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Original Research Paper

Development of a novel electrospun nanofibrous delivery system for poorly water-soluble β-sitosterol

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ABSTRACT

Electrospinning was used as a novel technique for fabricating polymeric nanofibers of a serum cholesterol lowering and poorly water-soluble plant sterol, β-sitosterol. Chitosan was used as a stabilizer/carrier polymer. The mean diameters of nanofibers ranged from 150 nm to 218 nm. β-sitosterol was in an amorphous form and homogeneously dispersed in the nanofibers. The β-sitosterol-loaded nanofibers were freely water-soluble and exhibited very short lag-time in releasing the plant sterol. The dissolution was associated with an immediate recrystallization of β-sitosterol in submicron level. In conclusion, electrospinning is a promising future technology for the formulation of poorly water-soluble plant sterols.

1. Introduction

Plant sterols, such as β-sitosterol and its saturated form β-sitostanol (Fig. 1), have a serum cholesterol lowering effect when administered orally [1–3]. They are generally applied in phytosterol supplementation either as dissolved or dispersed in food ("functional food preparations"), or as pharmaceutical oral preparations [4]. The main challenges associated with the oral administration of the most common plant sterols (or phytosterols) are poor water solubility and high daily doses (i.e., up to 3 g/day for β-sitosterol) [3,4]. Furthermore, their solubility in edible fats and oils is very limited [5]. The physical state of the plant sterols has been shown to affect their cholesterol absorption reducing effect [3,5,6].

To date, only few strategies have been proposed for improving the oral administration and efficacy of plant sterols. These have included a microcrystalline β-sitosterol suspension

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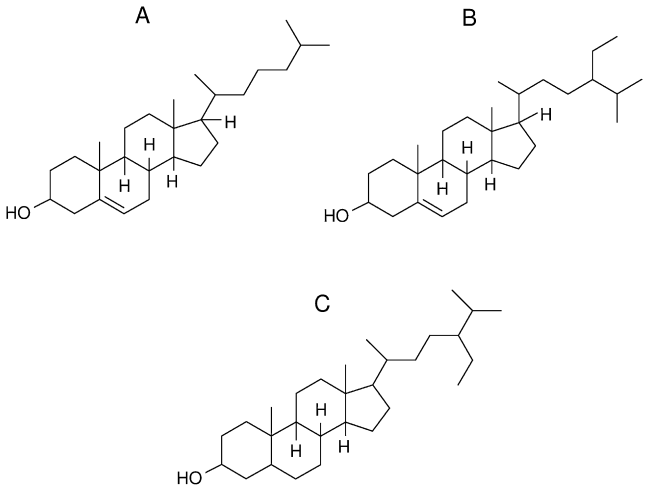


Fig. 1 – Molecular structure of (A) cholesterol, (B) β-sitosterol and (C) β-sitostanol.

in oil [3], β-sitostanol oil suspension in capsules [7], powdered plant sterol dissolved/dispersed in food (margarine) [8], esterification of plant sterols [5], and more recently plant sterols combined with, or esterified to, fish-oil fatty acids [9]. Each strategy has some advantages in clinical effectiveness, but there is still no ideal oral preparation for plant sterols. In spite of these novel formulation approaches, the oral administration of the plant sterols in the management of hypercholesterolemia has been questioned [10].

Nanofibers hold today great promises for a wide range of biomedical and pharmaceutical applications, including tissue engineering, drug delivery systems and solubility/stability enhancement [11–13]. Electrospinning (ES) is an effective and up-scalable continuous method to fabricate nanofibers with a diameter from a few tenths of nanometers to several micrometers, and a large surface area [12]. Recently, this technique has been used to fabricate high-energy amorphous solid dispersions (SDs) of poorly water-soluble drugs [14–16]. It is generally known that the amorphous form of a drug has an enhanced dissolution and bioavailability compared to the crystalline counterpart, but the amorphous form is physically very unstable. We hypothesize that the application of ES and hydrophilic carrier polymer(s) could be an interesting approach for stabilizing plant sterols in an amorphous form, and consequently, improving their dissolution and bioavailability. To the best of our knowledge, ES or any other related processes have not been applied to formulate plant sterols into nanofibers.

