Resveratrol proniosomes as a convenient nanoingredient for functional food

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Abstract

Proniosomes are free-flowing powders composed of water-soluble carriers blended with surfactants, which form niosomes upon hydration. In this work, proniosomal formulations containing the natural antioxidant resveratrol (RSV) were prepared and fully characterized. A pre-formulation study on RSV-loaded niosomes was carried out to determine the most promising ratio between the two surfactants, Tween 20 and Span 60, in terms of entrapment efficiency and antioxidant activity. The optimized formulae were subsequently adapted to be prepared as proniosomes by the slurry method, including lactose or maltodextrin as carriers. The impact of surfactants and carriers properties on size, entrapment efficiency and release kinetics of proniosomes were evaluated. In vitro release of RSV in simulated gastric and intestinal media was determined, as well as the vesicular stability. Moreover, the biocompatibility of the formulations was determined on intestinal cells in vitro. Overall, the developed proniosomes provide promising nanoingredient for functional food, improving resveratrol stability and bioavailability.

Keywords: resveratrol, niosomes, proniosomes, carrier, Span 60, Tween 20

List of compounds:

Resveratrol (PubChem CID: 445154)
Cholesterol (PubChem CID: 5997)
Lactose (PubChem CID: 440995)
Maltodextrin (PubChem CID: 107526)
Span 60 (PubChem CID: 16218600
Tween 20 (PubChem CID: 443314)

1. Introduction

Resveratrol (trans-3,5,4′-trihydroxystilbene, RSV) is a polyphenol compound, which is produced by several plant species to defend them from injuries. It is naturally present in high levels in some fruits, which are part of the human diet, such as blueberries (Vaccinium spp.), blackberries (Morus spp.), and peanuts (Arachishypogaea). Red wine is the main source of RSV in the Mediterranean diet, since *Vitis vinifera* synthetize this polyphenol in response to environmental stress such as UV radiation, drought, parasitic or fungal attack (Sinico et al., 2016). In addition to the well-known potent antioxidant and anti-microbial activity, RSV exerts anti-inflammatory, neuro-protective, anti-aging, and anti-cancer effects (de Sá Coutinho, Pacheco, Frozza, & Bernardi, 2018; Salehi et al., 2018; Smoliga, Baur, & Hausenblas, 2011; Zupančič, Lavrič, & Kristl, 2015). Food supplementation with RSV has proved to reduce oxidized LDL, as well as blood sugar levels and oxidative stress, thus decreasing the risk for cardiovascular and metabolic disease. More recently, the preventive effect of RSV on body fat accumulation has been demonstrated (Wang, Zhu, & Du, 2015). Consequently, RSV could be a useful drug as well as ingredient for functional foods (Breuss et al., 2019).

Despite of the great beneficial potentials for human health, the use of RSV in both nutritional and therapeutic context is strongly affected by the combination of several limiting factors including poor water solubility, low chemical stability and high metabolization (Gambini et al., 2015; Zupančič et al., 2015). When RSV has been used in its free form as solid dietary supplements in capsules, it presented a very low bioavailability as a consequence of poor hydrosolubility. On the other hand, when RSV has been administrated in vegetable matrices or in liquid vehicles promoting an high oral absorption, results from pharmacokinetic studies indicated a limited bioavailability, since the majority of the absorbed RSV was rapidly conjugated and transformed into metabolites (Gandolfi et al., 2017). Moreover, RSV is a photosensitive molecule that exists in cis and trans structural isomers. Many studies have shown that the main antioxidant action is exerted by trans-resveratrol, which is converted into its cis isomer, a less active form, upon light exposure (Amri, Chaumeil, Sfar, & Charrueau, 2012).

In this context, an increasing number of recent studies have focused on novel formulation approaches to overcome RSV weak oral bioavailability, including nanosizing technologies, complexation with macromolecules and encapsulation in nanocarriers (Lai, Schlich, Pireddu, Fadda, & Sinico, 2018; Pandita, Kumar, Poonia, & Lather, 2014; Sessa et al., 2014; Singh et al., 2017; Sinico et al., 2016).

Among various nanocarriers, vesicular systems based on a bilayer membrane such as liposomes and niosomes have been proposed as convenient oral delivery systems for RSV (Bonechi et al., 2012; Pando, Beltrán, Gerone, Matos, & Pazos, 2015). Indeed, these colloidal nanocarriers are able not only to increase the drug solubility, but also to protect the encapsulated molecules from degradation and to provide a controlled release, which can be tuned changing the vesicle composition and/or decorating their surface. Particularly, RSV-loaded niosomes have been suggested as a food additive in enriched yogurts, since they did not involve changes in the textural properties of this food (Pando et al., 2015).

