvents were removed under nitrogen flow, and the resulting films were freeze-dried under vacuum in order to eliminate eventual solvent residues. MLVs were obtained adding 168 μL of 50 mM TRIS buffer solution (pH 7.4), heating at 37 °C (a temperature higher than the DMPC phase transition temperature, 24.8 °C), shaking three times for 1 min, and keeping for 1 h at 37 °C in order to homogenize them, allowing them to reach the partition equilibrium between aqueous and lipidic phases. Afterward, 120 μL of MLV (0.007375 mmol of lipid) was transferred in 160 μL aluminum pans, hermetically sealed, and submitted to DSC analysis.

DSC. DSC was performed by using a Mettler TA Star^e equipped with a DSC 30 calorimetric cell and a Mettler STAR^e V 6.10 SW software. The scan heating rate employed was 2 °C/min in the temperature range of 2−37 °C.

Each sample was heated and then cooled three times in the temperature range chosen, in order to evaluate the data reproducibility demonstrating that the partition equilibrium between aqueous and lipidic phases was reached. The sensitivity of the calorimetric system was automatically chosen as the maximum possible by the calorimetric system, and the reference pan was filled with 120 μL of Tris buffer solution. The DSC was calibrated, in transition temperature and enthalpy changes, by using indium, stearic acid, and cyclohexane by following the procedure of the Mettler TA STAR^e Software. After the calorimetric analysis was carried out, aliquots of all samples were extracted from the calorimetric aluminum pans and used to determine, by the phosphorus assay (*17*), the exact amount of phospholipids present in each sample.

Permeation Kinetic. These experiments were carried out according to three experimental patterns: (i) in order to evaluate the β-sitosterol ability to go through the aqueous medium and reach and cross the membrane model, an exact amount of powdered β-sitosterol (to have a 0.12 molar fraction of compound with respect to phospholipid) was weighted in the bottom of the DSC pan and then added with 120 μL of DMPC MLV aqueous dispersion (0.007375 mmol of lipid); (ii) in order to evaluate the possibility to increase the rate and extent of these processes, the previously described kinetic measurements were also done in the presence of solid powdered β-CDs, keeping unchanged the β-sitosterol molar fraction (0.12) and varying the amount of β-CDs in order to get 1:0.5, 1:1, 1:2, and 1:20 mol:mol β-sitosterol/β-CDs ratios; (iii) to monitor eventual interactions between MLV and β-CDs, powdered β-CD amounts (the same used in the above experiments) were weighted in the bottom of the DSC pan and added with 120 μL of DMPC MLV aqueous suspension (0.007375 mmol of lipid).

The samples were hermetically sealed, and the interaction between β-sitosterol and DMPC (with or without β-CDs) and between β-CDs and DMPC was detected submitting the samples to the following calorimetric analysis: (i) a heating scan between 2 and 37 °C at the rate of 2 °C/min; (ii) an isothermal period (1 h) at 37 °C; and (iii) a cooling scan between 37 and 2 °C at the rate of 2 °C/min.

The procedure was repeated eight times to follow the variations in the calorimetric curves in comparison with that of pure DMPC, which indicates the occurrence of interactions between the tested compounds and the DMPC bilayers.

RESULTS AND DISCUSSION

The effect of phytosterols on phospholipid model biomembranes has received some attention, and several studies have been carried out. The effect of phytosterols on the surface pressure area isotherm of a monolayer on water and on the permeability, fluidity, and polarity of a DPPC bilayer membrane has been reported (*18*). The ability of some plant sterols to