The present work aims to investigate ES as a novel technique for preparing amorphous polymeric nanofibers of poorly water-soluble β-sitosterol and a hydrophilic polymer (chitosan). In this proof-of-the-concept study, the main hypothesis was that the electrospun nanofibers could improve the solubility and dissolution behavior of β-sitosterol by affecting the solid state of the active substance. Special attention was paid to the effects of a carrier polymer and solvent system on the solid-state properties, wetting and early-stage dissolution of the nanofibers.

2. Materials and methods

2.1. Materials

β-sitosterol (Calbiochem®, Merck Millipore, Merck KGaA, Darmstadt, Germany) contained 75.5% β-sitosterol, β-sitostanol (13%), campesterol (8.4%), campestanol (1.3%), and contaminants (1.8%) as related substances. Chitosan Ch 90/160 (Primex BioChemicals AS, Haugesund, Norway) was used as a stabilizer and carrier polymer in nanofibers. The solvents tested for ES were ethanol (96% w/V) (Lach-Ner s.r.o., Brno, Czech Republic), acetic acid (Sigma-Aldrich Chemie GmbH, Munich Germany), trifluoroacetic acid (TFA, Riedel-de Haën®, Seelze, Germany) and 1,1,1,3,3,3-hexa-fluoro-2-propanol (HFIP, Sigma-Aldrich BVBA Diegem, Belgium).

2.2. Selection of solvent system for electrospinning

The solubility/miscibility of crystalline β-sitosterol and the carrier polymer (chitosan) in different solvent systems was tested based on the visual inspection and based on the values obtained from the literature. A total of 350.0 mg (175 mg β-sitosterol + 175 mg chitosan) of solid material was placed into a glass vial and the organic solvent (5.0 ml) was added by manually stirring with a glass rod. The total concentration (w/V) of solids was 7.0% (w/V), and the ratio of β-sitosterol and chitosan was 1:1. Alternatively, the corresponding binary solvent mixtures (2.5 + 2.5 ml or 1.67 + 3.33 ml) were added by stirring with a glass rod. The appearance of solution/dispersion was visually inspected within regular intervals for subsequent 24 hours.

2.3. Electrospinning of the nanofibers

The β-sitosterol-chitosan SD nanofibers were prepared using an ESR200RD robotized ES system (NanoNC Co. Ltd., Seoul, Republic of Korea). A schematic diagram of the ES process is shown in Fig. 2. The ES set-up consisted of a high-voltage power supply (Model HV30; maximum voltage 30 kV), a robotic-controlled automatic syringe pump (type X1EA, min. 0.01 μm/min, 10 ml installation), a plastic Norm-Ject® syringe (Henke Sass Wolf, Tuttlingen, Germany) with a needle size of 23G (inner diameter 0.34 mm, outer diameter 0.64 mm), and a grounded collector plate. The flow rate of the solution was 0.5 ml/h and the voltage applied was 10–12 kV. The distance between the spinneret and the fiber collector was 10 cm. All experiments were carried out at room temperature (22 ± 2 °C) and 18–25% RH.

The ratio of β-sitosterol and chitosan used in the nanofibers was 1:1 (w/w), and the total solid concentration in the organic solvent system was 7.0% (w/w). The final theoretical concentration of β-sitosterol in the electrospun nanofibers at a dried state was 50% (w/w).

2.4. Characterization of the nanofibers

The size (diameter) and morphology of the nanofibers were investigated with a scanning electron microscope (SEM, Zeiss EVO® 15 MA, Germany). All samples were mounted on aluminum stubs with a double-sided tape and then coated (Agar Sputter Coater, Agar Scientific, Stansted, United Kingdom) with