Niosomes are lipid nanoparticles with an aqueous core delimited by close concentric bilayers composed of hydrated non-ionic surfactants, with or without incorporation of cholesterol or its derivatives (Marianecci et al., 2014). Non-ionic surfactants are amphiphilic molecules that have two distinct regions in their chemical structure, one of which is hydrophilic and the other is hydrophobic. Their aggregation structures, namely niosomes, are analogous to phospholipid vesicles and, like liposomes, they are capable of encapsulating both hydrophilic and lipophilic molecules. The loading can be achieved by entrapping hydrophilic compounds in the aqueous core while the lipophilic substances are incorporated into the lipophilic domain of the bilayers (Uchegbu & Florence, 1995).

Niosomes were originally developed as an alternative drug delivery system to liposomes, in order to overcome the problems associated with high cost and low stability of phospholipids. Indeed, there is a great variety of suitable surfactants, which show high chemical stability and affordable cost.

Despite the reported advantages over liposomes, also niosomal dispersions present problems related to physical instability including vesicle fusion, aggregation, sedimentation and drug leakage during storage. Moreover, the traditional method for producing vesicles, such as niosomes or liposomes, involves drying the lipid to a thin film from an organic solvent solution, and then hydrating it with an aqueous medium. The resulting multilamellar lipid nanoparticles can be further processed by sonication, extrusion, or other treatments to optimize size, morphology and drug entrapment. All other methods reported in the literature involve specialized apparatus and are time consuming.

Proniosomes, which are the dry powder of the vesicular systems, have been developed by simple preparation procedures for circumvent all of the above reported complications, while preserving composition and features of niosomes. Indeed, they are dry and free flowing powders of a surfactant-coated carrier, which can be

hydrated in aqueous food matrices (such as milk, yogurt or functional beverages) to form niosomes immediately before use (Hu & Rhodes, 1999). Moreover, proniosomal powders offer several further advantages over their liquid counterparts, such as ease of dosing and transportation and reduced risk for microbiological contamination (Ravaghi et al., 2017; Shehata, Abdallah, & Ibrahim, 2015).

In the present work, novel RSV formulations based on proniosomes technology were developed with the dual aim of increasing solubility while keeping a solid form. Moreover, as RSV is particularly instable in aqueous environment, its inclusion in lipid nanoparticles would increase its shelf life.

For this purpose, at first, six niosomal formulations have been prepared using Tween 20 or Span 60 or different combinations of two surfactants. Then, the two best formulations in term of RVS encapsulation efficiency and antioxidant activity, have been selected for developing proniosomal powder by using lactose or maltodextrin as carrier.

2. Material and methods

2.1. Materials

Polysorbate 20 (Tween 20), sorbitan monostearate (Span 60) and cholesterol were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Trans*-resveratrol (RSV) and spray dried lactose were purchased from Galeno (Prato, Italy). Maltodextrin (Glucidex 6-D) was a kind gift from Roquette (France). All other chemicals used in the experiments were of analytical grade. Ultrapure water was obtained from a Millipore system and was used throughout the experiments.

2.2. Preparation of niosomes

Niosomes were prepared by the thin film hydration method, combined with probe sonication (complete compositions are reported in Table 1-A). Briefly, weighed amounts of surfactant, cholesterol and RSV were dissolved in 10 mL ethanol in a round bottom flask. Ethanol was evaporated under reduced pressure and a thin film was formed. The thin film was hydrated with 10 mL ultrapure water at 60°C under continuous mixing triggered by an overhead stirrer and palette (Eurostar 40, IKA, Germany). After hydration, probe sonication was applied to niosome dispersions (Soniprep 150 plus, MSE Crowley, UK) to reduce the diameter of lipid

nanoparticles. The obtained niosome dispersions were stored at room temperature and characterized within 24 hours from the preparation.

2.3. Preparation of proniosome powders

Proniosomal powders were prepared by the slurry method. The exact composition of each proniosomal formulation is reported in Table 1-B. Briefly, weighted amounts of Tween 20, cholesterol, Span 60 (if present) and resveratrol were mixed in a round bottom flask. After complete dissolution of the components in 10 ml ethanol, the carrier (lactose or maltodextrin) was added to form a slurry. The organic solvent was removed under vacuum to yield a free flowing powder. Just before the following experiments, proniosome powders were hydrated with 10 mL deionized water, the mixture was vortexed and probe sonicated to obtain niosomes. Unloaded proniosome powders (blank), without RSV, were prepared using the same method.

2.4. Size analysis, size distribution and zeta potential measurement

Mean diameter, size distribution (polydispersity index, PDI) and zeta potential of proniosomes-derived niosomes were determined by dynamic light scattering (DLS) and Electrophoretic Light Scattering (ELS), respectively, using a Malvern Nano ZS (Worcestershire, Malvern Instruments Ltd., UK) at 25 °C. For size and PDI determination, the niosomal dispersion was diluted (1:100) with water, while for Z potential analysis a 1:10 dilution was employed to prepare the sample.