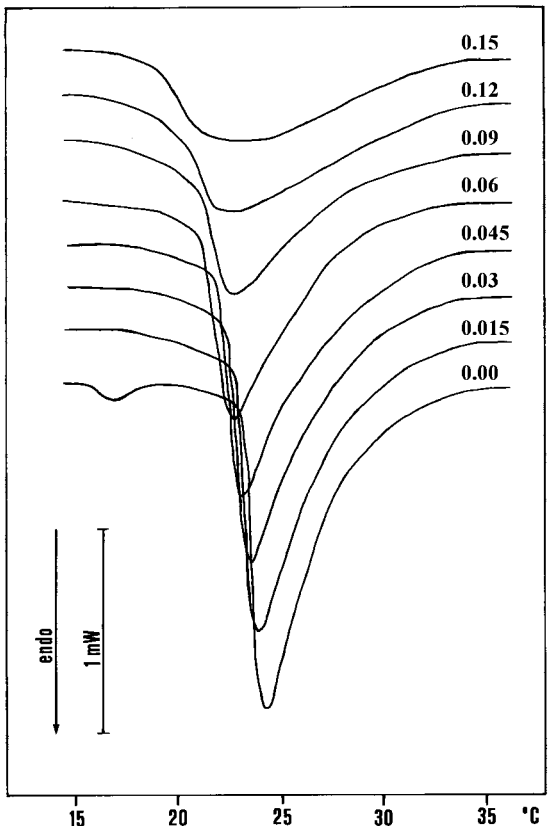

Figure 1. Calorimetric curves, in heating mode, of DMPC MLV prepared in the presence of increasing molar fraction of β-sitosterol.

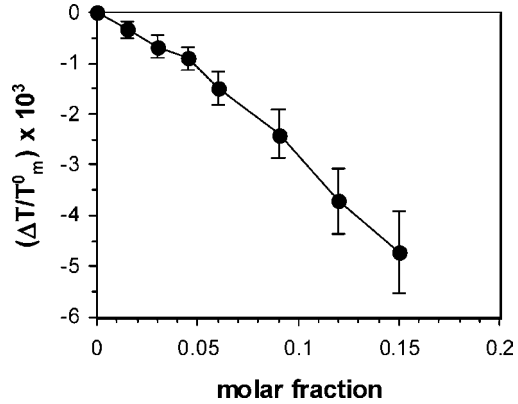

Figure 2. Transition temperature variations of DMPC MLV prepared in the presence of increasing molar fractions of β-sitosterol, expressed as $\Delta T/T_m^0$ ($\Delta T = T_m - T_m^0$, where T_m^0 is the transition temperature of pure DMPC MLV and T_m is the transition temperature of DMPC MLV prepared in presence of β-sitosterol), as a function of β-sitosterol molar fraction in the MLV aqueous dispersion.

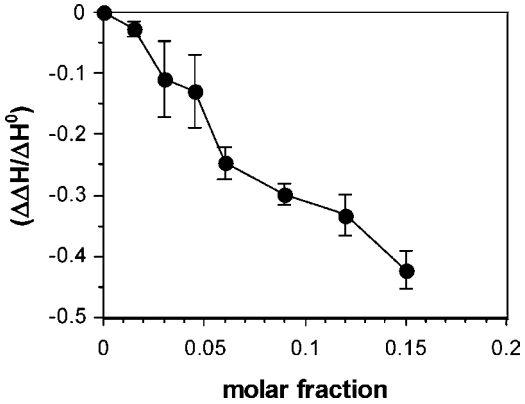

Figure 3. Enthalpy change variations of DMPC MLV prepared in the presence of increasing molar fractions of β-sitosterol, expressed as $\Delta\Delta H/\Delta H^0$ ($\Delta\Delta H = \Delta H - \Delta H^0$, where ΔH^0 is the enthalpy change of pure DMPC MLV and ΔH is the enthalpy change of DMPC MLV prepared in presence of β-sitosterol), as a function of β-sitosterol molar fraction in the MLV aqueous dispersion.

regulate water permeability and acyl chain ordering of soybean phosphatidylcholine bilayers has been investigated (*19*). Halling and Slotte have compared the effect of different phytosterols and their interaction with DPPC bilayers (*20*). However, nothing has been reported on the interaction between β-sitosterol and DMPC as well as on its ability to interact with biomembranes in the presence of β-CDs. In this paper, we have investigated by means of DSC the capability of β-sitosterol to interact with a biomembrane model constituted by DMPC MLV and the transfer kinetics from the aqueous medium surrounding MLV to the bilayer surface, also in the presence of different amounts of β-CDs in the aqueous medium. The procedure previously described to study the interaction of bioactive compounds with DMPC MLV (*21*−*24*) has been followed here.

