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Title: Formulation of resveratrol entrapped niosomes for topical use

Article Type: Full Length Article

Keywords: niosomes, resveratrol, thin film hydration method, ethanol injection method, penetration enhancer, topical use

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Niosomes were formulated with Gelot 64 (G64) as surfactant, and two skin-compatible unsaturated fatty acids (oleic and linoleic acids), commonly used in pharmaceutical formulations, as penetration enhancers.
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13. Is your size measurement method sensitive enough to differentiate between the stated mean size values? Please also consider the PdI. I would rather say that the size of the particles is comparable in all of your samples.
Yes, the method is sensitive enough since, as we mentioned in the previous question, the accuracy of the method is ± 2% for the aforementioned diameters range (0.3 nm - 10 microns).

14. You state that RSV penetration corresponds to niosome size. How can you be sure that the effect is size dependent? You compare niosomes with different formulations. To test this hypothesis you would need to prepare niosomes of the same formulation with different sizes.
In the present work, we compare niosomes with different formulations, but all niosomes were prepared for each formulation tested with two different preparation methods (see section 2.2 of the manuscript). Therefore, we consider that niosomes of the same formulation with different sizes were properly prepared. From the results obtained, it was concluded that "EIM method produces niosomes with smaller mean sizes, narrower size distributions, higher EE and stability than those prepared with the TFH-S method."
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RESPONSE TO REVIEWER'S COMMENTS

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We would like to thank the reviewers for their useful comments and suggestions on our manuscript entitled *Formulation of resveratrol entrapped niosomes for topical use* (Ms. Ref. No.: COLSUB-D-14-01483). They pose practical and stimulating questions. After careful revision and taking into consideration those comments, several changes have been made. These changes are written in blue color in the revised manuscript and are properly discussed in the following paragraphs.

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*Graphical Abstract (for review)

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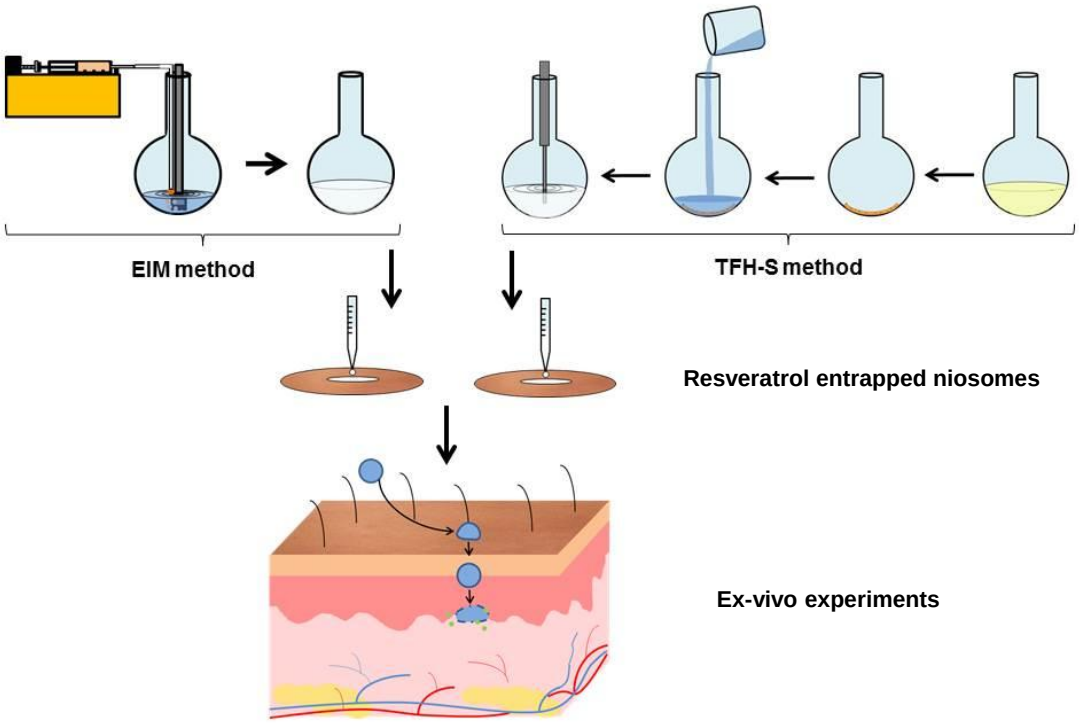

Highlights

- Resveratrol entrapped niosomes for topical use were formulated
- Thin film hydration and ethanol injection (EIM) were used as preparation methods
- Oleic and linoleic acids were compared as skin penetration enhancers
- EIM produced small size niosomes with high encapsulation efficiency
- Smaller niosomes were more effective for resveratrol skin penetration

Formulation of resveratrol entrapped niosomes for topical use

Daniel Pando, María Matos, Gemma Gutiérrez, Carmen Pazos*

Department of Chemical and Environmental Engineering, University of Oviedo, Julián Clavería 8, 33006 Oviedo, Spain

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Abstract

A new approach to the formulation of resveratrol (RSV) entrapped niosomes for topical use is proposed in this work.