2.5. Entrapment efficiency

The entrapment efficiency (EE%) of proniosome-derived niosomes was evaluated on the same day of the hydration. The unencapsulated RSV was separated from niosomes by exhaustive dialysis. Briefly, 1 mL of niosomal suspension was loaded into a dialysis bag (Spectra/Por membranes: 12–14 kDa MW cut off, 3 nm pore size; Spectrum Laboratories Incorporation, USA) and dialysed against 1000 ml of distilled water for 2 hours, at 4°C, replacing the water after 1 hour. Purified and unpurified lipid nanoparticles were disrupted by the addition of 9 parts of ethanol to 1 part of niosomes. The obtained mix was vortexed and centrifuged at 8000 RPM for 5 minutes on a Mikro 200 Centrifuge (Hettich, Tuttlingen, Germany). The supernatant (1 mL) was recovered and transferred in HPLC vials for subsequent analysis.

167 **2.6. DPPH assay**

168 The antioxidant activity of niosomal RSV was assessed by evaluating its ability to scavenge the stable radical
169 2,2-diphenyl-1-picrylhydrazyl (DPPH). A methanolic solution of DPPH (0.04 mg/mL) was mixed with
170 appropriate amount of niosomes to yield final RSV concentrations in the range 0.005-0.05 mg/mL. A negative
171 control was prepared by mixing 980 µL of DPPH solution with 20 µL of deionized water. After 30 minutes of
172 incubation in the dark at room temperature, the absorbance was read at 517 nm by a Synergy 4 multiplate
173 reader (BioTek, Winooski, USA). The percent antioxidant activity was calculated according to the following
174 equation: % antioxidant activity = $100*(ABS_{control} - ABS_{sample})/ ABS_{control}$.

175

176 **2.7. In vitro release in SGF and SIF**

177 Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to United States
178 Pharmacopoeia (USP) XXIV (Cohen et al., 1990). To determine the RSV release from the formulations, 4 mL
179 of niosomal suspension was loaded into a dialysis bag and incubated in a dissolution apparatus type 2 (paddle),
180 filled with 800 ml of SGF or SIF and thermostated at 37°C. The dialysis bags were stirred at 180 rpm
181 throughout the experiment, and aliquots were withdrawn after 15, 30, 60, 90 and 120 minutes (release in gastric
182 medium) or 1, 2, 4, 6 and 8 hours (release in intestinal medium). The samples were prepared for HPLC analysis
183 as previously reported.

184

185 **2.8. HPLC method**

186 RSV content was quantified at 283 nm using a chromatograph Alliance 2690 (Waters, Italy). The column was
187 a Sunfire C18 (3.5 µm, 4.6 × 150 mm, Waters). The mobile phase was a mixture of 20 % water and 80%
188 acetonitrile (v/v), delivered at a flow rate of 0.5 ml/min. A standard calibration curve (R^2 of 0.999) was built
189 up by using working, standard solutions (1.0–0.01 mg/ml). The retention time of RSV was 4 min, and the
190 minimum detectable amount was 2 ng/µl.

191

192 **2.9. FTIR spectroscopy (FT-IR)**

193 Fourier-transform infrared measurement were performed using reflectance FT-IR spectroscopy and attenuated
194 total reflettance (ATR-FT-IR) spectroscopy. FT-IR analyses were collected using Perkin Elmer FTIR

Spectrometer Spectrum One (PerkinElmer Waltham, MA, USA) in a spectral region between 4000 and 600 or 450 cm^{-1} for solid or liquid compounds respectively and analysed by transmittance technique with 8 scansions and 4 cm^{-1} resolutions. Solid samples were mixed in a mortar with KBr (1:100) and pressed in a hydraulic press (14 tons) to small tablets, while for liquid samples one drop was placed between two widows of sodium chloride. RSV, Lactose, Maltodextrin, Span 60, cholesterol, proniosomal powder and unloaded formulation of the optimized proniosome powder were also analyzed in ATR-FT-IR using a Perkin Elmer Universal ATR Sampling Accessory with a resolution of 4 cm^{-1} and 32 scans in the range 4000–650 cm^{-1}.

2.10. X-ray powder diffractometry (XRPD)

For further characterisation of the crystalline state XRPD diffractograms of RSV, Lactose, Maltodextrin, Span 60, cholesterol, and unloaded formulation of the optimized proniosome powder were recorded using a Rigaku Miniflex diffractometer (Rigaku Corporation, Tokyo, Japan) with a Ni-filtered CuKa radiation detector (λ = 1.5405 Å) operating at a voltage of 30 kV and a current of 15 mA in the 2θ range from 3° to 60° with a scan angular speed of 2°/min and a scan step time of 2.00s. The results were then obtained as peak height (intensity) versus 2θ.