The calorimetric analyses have been taken in the temperature range between 2 and 37 °C, where DMPC exhibits a main calorimetric peak at 24.8 °C, associated with the phase transition from an ordered to a disordered state, and a small calorimetric peak (the pretransition peak) at about 16.5 °C, related to the hydrophobic chains tilt (*25*, *26*). The calorimetric curves of DMPC MLV alone or in the presence of increasing molar fraction of β-sitosterol are reported in **Figure 1** to demonstrate the sterol interaction with the membrane model through the variation of the membrane thermotropic behavior. The addition of β-sitosterol to MLV causes the disappearance of the pretransition peak and the broadening and the shift toward lower temperature of the main calorimetric peak. The T_m values as well as the ΔH values obtained from the calorimetric curves have been plotted in **Figures 2** and **3** as respectively $\Delta T/T_m^0$ ($\Delta T = T_m - T_m^0$, where T_m is the value of transition temperatures of DMPC vesicles in presence of β-sitosterol, while T_m^0 is the transition temperature of pure DMPC MLV) and $\Delta\Delta H/\Delta H^0$ ($\Delta\Delta H = \Delta H - \Delta H^0$, where ΔH is the value of enthalpy change of DMPC vesicles in the presence of β-sitosterol, while ΔH^0 is the enthalpy change of pure DMPC MLV) against β-sitosterol molar fraction present in the aqueous lipid dispersion. It is evident as β-sitosterol provokes a decrease of the T_m and the ΔH proportional to its concentration inside the lipid membrane (related to the amount of compound present in the MLV aqueous dispersion); a similar trend has been previously reported for phosphatidylcholine bilayers containing β-sitosterol by Halling and Slotte (*20*). These results indicate that β-sitosterol interacts with the membrane model in a concentration-dependent way.

It is known that plant sterols are absorbed from the intestine to a lower extent in comparison to cholesterol. When absorbed, plant sterols are transported in lipoproteins and may be get incorporated in cellular membranes. Several studies have evidenced the effects of plant sterols in phospholipid membranes, such as effects on phospholipid condensation, membrane permeability, phospholipid order, membrane interfacial qualities, and membrane domains (*20*). In particular, a change in sterol content could be critical for the membrane functions of erythrocytes, which lack the ability to adjust the sterol content of their plasma membrane.

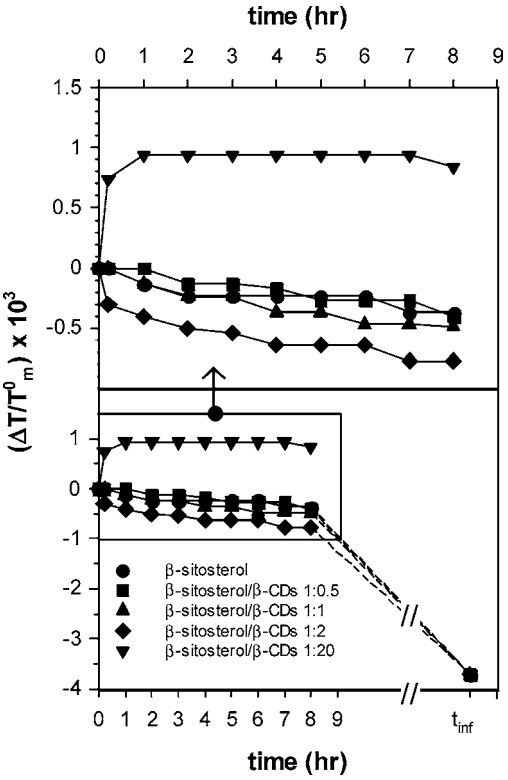

Figure 4. Transition temperature variations of DMPC MLV left in contact with β-sitosterol (0.12 molar fraction) or with β-sitosterol and β-CDs, at different ratios, as a function of the incubation time. t_{inf} value represents the T_m variation of DMPC MLV prepared in presence of 0.12 molar fraction of β-sitosterol homogeneously dissolved inside the lipid bilayer and is considered as the maximum interaction between the compound and the MLV.