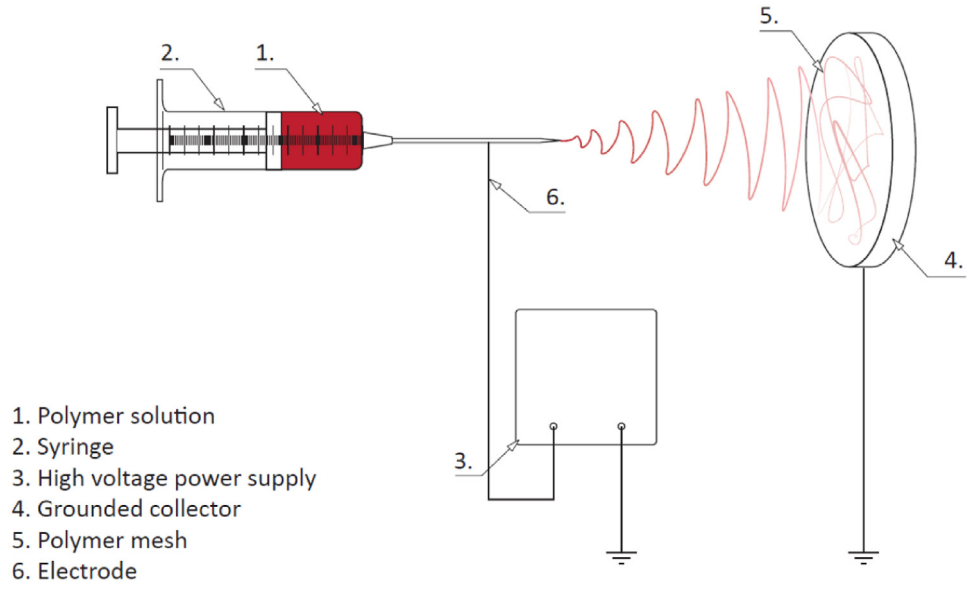


Fig. 2 – Schematic diagram of the electrospinning set-up.

a 3-nm gold layer (100 sec/25 mA) in an argon atmosphere prior to microscopy. The ImageJ software (Version 1.49) was used to measure the nanofiber size distribution.

2.5. *Solid-state properties analyses*

The solid-state properties of the starting materials and the electrospun nanofibers were studied by X-ray powder diffraction (XRPD, D8 Advance, Bruker AXS GmbH, Germany). The XRPD experiments were carried out in a symmetrical reflection mode (Bragg-Brentano geometry) with CuK_α radiation (1.54 Å). The scattered intensities were measured with a LynxEye one-dimensional detector with 165 channels. The angular range was from 3° 2θ to 70° 2θ with steps of 0.0185° 2θ. The total measuring time was 83 seconds per step. The operating current and voltage were 40 mA and 40 kV, respectively.

Raman spectra were collected using a Raman spectrometer (B&W TEK, Inc., Newark, DE, USA) equipped with a fiber optic probe. The laser source was a 300-mW diode laser system which is operating at 785 nm.

Differential scanning calorimetry (DSC, DSC4000, PerkinElmer Ltd., Waltham, Massachusetts, USA) was used for investigating the thermal behavior of both the pure materials and the nanofibrous samples.

2.6. *Wetting and dissolution measurements*

The wetting and dissolution properties of the SD nanofibers were monitored *in situ* by using an optical microscope (magnification 50×) (Leica DMLB, Germany) equipped with a digital camera (Canon PowerShot S50, Canon, Japan). A nanofibrous sample was placed onto the microscope glass plate, and subsequently 1–2 standard drops of aqueous solution were carefully added onto the samples at room temperature (22 ± 2 °C). The solutions studied were (1) an aqueous buffer solution pH 1.2 (KCl/HCl), (2) phosphate buffer pH 7.2, and (3) purified water. Micrographs were taken in regular intervals every 2 seconds, and for some experiments 10 s video clips were recorded.

3. Results and discussion

3.1. *Electrospinning of β-sitosterol loaded nanofibers*

So far, there are no studies in the literature describing the formulation of plant sterols into nanofibers in order to improve their physical solid-state stability, dissolution and bioavailability. This is obviously due to the challenging physicochemical properties of such plant sterols and their derivatives, including crystallinity (and polymorphism), poor water solubility, limited solubility in oils and fats, and rather large doses needed for effective lowering of the serum cholesterol levels. The major ES process-related challenge is also to find a proper carrier polymer and solvent system.