2.11. Biocompatibility

The biocompatibility of the formulations was tested on human epithelial intestinal cells (Caco-2) kindly gifted by Prof. Caitriona O'Driscoll (School of Pharmacy, UCC, Ireland). The cells were cultured in Dulbecco Modified Eagle Medium (DMEM, high glucose, Sigma Aldrich, Milan, Italy) supplemented with 50 units/mL penicillin and 50 mg/mL streptomycin and 10% fetal bovine serum (FBS) and maintained in a humidified, 5% CO_2 atmosphere tissue culture incubator at 37 °C. Caco-2 were seeded in 96-well plates 24 h prior to the treatment with the formulations (10^4 cells/well). Proniosomes-derived niosomes were mixed with complete medium to achieve different RSV concentrations (in the range 37-365 µM) and cells were exposed to the formulations for 4 h. After this time the medium was discarded and replaced with 100 µL MTT in PBS (0.5 mg/ml). Cells were incubated for 4 h at 37°C after which the MTT was removed and 100 µL DMSO was added to each well. Absorbance was measured at 517 nm using a Synergy 4 plate reader (BioTek, Winooski, USA). The results are expressed as the percentage cell viability relative to untreated cells.

223

224 **2.12. Statistical analysis**

225 Data are expressed as mean ± standard deviation of at least 3 independent determinations if not otherwise
226 stated. Data analysis was carried out using the software package R, version 2.10.1. Student's t-test was used to
227 substantiate statistical differences between two samples at $p < 0.05$. Multiple comparisons of means (Tukey
228 test) was used to carry out comparisons between groups at $p < 0.05$ as a minimal level of significance.

229

230 ## 3. Results and discussion

231 At first, a preliminary formulation study of RSV niosomes was carried out to investigate the effect of surfactant
232 properties on RSV encapsulation and antioxidant activity. To this aim, Tween 20 and Span 60, a hydrophilic
233 and a hydrophobic surfactant, respectively, were used alone or blended in different ratios to prepare niosomes
234 (Table 1-A).

235

236 **Table 1.** Composition of niosome dispersions (mg for 10 ml of formulation) **(A)** and proniosomal powders (mg) **(B)**

237

A

Niosome Formulations	**HLB**	**Components**					
		Resveratrol	**Tween 20**	**Span 60**	**Cholesterol**	**Lactose**	**Maltodextrin**
N-HLB-16.7	16.7	5	184	-	58	-	-
N-HLB-13.5	13.5	5	135	17.2	58	-	-
N-HLB-11.0	11.0	5	96.6	30.6	58	-	-
N-HLB-8.5	8.5	5	53.5	44	58	-	-
N-HLB-4.5	4.5	5	-	64.5	58	-	-

B

Proniosomal Formulations							
PL16.7	16.7	5	184	-	58	736	-
PM16.7	16.7	5	184	-	58	-	736
PL13.5	13.5	5	135	17.2	58	736	-
PM13.5	13.5	5	135	17.2	58	-	736

238

239

240 Tween 20 and Span 60 have a hydrophilic-lipophilic balance (HLB) of 16.7 and 4.5, respectively, thus
241 providing the ideal starting material to obtain a range of intermediate HLB values through mixing in

appropriate ratios. In addition to surfactants, cholesterol represents an essential component to induce the formation and stabilization of bilayers (Uchegbu & Florence, 1995). Indeed, when cholesterol is not included in the formulation, a micelle or vesicle dispersion is obtained depending on the type and ratio between surfactant mixture. Surfactants with a single alkyl tail normally form micelles in diluted aqueous solutions and need additives such as cholesterol to achieve suitable molecular geometry and hydrophobicity for bilayer vesicle formation. However, non-ionic surfactant with a single alkyl tail such as Span 60 can form vesicular structure since it has relatively large hydrophobic moiety (HLB=4.7) with low water solubility. Some surfactants cannot form vesicle without cholesterol, due to the higher HLB (hydrophilicity), such as Tween 20 that has a large hydrophilic head group and a HLB of 16.7.