These curves are considered as the maximum effect caused by the sitosterol when homogeneously dispersed inside the lipid model membranes; as they relate, amounts of sterol and interaction are employed (the T_m data) as reference in the following experiments to monitor the sitosterol transfer from the solution to the model membranes.

Permeation kinetic experiments have been carried out leaving DMPC MLV in contact with β-sitosterol to evaluate the capability of the sterol to pass through the aqueous medium, reach the MLV surface, and interact with phospholipid bilayers. Given that β-sitosterol is a very hydrophobic compound, we have carried out kinetic experiments with MLV and β-sitosterol in the presence of increasing ratios of β-CDs, as a solubilizing agent, to determine if β-CDs favors the sterol dissolution in the aqueous medium and the migration rate (through the medium), by the formation of inclusion complexes (*27*, *28*) "on situ" avoiding the formation of the complexes during a formulation process. This procedure should allow the sterol to more easily reach the MLV outer lipid layer, improving its interaction with the membranes. The interaction between a compound and the MLV is revealed by a change in the shape and/or in the thermodynamic parameters of the calorimetric curves with respect to that of pure DMPC. To state that any variation in the calorimetric curves (when β-sitosterol was employed together with β-CDs) was due to β-sitosterol and not to β-CDs, control kinetic experiments have been done in the presence of β-CDs alone at the same concentration used in the kinetic experiments with both β-sitosterol and β-CD. We will call these β-CDs samples as β-CDs$_{0.5}$, β-CDs$_1$, β-CDs$_2$, and β-CDs$_{20}$; the subscript number indicates that β-CDs have been

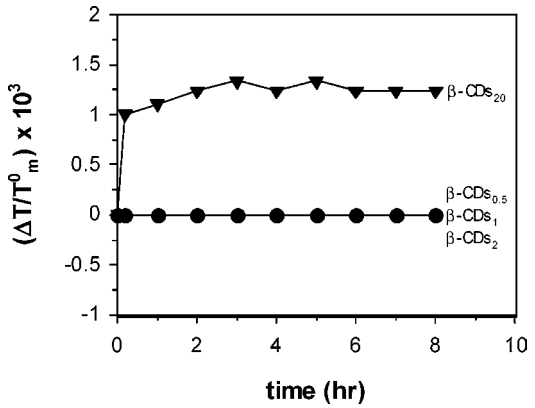

Figure 5. Transition temperature variations of DMPC MLV left in contact with different amounts of β-CDs as a function of the incubation time.

used in the experiments with MLV and β-CDs at the same molar ratio used in the experiments with MLV, β-sitosterol, and β-CDs.

A negligible shift toward a lower value of temperature of the main calorimetric peak and no evident change in the pretransition peak is seen in curves related to β-sitosterol transfer to MLV. The curves related to MLV in the presence of β-sitosterol/β-CDs show the gradual disappearance of the pretransition peak and a shift of the main calorimetric peak toward lower temperatures (1:0.5, 1:1, and 1:2 ratio) or toward higher temperatures (1:20 ratio). Interestingly, the shift depends on the β-sitosterol/β-CDs ratio. The curves of the control experiments obtained by employing MLV and free β-CDs (β-CDs$_{0.5}$, β-CDs$_1$, and β-CDs$_2$) show no variation of the peak shape and T_m with respect to the pure DMPC even after a long incubation time. Conversely, when β-CDs$_{20}$ have been used, the disappearance of the pretransition peak together with the shift of the main peak toward higher temperature has been seen in the calorimetric curves (data not shown).