Niosomes were formulated with Gelot 64 (G64) as surfactant, and two skin-compatible unsaturated fatty acids (oleic and linoleic acids), commonly used in pharmaceutical formulations, as penetration enhancers.

Niosomes were prepared by two different methods: a thin film hydration method with minor modifications followed by a sonication stage (TFH-S), and an ethanol injection modified method (EIM). Niosomes prepared with the EIM method were in the range of 299-402 nm, while the TFH-S method produced larger niosomes in the range of 293-496 nm. Moreover, niosomes with higher RSV entrapment efficiency (EE) and better stability were generated by the EIM method.

Ex-vivo transdermal experiments, carried out in Franz diffusion cells on newborn pig skin, indicated that niosomes prepared by the EIM method were more effective for RSV penetration in epidermis and dermis (EDD), with values up to 21% for both penetration enhancers tested.

The EIM method, yielded the best RSV-entrapped niosomes, seems to be the more suitable for scaling up.

Keywords: niosomes, resveratrol, thin film hydration method, ethanol injection method, penetration enhancer, topical use.

1. Introduction

Resveratrol (RSV) is a natural polyphenol found in a wide variety of plants that has both chemopreventive and therapeutic effects, because of its anti-oxidant, anti-inflammatory, cardioprotective, and anti-tumour properties. However, its applications are restricted because it is easily oxidizable, has low solubility in water, short biological half-life, and rapid metabolism and elimination (Caddeo, *et al*. 2013; Pando, *et al*. 2013a; Pando, *et al.* 2013b; Scognamiglio, *et al.* 2013; Matos, *et al.* 2014). Moreover, it is an extremely photosensitive molecule, and exposure to light leads to an irreversible change from the active *trans* isomer to the inactive *cis* isomer. Thus, *trans*-resveratrol should be encapsulated before being administered either for food or topical applications.

Because of its chemopreventive and antioxidant properties, RSV is considered to be an interesting drug for incorporation into dermatological preparations. Special attention has been paid to its topical application in different physiological and pathological conditions, such as skin cancer prevention or psoriasis treatment (Jang, *et al*. 1997; Caddeo, *et al*. 2013; Scognamiglio, *et al*. 2013). The drug delivery into the skin has the advantage that high drug concentrations are located at specific sites of action. For this reason, the *ex-vivo* percutaneous absorption of RSV in different nanocarriers has been widely investigated in the last years (Sinico and Fadda 2009, Pando, *et al*. 2013b, Scognamiglio, *et al*. 2013, Marianecci, *et al*. 2014).

The intercellular lipids of the human stratum corneum consist mainly of cholesterol, ceramides and free fatty acids, structurally organized into multilamellar bilayers, which dictate the overall skin permeability properties.

Liposomal delivery systems have been used as a promising approach to overcome the limited permeability of drug across the stratum corneum of skin (Srisuk, *et al*. 2012). However, over the last two decades, niosomes are preferred over liposomes because of their higher chemical stability, lower cost, and the lower efficiency of liposomes for drug delivery across the skin (Sinico and Fadda 2009, Marianecci, *et al*. 2012). Niosomes are vesicles formed by the auto-assembling of non-ionic surfactants in aqueous media resulting in closed bilayer structures (Uchegbu, and Vyas, 1998). Non-ionic surfactants can improve the solubility of some poorly soluble drugs enhancing transdermal delivery by incorporation. These vesicular systems also provide sustained drug release to prolong its action (Kumar and Rajeshwarrao, 2011).

The purpose of this work is to propose a new approach to formulate RSV-entrapped niosomes for topical delivery by comparing two specific methods of preparation: a thin film hydration method with minor modifications, followed by a sonication stage (TFH-S), and an ethanol injection modified method (EIM).

Niosomes were formulated with Gelot 64 (G64) as surfactant, and two skin-compatible unsaturated fatty acids as penetration enhancers, commonly used in pharmaceutical

formulations: oleic acid (OA) and linoleic acid (LA) (Rita and Lakshmi, 2012). Niosomes were characterized in terms of size, morphology, and stability. *Ex-vivo* transdermal experiments, carried out in Franz diffusion cells on newborn pig skin, enabled to study the influence of niosomes formulation and preparation method on RSV skin delivery.