In this study, the total surfactant concentration was kept constant at the value of 15 mM, fixing the cholesterol/surfactant molar ratio at 1:1. The preparation of niosomes was carried out exploiting the thin film hydration method followed by probe sonication, which yielded low dispersity (PDI < 0.2) populations of lipid nanoparticles having mean diameters in the range 110-190 nm. The DLS analysis of niosomes did not show a statistically significant correlation between size of nanoparticles and HLB (data not shown), thus these data did not contribute to the choice of the formulations to be prepared as proniosomes. The entrapment efficiency (EE%) of RSV in the formulated niosomes was assayed by HPLC, after removal of the un-encapsulated drug by exhaustive dialysis. Also in this case it was not possible to detect any significant difference in term of EE% between the analyzed formulations. However, niosomes prepared with the surfactant blends at HLB of 4.5, 8.5 and 11 showed a high variability in the RSV EE%, while niosomes having more hydrophilic HLB values were characterized by highly reproducible data (Figure 1, A). Lower reproducibility of data were detected as the amount of Span 60 in the formulation increased, thus suggesting that this highly hydrophobic surfactant may be not suitable for the incorporation of RSV in stable niosomes in the applied conditions. Indeed, previous published formulation studies on RSV-containing Span 60 niosomes revealed a wide range of entrapment efficiencies (from 25 to 72%) when minor changes to the process parameters and/or components are applied (Pando et al., 2015). Conversely, in addition to the high and reproducible EE%, RSV-niosomes having HLB of 13.5 and 16.7 also displayed the highest antioxidant activity in vitro, assessed by the DPPH assay (Figure 1, B). Indeed, RSV loaded on HLB 4.5 and 8.5 niosomes showed a remarkably lower antioxidant activity as compared to more hydrophilic niosomes and to a methanolic solution, used as control. Once again, Span 60 is

probably responsible of such results, since its highly hydrophobic character may induce a strong interaction with lipophilic RSV, which is prevented from reaching and scavenging the DPPH radical. On the other hand, niosomes prepared with surfactant blends with HLB higher than 11 resulted in an antioxidant activity of RSV equivalent or superior to the control.

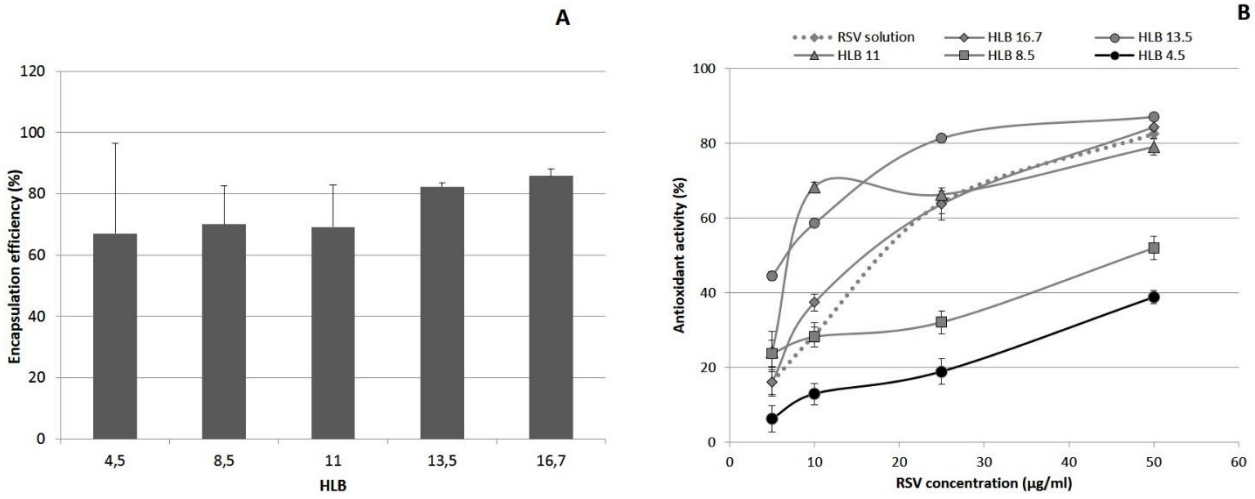

Figure 1. Characterization of niosomes. Entrapment efficiency (**A**) and antioxidant activity of RSV-loaded niosomes prepared with surfactant blends having different HLB (**B**). Results are expressed as means of 4 independent measurements ± standard deviations

In light of these results, niosomes with an HLB of 13.5 and 16.7 were chosen to be formulated as proniosomes. The niosomes formulae were adapted to the preparation of proniosomes by including a quantity of inert carrier sufficient to obtain a free flowing powder (Table 1-B).

For the production of proniosomes, the slurry method was employed using environmentally friendly ethanol as the only non-aqueous solvent. Moreover, the slurry method allows the preparation of proniosomal powders through a faster and cheaper procedure, as compared to the freeze-drying method. Spray-dried lactose and maltodextrin were selected as carriers, among several water-soluble excipients, for their convenient flow and compressibility properties. In particular, maltodextrin due to high surface area and the porous structure was chosen as carrier to prepare proniosomes with high surfactant to carrier mass ratios (Sahoo, Biswas, Guha, & Kuotsu, 2014). A detailed physico-chemical analysis of the proniosomal powders was carried out to describe

the main features of the formulations, such as interactions between the components, chemical identity of the active ingredient (including its isomeric form), and crystalline or amorphous state.