Figure 4 shows the results of the previously described kinetic experiments with MLV and β-sitosterol (0.12 molar fraction) in the absence and in the presence of increasing concentrations of β-CDs. In the figure, t_{inf} is the transition temperature variation of MLV prepared in the presence of a 0.12 molar fraction of β-sitosterol homogeneously dissolved in the lipid matrix (as reported in Liposomes Preparation) and represents the maximum effect induced by the interaction between MLV and β-sitosterol at a given molar fraction. This figure shows the T_m variation (as $\Delta T/T_m^0$) and compares the t_{inf} and the variation recorded after 8 h of incubation between MLV and β-sitosterol. An *increase* of the $\Delta T/T_m^0$ value is observable at 1:20 β-sitosterol/β-CDs ratio; in all of the remaining cases, a *decrease* in the temperature variation is seen. It is worth noting that none of the β-sitosterol/β-CDs mixtures reaches the value observed for the pure sterol homogeneously dissolved in the lipid matrix (t_{inf}). The higher the β-CDs amount is, the more evident the $\Delta T/T_m^0$ decrease up to 1:2 β-sitosterol/β-CDs ratio is. In fact, a small $\Delta T/T_m^0$ decrease is visible for β-sitosterol and is not changed by the presence of β-CDs in the ratio 1:0.5. A 1:1 ratio is needed to provoke a bigger $\Delta T/T_m^0$ decrease but just after 4 h of incubation. Finally, a 1:2 ratio causes a well-defined decrease in the $\Delta T/T_m^0$. At a 1:20 β-sitosterol/β-CDs ratio, a $\Delta T/T_m^0$ increase is obtained, indicating an anomalous behavior at this ratio.

In **Figure 5**, the results related to the interaction between MLV and β-CDs are reported. While a flat line is obtained with β-CDs$_{0.5}$, β-CDs$_1$, and β-CDs$_2$, an increase in the transition

temperature variation value is observable when using β-CDs$_{20}$. The results demonstrate that the low amounts of CDs chosen by us and usually employed to form inclusion complexes (1:1 molar ratio) (*29*, *30*) can be added without modifying the DMPC phase transition, a positive $\Delta T/T_m^0$ shift being, instead, observed when very high amounts of β-CDs are employed. Thus, we can exclude any effect of β-CDs on DMPC when low ratios of β-CDs are employed together with β-sitosterol, allowing us to attribute only to β-sitosterol the observed effects on the DMPC phase transition.

Several mechanisms responsible for the limited absorption rate of plant sterols have been suggested, evidencing that it is due to a combination of factors. In particular, micellar solubility and the slow transfer rate from the cell surface to intracellular sites are the major factors affecting sitosterol absorption rate (*31*, *32*).

Taking together our findings, we can confirm that β-sitosterol by itself, being very hydrophobic and thus unable to pass through the aqueous medium, interacts only at a slight extent with the phospholipid bilayers. This is consistent with the well-known poor availability of orally ingested β-sitosterol. Furthermore, the presence of suitable concentrations of β-CDs can improve the MLV/β-sitosterol interaction. In fact, β-CDs encapsulate β-sitosterol and make it more suitable to diffuse in the aqueous medium and to come in contact with the MLV surface, allowing its permeation through biomembranes (model mimicking a cellular absorption). While a high β-CDs concentration causes changes in the thermotropic behavior of DMPC with an increase of the T_m. The T_m increase of the dipalmitoylphosphatidylcholine biomembrane model due to the interaction with β-CDs was already reported by some researchers studying the effects of sugars on phospholipid membranes (*33*−*35*). Our results are in agreement with the literature data considering that the increase of the temperature of the gel−liquid crystalline phase transition is due to the stabilizing effect of carbohydrates on phospholipid bilayers through the formation of hydrogen bonding most likely at the headgroup phosphate group (*36*). Moreover, it has also been demonstrated that a high CDs concentration can catalyze sterol desorption from monolayer and bilayer membranes, hindering in our experimental conditions to the sitosterol to interact with phospholipids (*37*, *38*). Thus, the choice of the opportune β-sitosterol/β-CDs molar ratio might also be important to avoid modifications in lipid biomembranes challenged by excessive β-CD concentrations.

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