2. Materials and methods

2.1. Materials

RSV, OA and LA, all of them with purity >99%, were supplied by Sigma-Aldrich (Germany). G64 was kindly supplied from Gattefossé (France). It consists of a mixture of glycerol monostearate EP/NF and PEG-75 stearate NF/JPE, supplied as semi-solid pellets, and it has a HLB value of 10. Methanol, acetonitrile, 2-propanol, and acetic acid of HPLC-grade were purchased from Sigma-Aldrich (USA). Deionized water was used in all experiments.

2.2. Niosomes preparation

Niosomes containing RSV were prepared by the following methods:

2.2.1. Thin film hydration-sonication method (TFH-S)

The TFH method (Bangham, *et al.* 1965, Baillie, *et al.* 1985) was utilized with minor modifications, followed by a sonication stage (TFH-S).

Accurately weighed amounts of G64 and penetration enhancer (OA or LA) in different weight ratios, in the range of 1:0.5 to 1:1.5, were dissolved in 6.25 mL of an absolute ethanol solution containing a known concentration of RSV, and placed in a 100 mL round bottom flask. Then, ethanol was removed at 40 ºC under reduced pressure in a rotary evaporator (Buchi, Switzerland). The dried film was hydrated with 12.5 mL of deionized water at 60 ºC to achieve a RSV concentration of 1 mg/mL. The resulting solution was further sonicated for 30 minutes (CY-500 sonicator, Optic Ivymen System, Spain), using 45% amplitude, 500 W power, and 20 kHz frequency.

2.2.2. Ethanol injection modified method (EIM)

The conventional ethanol injection method, first described in 1973 (Batzri and Korn, 1973), offers advantages such as simplicity, absence of potentially harmful chemicals, and suitability for scaling-up (Wagner, *et al*. 2002; Pham, *et al.* 2012).

Appropriate weighed amounts of G64 and stabilizer (OA or LA) in different weight ratios, from 1:0.5 to 1:1.5, were dissolved in 6.25 mL of an absolute ethanol solution containing a known concentration of RSV. Then, this solution was injected, with a syringe pump (KDScientific, USA) at a flow of 120 mL/h, into deionized water at 60ºC, stirring at 15000 rpm with a homogenizer (SilentCrusher M, rotor model 22G, Heidolph,

Germany). Although spontaneous niosomes formation occurs as soon as the organic solution is in contact with the aqueous phase (Pham, *et al*. 2012), vigorous agitation is needed to obtain narrower size distributions. Once niosomes were formed, ethanol was removed at 40 ºC under reduced pressure in a rotary evaporator.

G64/stabilizer:RSV ratio was 30:1 for both preparation methods.

2.3. Niosomes size

Mean (Z-Average) size and polydispersity index (PDI) of niosomes were determined via Dynamic Light Scattering (DSL) using a Zetasizer Nano ZS (Malvern Instruments Ltd, UK). Three independent samples were taken from each formulation, and measurements were carried out three times for each sample at room temperature without dilution.

2.4. Niosomes morphology

Morphological analysis of niosomes was carried out by negative staining transmission electron microscopy (NS-TEM), using a JEOL-2000 Ex II TEM (Japan). A drop of the niosomal formulation was placed on a carbon-coated copper grid, and the sample excess was removed with filter paper. Then a drop of 2% (w/v) PTA (phosphotungstic acid solution) was applied to the carbon grid and left to stand for 2 minutes. Once the excess staining agent was removed with filter paper, the sample was air-dried and the thin film of stained niosomes was observed with the transmission electron microscope.

2.5. Niosomes stability

The stability of niosomes was determined by measuring backscattering (BS) profiles in a Turbiscan Lab® Expert apparatus (Formulaction, France) provided with an Ageing Station (Formulaction, France). Undiluted niosomes samples were placed in the cylindrical glass test cells and backscattered light was monitored as a function of time and cell height for 15 days, every 3 hours, at 30 °C. The optical reading head scans the sample in the cell, providing BS data every 40 μm in % relative to standards as a function of the sample height (in mm). These profiles build up a macroscopic fingerprint of the niosomes at a given time, providing useful information about changes in niosomes size distribution or appearance of a creaming layer or a clarification front with time (Pando, *et al*. 2013a).