The ATR-FTIR method makes it possible to monitor the slightest changes in the state of the sample at molecular level, since there is no sample preparation, unlike in the case of the KBr pellet method, when grinding and pressing mostly unifies the samples with weak interactions.

In the spectra the main absorption band of resveratrol are at 3270 cm^{-1} (O-H stretching), at 3019 (H-Csp3 stretching), at 1606, 1587, 1384 cm^{-1}, the three characteristic strong bands corresponding to C-C aromatic double bond stretching, C-C olefinic stretching and C-O stretching, respectively. Moreover, the band at 965 cm^{-1} demonstrated the *trans* form of RSV. The characteristic peak corresponding to either excipients or drug did not show any significant shift in the spectrum suggesting no interactions between them.

The XRPD pattern of RSV evidenced its crystalline pattern with intense reflection peaks at 6.52, 13.16, 16.26, 19.08, 20.84, 22.22, 28.20, 31.50 2θ. X-ray diffraction analyses of PL formulation showed a crystalline pattern with reflection peaks attributable to RSV, even if some of them with lower intensities (Figure 2B). Otherwise, PM has an amorphous profile due to maltodextrins.

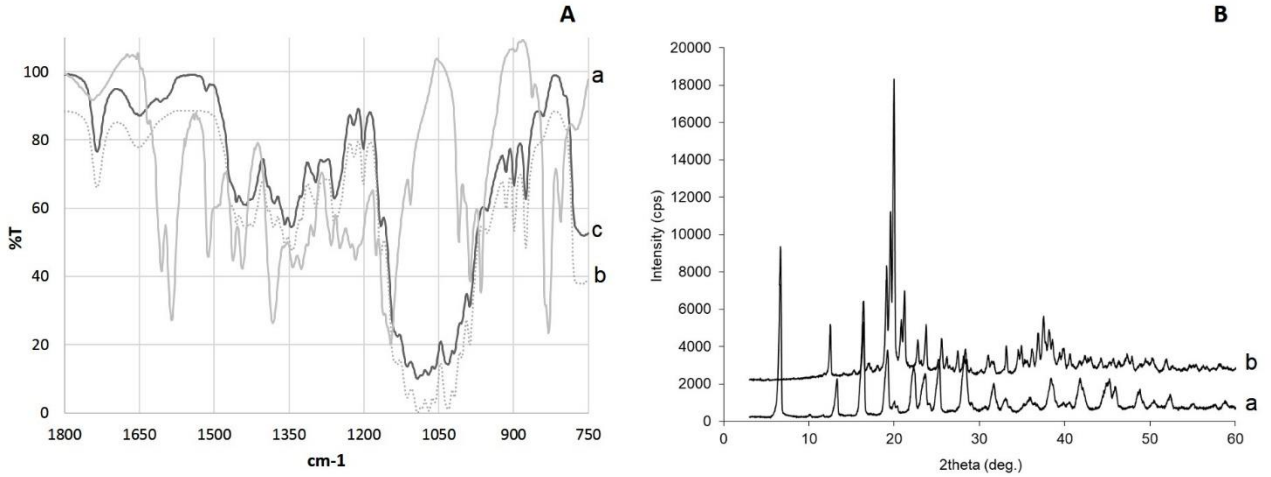

Figure 2. ATR-FTIR spectra in the 1800-750 cm-1 range: RSV (a), RSV loaded PL16.7 proniosome (b), empty PL16.7 proniosome (c) (**A**). Diffraction patterns of RSV (a) and RSV loaded PL16.7 proniosome (b) **(B)**

The physico-chemical and biopharmaceutical properties of niosomes generated by the hydration of proniosomal powders were subsequently evaluated.

As reported in table 2, the hydration and sonication of proniosomes resulted in the formation of lipid nanoparticles with a mean diameter in the range 180-300 nm and highly negative surface charge. Of note, the different Span60/Tween20 ratio had an impact on the physico-chemical properties of proniosome-derived niosomes. Indeed, formulations having HLB of 13.5 were significantly less polydisperse than PL16.7 and PM16.7, which also showed higher batch to batch variability in terms of mean diameter, which resulted in higher standard deviation. However, these differences in size and PDI did not have consequences on the entrapment efficiency of RSV within the formed nanoparticles. Indeed, no statistically significant differences on the amount of incorporated RSV were determined between the hydrated proniosomes, thus suggesting that the polyphenol could be efficiently loaded on the nanoparticles regardless of their size. On the other hand, the carrier used did not influence the mean size and polydispersity of niosomes. This can be especially noted by comparing the physicochemical attributes of PL13.5 and PM13.5, where the latter only shows a slightly larger mean diameter, which however becomes not significant if the PDI is considered. Apparently, the different molecular weight and chemical structure of lactose and maltodextrin do not have an influence on lipid nanoparticles properties. Indeed, it has been reported that the dissolution behavior of the carrier play a key role in the efficient hydration of the surfactant and the consequent formation of niosomes from proniosomal powders, and both lactose and maltodextrin show excellent dissolution properties (Ravaghi et al., 2017).