Table 1 presents the results of the solubility/miscibility of β-sitosterol and chitosan in purified water and different organic solvents/solvent systems. The test was based on visual inspection. Both β-sitosterol and chitosan were practically insoluble in purified water, but they dissolved in HFIP and TFA and in their mixtures. Consequently, non-aqueous and readily evaporating HFIP and TFA were selected for further ES experiments with β-sitosterol and chitosan. The 1/1 TFA/HFIP mixture was selected, since HFIP is more readily evaporating solvent, which is an advantage in ES. In addition, by increasing the amount of TFA (the 2/1 mixture), the viscosity of the solvent system decreased and the formation of nanofibers was prevented (only dots were obtained). Regarding the use of TFA/acetic acid, the present solvent system was not electrospinnable, even though the viscosity of the mixture was at a proper level (both TFA and acetic acid are not readily evaporating). The formation of nanofibers was not successful, resulting in very brittle nanofibers.

Table 1 – Solubility/miscibility of crystalline β-sitosterol and chitosan in different solvent systems.

Exp.	Material	Solvent	Concentration (w/w)	Comments
1	β-sitosterol	Ethanol	3.5%	White dispersion with sedimentation, not viscous
2	β-sitosterol	HFIP	3.5%	Violet, clear solution, not viscous
3	β-sitosterol	TFA	3.5%	Dark, brownish-purple dispersion, not viscous
4	Chitosan	TFA	3.5%	Clear, yellow, viscous solution
5	Chitosan	Acetic acid	3.5%	White-yellow (creamy), cloudy, low viscous dispersion
6	β-sitosterol/chitosan 1:1 (w/w)	Ethanol/acetic acid	3.5%	Creamy, white dispersion with yellow sedimentation, low viscosity
7	β-sitosterol/chitosan 1:1 (w/w)	HFIP/acetic acid	3.5%	Yellowish-white gel, high viscosity dispersion
8	β-sitosterol/chitosan 1:1 (w/w)	TFA/acetic acid	3.5%	Dark brown, clear, high viscous solution
9	β-sitosterol/chitosan 1:1 (w/w)	TFA	3.5%	Brownish-purple, high viscous solution
10	β-sitosterol/chitosan 1:1 (w/w)	TFA/ethanol	3.5%	Yellowish-white dispersion (with sedimentation), low viscosity
11	β-sitosterol/chitosan 1:1 (w/w)	TFA/HFIP 2:1 (w/w)	3.5%	Dark purple, clear solution, high viscosity
12	β-sitosterol/chitosan 1:1 (w/w)	TFA/HFIP 1:1 (w/w)	3.5%	Dark purple, clear solution, high viscosity

HFIP = hexafluoroisopropanol; TFA = trifluoroacetic acid.

ES of β-sitosterol-loaded chitosan nanofibers using TFA and HFIP as solvents was performed successfully without any significant drawbacks. The mean diameters measured by SEM of the electrospun SD nanofibers were 217.6 ± 98.6 nm (TFA) and 149.7 ± 69.1 nm (TFA + HFIP) depending on the solvent system used (Figs. 3 and 4). Individual fiber diameters ranged from 50 nm to 750 nm. It is evident that changing the solvent system from a single solvent (TFA) to a binary solvent mixture (TFA + HFIP) influences the viscosity of the jet, thus affecting the nanofibers' diameter (see also Table 1). According to Williams et al. [17], increasing the viscosity of the solvent system will allow the electrospun jet to withstand greater Coulombic repulsions, giving generally a large fiber diameter. The Coulombic repulsion forces are formed between the charges on the jet surface, and once these repulsive forces overcome the surface tension of the liquid a jet is ejected from the apex of the cone (the cone-jet) [17,18]. The Coulombic repulsion forces are directly proportional to the applied voltage and reduce as the applied voltage on the emitter needle decreases [19].