2.6. Niosomes entrapment efficiency (EE)

Entrapped RSV was removed from free RSV by dialysis. A 2 mL sample was placed into a dialysis bag, immersed in 1000 mL of deionized water at room temperature, and stirred at 500 rpm for 2 hours. Dialyzed and non-dialyzed samples were diluted 1:10 (v/v) with methanol to facilitate the rupture of vesicle membrane and to extract RSV from vesicles. Then, RSV was analysed by chromatography (RP-HPLC) (HP series

1100 chromatograph, Hewlett Packard, USA). The system was equipped with a UV/VIS absorbance detector HP G1315A and a fluorescence detector 1260 Infinity A (Agilent Technologies, USA). A 305 nm wavelength was used for UV/VIS detector while fluorescence detector used 310/410 nm of $\lambda_{excitation}/\lambda_{emission}$ at 310/410 nm. The column was a Zorbax Eclipse Plus C_{18} of 5 µm particle size, 4.6 mm × 150 mm (Agilent Technologies, USA).

The mobile phase consisted of a mixture of (A) 100% milliQ-water and (B) 100% methanol with gradient elution at 0.8 mL/min. The step gradient started with a mobile phase of 80% (A) running 100% mobile phase (B) in minute 5 for 10 minutes. The mobile phase (B) was fed for 2 minutes after each injection to prepare the column for the next sample. The separation was carried out at 30°C.

2.7. Ex-vivo skin penetration and permeation studies

Experiments were carried out in vertical Franz cells with an effective diffusion area of 0.785 cm^2, and using the skin of newborn pig. The skin, previously frozen at -80°C, was pre-equilibrated in saline solution at 25°C for 1 hour. Then, the skin was placed onto the Franz cell and sandwiched with the stratum corneum (SC) side facing the donor compartment. The receptor container, thermostated at 37 ± 1°C, was filled with 5.5 mL saline solution (0.9% w/v NaCl), and was continuously agitated with magnetic stirring.

A 100 µL sample was applied onto the skin, in the donor compartment (*n* = 3 per formulation) during 8 hours. After this period of time and once the skin was removed from the Franz cell, it was gently rinsed with deionized water and dried.

To determine the amount of RSV that did not penetrate into the deeper layer of the skin (*i.e.* epidermis and dermis (EDD) passing through the SC), a separation of SC from skin was carried out. This was carried out by stripping the SC layer with adhesive tape (Tesa AG, Germany). The RSV present in SC and EDD was extracted with methanol. This method had been previously validated by histological examination of stripped skin (Manconi, *et al*. 2005).

Receiver compartment samples were lyophilized and then methanol was added to extract resveratrol. RSV content, both in skin layer (SC and EDD) and receiver compartment samples, was finally determined by RP-HPLC.

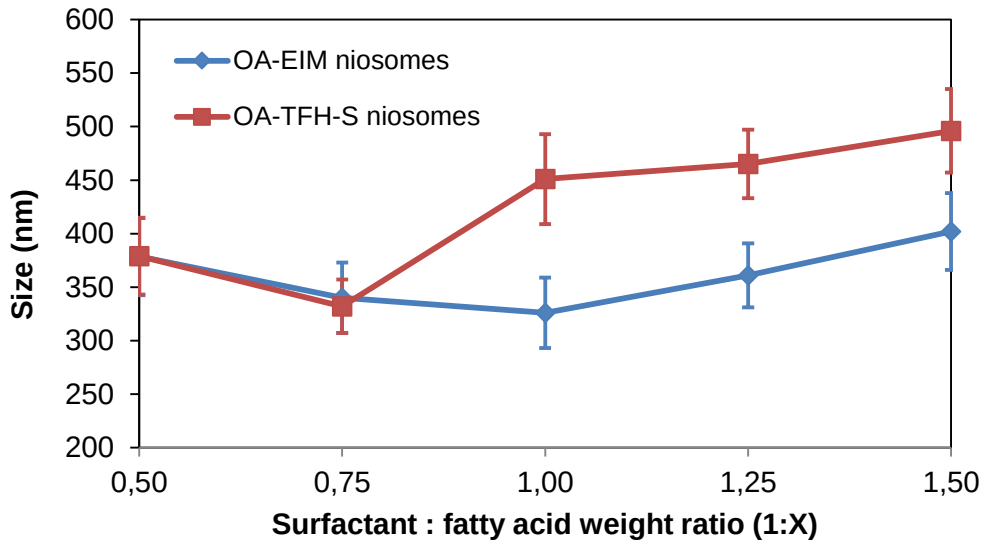
2.8. Statistical analysis

All data were expressed as the mean ± SD (standard deviation) of three independent experiments, and statistical analysis of the data was carried out (ANOVA). Fisher's test ($p<0.05$) was used to calculate the least significance difference (LSD) using statistical software (Microsoft Excel 2010).