Table 2. Mean diameter, PDI, Z potential and entrapment efficiency of the proniosome-derived niosomes. Results are expressed as means of 4 independent measurements ± standard deviations

Formulations	Mean diameter (nm)	PDI	Z potential	Entrapment efficiency (EE%)
PL16.7	282 ± 142	0.30 ± 0.01	- 38.3 ± 6.7	84 ± 3
PM16.7	194 ± 55	0.28 ± 0.11	- 37.3 ±4.7	76 ± 5
PL13.5	180 ± 2	0.14 ± 0.11	- 47.5 ± 2.2	75 ± 4
PM13.5	194 ± 3	0.20 ± 0.11	- 43.7 ± 5.0	81 ± 1

The RSV release from proniosome-derived niosomes was investigated in simulated gastrointestinal environments. To this aim, the formulations were loaded into a dialysis tubing and incubated at 37°C in a type 2 dissolution apparatus, using either simulated gastric or intestinal fluids as release media. Since no regulatory

guideline exists regarding the release studies from nanoparticles, the dialysis bag method was used in combination to the type 2 apparatus to adapt the official dissolution method reported by the US and EU pharmacopoeias to the developed nanoformulations (Souza, 2014). Results reported in Figure 3A show a slightly slower release rate of RSV from PM16.7 in gastric environment, as compared to all the other formulations, which do not differ significantly from one another. Indeed, after two hours of incubation in simulated gastric fluid PM16.7-derived niosomes released 26% of the initial RSV dose, while all the other formulations triggered the release of 39%, 41% and 48% (PM13.5, PL13.5 and PL16.7, respectively) of the loaded dose. A stronger interaction between the hydrophilic surfactant tween 20 and the bulky maltodextrin may be responsible for this reduced release rate, which was also partially observed in simulated intestinal fluid (Figure 3B). The behavior of lactose-based (PL) formulations in the simulated intestinal environment is characterized by an initial burst release of 25-31% of the dose after 1 hour of incubation, followed by a decrease in the release rate for the rest of the experiment. Interestingly, PL16.7 showed a markedly slower release rate as compared to PL13.5, which ultimately resulted in 53% and 69% of RSV released at the end of the incubation time, respectively. The faster release from PL13.5 may be attributed to the blend of surfactants, which is the only difference between the cited formulations. Indeed, also the other formulation with HLB of 13.5 (PM13.5) reached the same amount of total RSV released (68%) while the lipid nanoparticles with higher HLB (PM16.7) liberated only 48% of RSV dose. It is interesting to note that a lower HLB value seem to trigger a faster release in simulated intestinal fluid but not in the gastric one. Although none of the employed surfactants are charged molecules, their behavior seems to be influenced by pH of the release medium, where more alkaline environment probably loosen the interactions of Span 60 with the other nanoparticles components, ultimately leading to a faster release compared to the HLB16.7 formulations.