The solvent system affected also the distribution of fiber diameter and the surface morphologies of the nanofibers (Figs. 3 and 4). Interestingly, the SEM images displayed individual nanoparticles (with an average diameter of ~ 100–200 nm) embedded in the network of the nanofibers. This suggests concomitant ES and electrospraying, resulting in the formation of nanoparticles in the nanofibrous network as "by products." This phenomenon can be controlled by varying the electrospun solution (e.g. polymer concentration) or processing (voltage, collection distance parameters). Since the present study is more like a proof-of-the-concept work, the experimental parameters of ES were not fully optimized. However, this would be an important topic area for future studies. As seen in Fig. 3B, some individual spherical beads ("bulbs") were observed in the nanomats electrospun with the binary solvent system TFA + HFIP. According to the literature, the beads are related to the instability of the jet of polymer solution, and they are generally formed if the surface tension of a solution is high or if the viscosity of a solution is low [17,20,21]. The solution surface tension and viscosity are mainly dependent on the solvent system and polymer concentration, respectively. Moreover, the net charge density carried by the jet of ES enhances

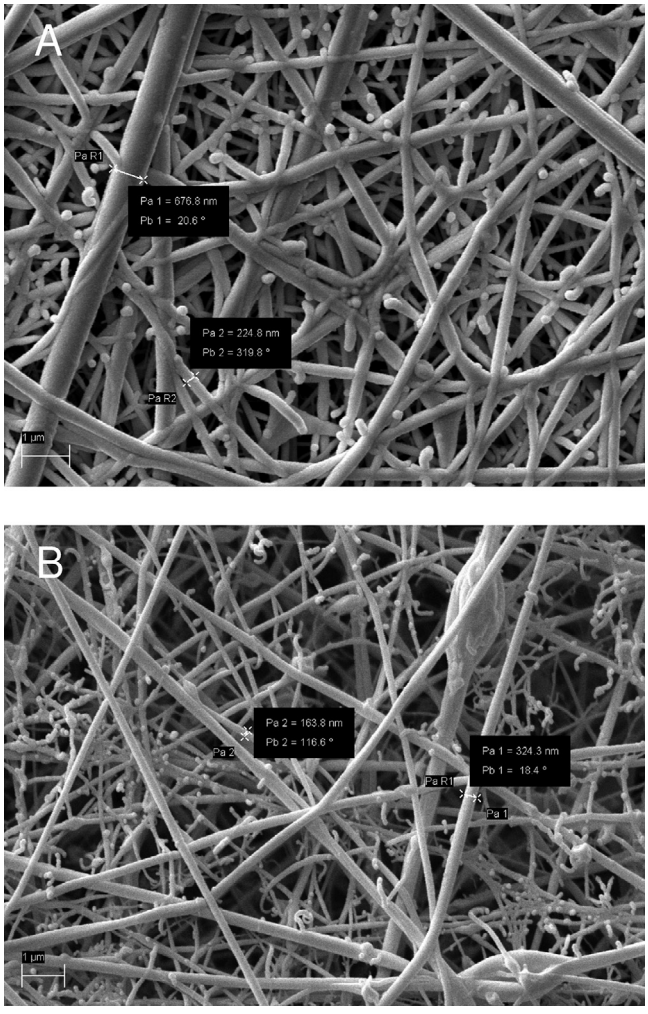
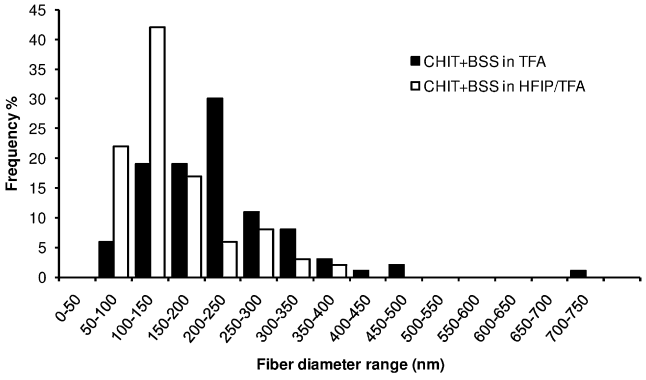


Fig. 3 – Scanning electron microscopy (SEM) micrographs of the electrospun chitosan nanofibers loaded with β-sitosterol. Key: (A) Single solvent system (TFA); (B) binary solvent system (TFA + HFIP). Magnification 20,000×.



(nm)	CHIT+BSS in TFA	CHIT+BSS in HFIP/TFA
Median	208,4	130,1
Mean	217,6	149,7
SD	98,6	69,1
Min	62,0	51,8
Max	737,8	363,1

Fig. 4 – SEM fiber diameter size distributions of the electrospun chitosan nanofibers loaded with β-sitosterol (BSS) and the electrospun with a single solvent system (TFA) or binary solvent system (TFA + HFIP).

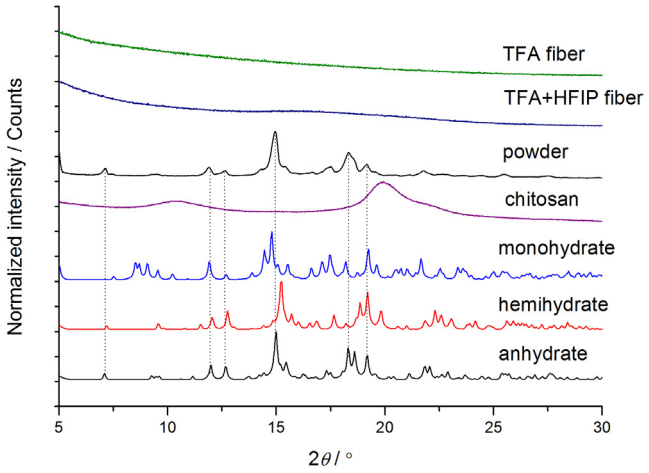


Fig. 5 – XRPD patterns for a commercial β-sitosterol powder (black line), chitosan (purple line) and electrospun nanofibers containing β-sitosterol and chitosan (1:1). TFA (green line) and the TFA/HFIP mixture 1:1 (blue line) were used as solvent systems for preparing the nanofibers. Theoretically calculated XRPD patterns of the three different crystal forms of β-sitosterol at room temperature are shown as references (obtained from the Cambridge Structural Database): anhydrate (LOFFET) (black line), hemihydrate (JOGMUP) (red line), monohydrate (TEXQOC) (blue line). Dotted line shows the peak positions of the characteristic β-sitosterol anhydrate XRPD reflections.

the formation of the beaded nanofibers [21]. In conclusion, the formation of nanoparticles and individual larger beads ("bulbs") as "by products" in the nanofibrous network in our study is most likely due to the following two reasons: relatively low viscosity of the polymer solution and voltage applied in the non-optimized process.

3.2. *Physical solid-state properties of the nanofibers*

According to the literature, free crystalline β-sitosterol exists in three different solid state forms with different water contents: anhydrated, hemihydrated and monohydrated crystals [3,22]. In the present study, β-sitosterol was originally in crystalline anhydrate form as verified by XRPD. As shown in Fig. 5, the characteristic major peaks for the crystalline anhydrate form of β-sitosterol were observed at the diffraction angles 2θ of 7.1°, 11.9°, 12.7°, 15.0°, 18.3°, and 19.1°. In our study, most likely the controlled storage conditions enabled the β-sitosterol to remain in an anhydrate form, and consequently the plant sterol did not undergo any solid-state changes to hemihydrate or monohydrate forms during a storage period prior to ES.

The XRPD patterns of the electrospun nanofibers loaded with β-sitosterol and chitosan at a weight ratio of 1:1 (TFA or the TFA/HFIP 1:1 mixture as solvent systems) showed an amorphous halo and absence of crystallinity (Fig. 5). The characteristic XRPD reflections of β-sitosterol anhydrate crystal form were not observed anymore in the XRPD patterns of the electrospun nanofibers. According to the literature, chitosan appears in partially crystalline solid phase with the characteristic broad peaks at diffraction angles 2θ of 9–10° and 19–20° in its XRPD pattern [23,24]. As seen in Fig. 5, the physical form of the chitosan used in the present study was in good accordance with that reported in the literature. As expected, the characteristic broad diffraction reflections of chitosan were not observed anymore in the XRPD patterns of the electrospun nanofibers (Fig. 5). The crystallographic properties of natural chitosan, however, can depend on several parameters, including its origin, molecular weight, deacetylation procedure and treatments used to condition the material (dissolving, precipitation, drying) [23]. It seems that dissolving chitosan to the organic solvents (the TFA/HFIP mixture is acidic) and the rapid evaporation of solvent in the ES process affected chitosan chain packing and decreased crystallinity. Hence, the present results suggest that ES with chitosan can be used for the amorphization of a poorly water-soluble crystalline β-sitosterol and also for fabricating amorphous solid dispersions (polymeric nanofiber matrices) of this plant sterol.