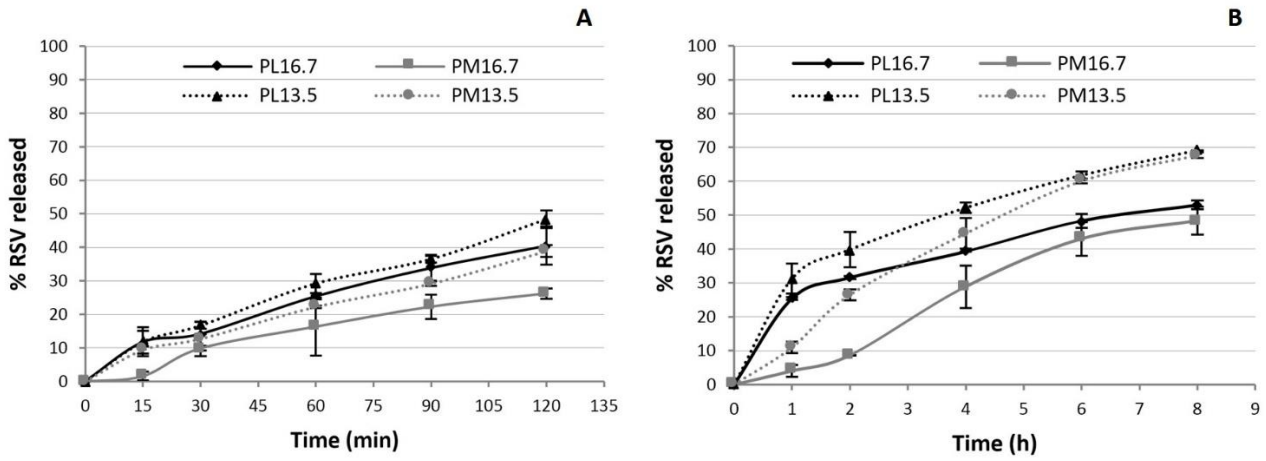

Figure 3. In vitro RSV release in simulated gastric fluid (SGF) (**A**), or simulated intestinal fluid (SIF) (**B**) from proniosome-derived niosomes. Values are expressed as % of the dose used for the experiments

All the components of the proniosomal formulations developed in this work are GRAS listed and accepted for oral use in pharmaceutical products by FDA and EMA. However, to evaluate the safety of the nanoformulations, a biocompatibility assay was carried out on intestinal cells (Caco2). The histrogram reported in Figure 4 represent the viability of cells after 4 hours of incubation with the proniosomal formulations, included in the medium in different amounts expressed as RSV concentration. Interestingly, proniosomes prepared with a HLB of 13.5 showed to be significantly less toxic than their HLB16.7 counterpart, at every concentration used. Indeed, given 80% viability as the lowest acceptable limit, PM13.5 and PL13.5 showed to be safe up to a RSV concentration of 146 µM, while the highest non-toxic dose reported for PL16.7 and PM16.7 was 73 µM. Of note, this experiment was carried out testing medium to high doses of formulations, as compared to other in vitro bioavailability testing of RSV containing nanoparticles (Caddeo et al., 2017)(Rocha et al., 2017). Thus, keeping in mind the higher risk for epithelial toxicity, we speculate that also the formulations having HLB of 16.7 could be employed safely at lower-but-therapeutically-relevant doses of RSV (Ramírez-Garza et al., 2018).

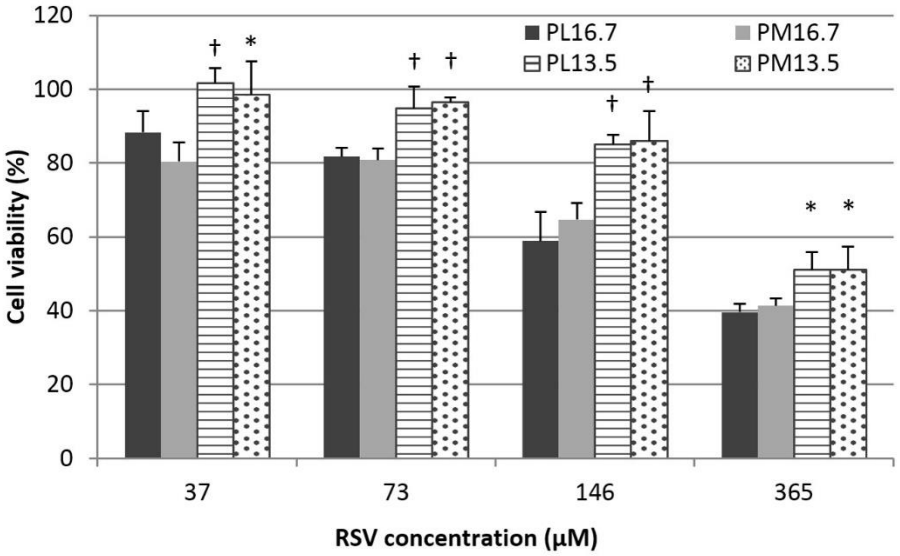

Figure 4. Biocompatibility of proniosome-derived niosomes on epithelial intestinal cells (Caco-2), compared to untreated cells (100%). Symbols represent level of significance between PL16.7 and PL13.5 or PM16.7 and PM13.5 formulations (* $P<0.03$; † $P<0.01$)

CONCLUSION

In this study RSV proniosomal powders containing Tween 20 and/or Span 60 as surfactants, and spray-dried lactose or maltodextrin as carriers, were successfully prepared by using the slurry method and characterised. The hydration and sonication of proniosome powders resulted in the formation of homogenous lipid nanoparticles dispersion with high entrapment efficiency values. *In vitro* studies indicated that the two surfactants ratio, and therefore the surfactant blend HLB, affected the release behaviour of RSV in both simulated gastric and intestinal fluid. Finally, biocompatibility assay carried out on intestinal cells clearly demonstrated that all the tested formulations could be employed safely at the doses commonly administrated by oral route. In conclusion, this study highlights the great potential of proniosomes as an effective strategy to improve bioavailability of poor water-soluble molecules, such as RSV, in enriched foods.

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