As seen in Fig. 6, the electrospun chitosan nanofibers containing β-sitosterol did not show the characteristic melting endotherm of crystalline β-sitosterol anhydrate in the DSC thermogram, confirming the presence of amorphous drug. Christiansen [3] reported that crystalline β-sitosterol anhydrate exhibits the melting endotherm at 133–138 °C (peak temperature). In our study, however, β-sitosterol powder exhibited a lower melting endotherm at 115–120 °C (peak temperature) (Fig. 6A). This difference in melting temperatures is most likely due to the different measurement settings (open *vs* closed pans, heating rate) as well as the fact that the β-sitosterol used in this study contained also other constituents, β-sitostanol (13%), campesterol (8.4%), campestanol (1.3%), and contaminants (1.8%), as related substances. In addition, a wide endothermic peak below melting could suggest a partial phase transition of β-sitosterol from anhydrate to monohydrate form (Fig. 6A). Chitosan thermal behavior (shown in Fig. 6B) was in good agreement with that reported in the literature [25].

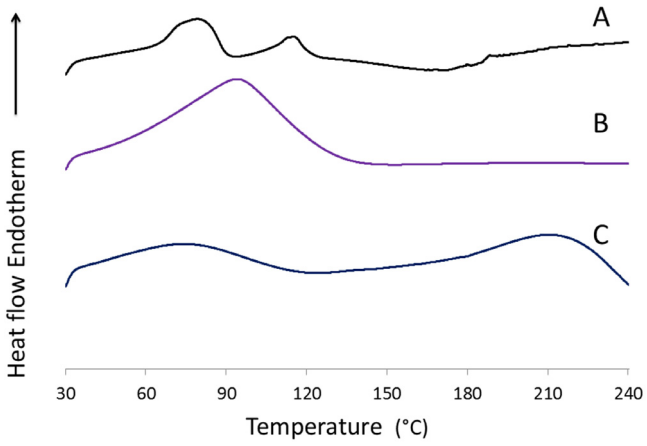


Fig. 6 – Differential scanning calorimetry (DSC) thermograms. Key: (A) β-sitosterol (black line), (B) chitosan (purple line), and (C) the electrospun nanofibers containing β-sitosterol and chitosan (1:1) (blue line). The TFA/HFIP mixture (1:1) was used as solvent systems for preparing the nanofibers.

3.3. *Wetting and dissolution of the nanofibers*

A prerequisite of cholesterol-lowering effect of oral plant sterols is that the sterol(s) have a rapid dissolution in intestinal fluids and bile, and that the uniform mixture of finely dispersed plant sterol(s) with the cholesterol containing food mass is simultaneously directed in the intestine [3]. This poses overwhelming challenges for the pharmaceutical formulation, and emerges the need for nanotechnology approaches to solve the problem.

According to the literature, the cholesterol absorption reducing effect of oral β-sitosterol and its saturated form, β-sitostanol, is dependent on their physical solid-state form, solubility and formulation (final dosage form) [3,6]. In our study, β-sitosterol was homogeneously and finely distributed in the nanofibers after fabrication (as shown in Fig. 3). Based on the *in situ* dissolution monitoring, the electrospun β-sitosterol-loaded nanofibers were freely water-soluble and exhibited very short lag-time in releasing the active substance (Fig. 7). β-sitosterol, however, tended to immediately recrystallize (or form submicron particles), when released from the polymeric nanofibers. The tiny crystals/particles of β-sitosterol were clearly visible in the vicinity of the nanofibers immediately after the addition of purified water or buffer solutions (Fig. 7). No significant differences were observed between the three different aqueous media studied in dissolving β-sitosterol-loaded nanofibers. As the composite nanofibers were further dissolved, the β-sitosterol crystals/particles still remained for 3–5 minutes in the buffer solution before they dissolved as well. This suggests that ES can be used for fabricating the composite nanofibers intended for templates of plant sterols (β-sitosterol), which allow the spontaneous *in situ* formation of submicron β-sitosterol particles when the nanofibers are added to water. The present approach could open a new alternative to formulate oral plant sterols for cholesterol lowering. In the literature, nanofibers have been successfully used for formulating the templates for self-assembled systems [26]. In addition, the utilization of advanced ES technologies (e.g., by preparing multi-active composites, ES printing for controlled patterning and porous capsules) could provide promising multiple platforms for plant sterols [27–29].

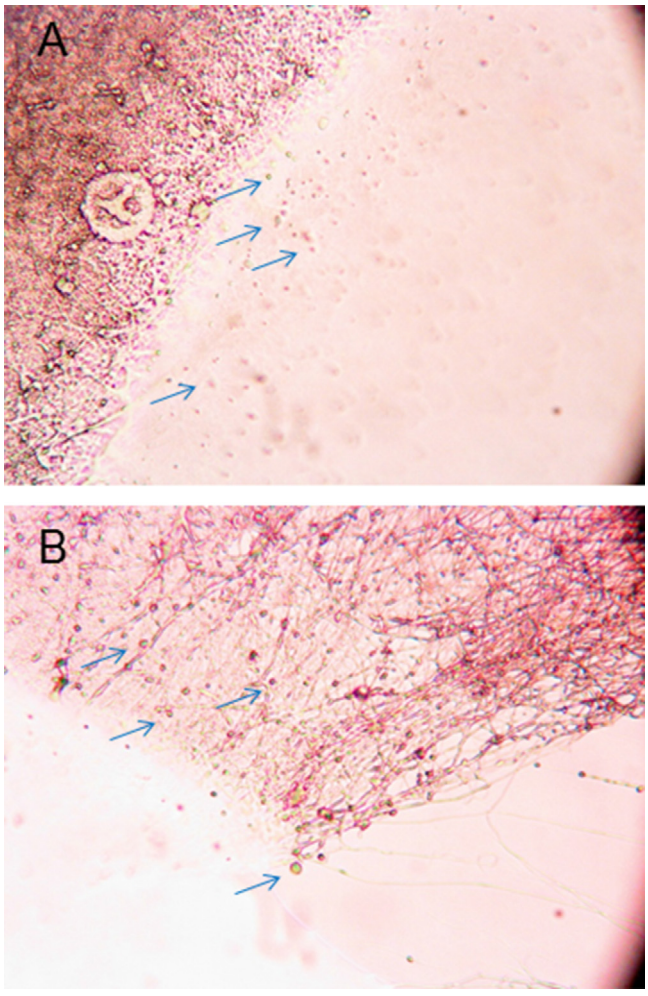


Fig. 7 – *In-situ* optical microscopy of the wetting and dissolution behavior of the nanofibers. The representative micrographs show chitosan-β-sitosterol nanofibers in contact with a buffer solution at pH 1.2. Key: (A) Immediately after wetting, β-sitosterol tended to recrystallize out from the nanofibers. (B) As the nanofibers were further dissolved, the β-sitosterol crystals still remained for 3–5 minutes in the buffer solution before they dissolved as well. Blue arrows indicate the tiny crystals of β-sitosterol in the vicinity of the nanofibers.

4. Conclusion

The present proof-of-the-concept study shows that electrospinning (ES) is a promising future technology for the formulation of poorly water-soluble crystalline plant sterols. ES is a rapid, continuous and up-scalable process, which provides non-destructive conditions for the formulation (vitrification) of plant sterols to amorphous solid dosage forms. Application of ES and a cationic carrier biopolymer (chitosan)

in highly acidic organic solvents could be an interesting approach for the formulation of poorly water-soluble plant sterols (such as β-sitosterol) in amorphous solid dispersions, and hence to improve their dissolution and cholesterol lowering effects via oral route. Using ES for fabricating nanofibrous templates of plant sterols (β-sitosterol), and consequently, allowing the spontaneous *in situ* formation of submicron β-sitosterol particles, can open a new avenue to formulate oral plant sterols. A major challenge, however, still remains in the solid-state stabilization (polymorphic changes) of a plant sterol in a nanofibrous solid dispersion matrix.

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