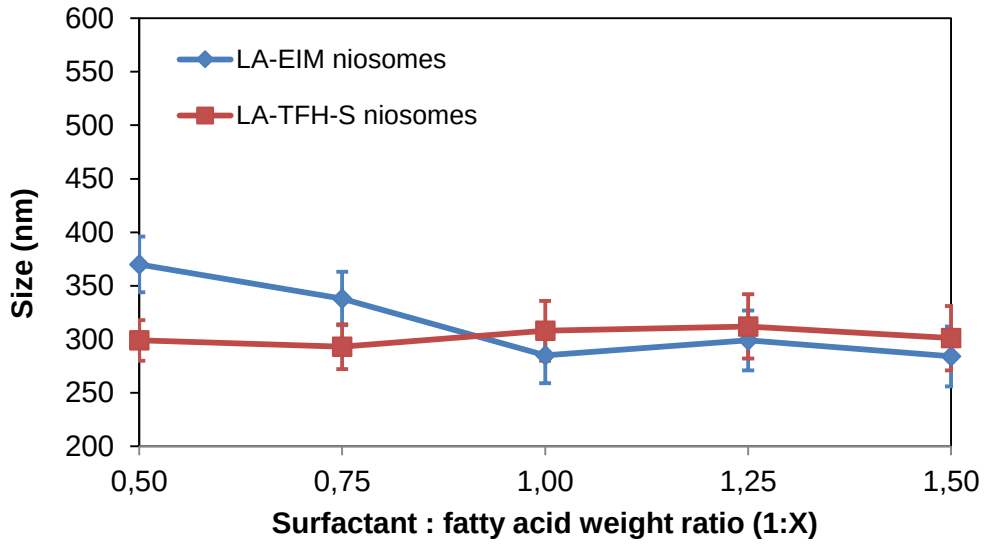
3. Results and discussion

3.1. Mean size and size distribution of niosomes

The mean sizes of niosomes formulated with G64 as surfactant, and OA or LA as penetration enhancer, at different weight ratios are shown in Figure 1.



(a)



(b)

Figure 1. Effect of surfactant (G64) to fatty acid weight ratio on niosomes size prepared by EIM and TFH-S methods. (a) Oleic acid (OA); (b) Linoleic acid (LA)

Niosomes prepared with LA as penetration enhancer showed smaller sizes than those prepared using OA ($p < 0.05$). Variations in niosome size up to 40% with TFH-S

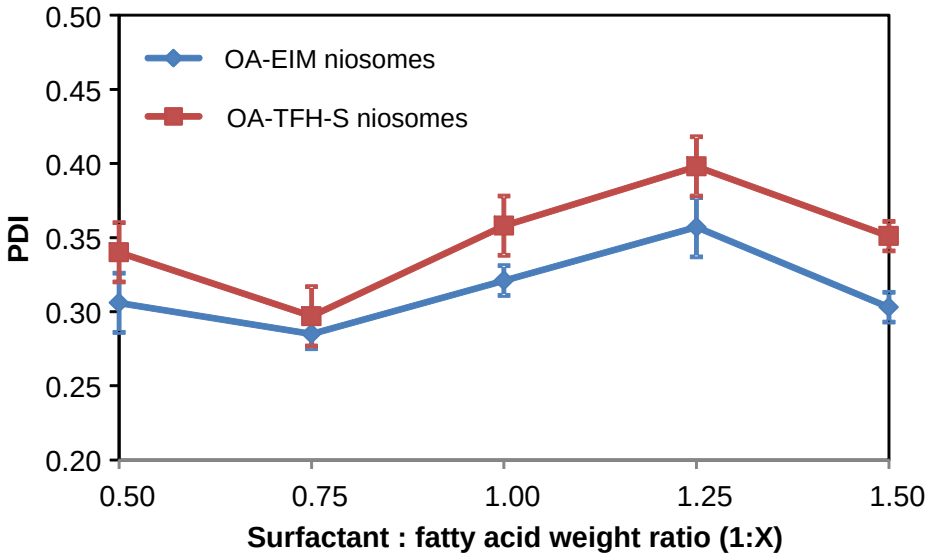
method, and up to 30% for EIM method were observed, depending on the type and concentration of fatty acid.

Figure 1(a) shows the niosome size as a function of the G64 : OA weight ratio for both preparation methods. It was observed that niosomes prepared by the EIM method had smaller size, in the range 326-402 nm. Niosomes prepared with the TFH-S method showed a higher variation with this parameter ($p < 0.05$). Moreover, while similar sizes were obtained with both methods for G64 : OA weight ratios of 1:0.5 and 1:0.75, big discrepancies were observed for larger weight ratios, and larger niosomes were obtained by the TFH-S method.

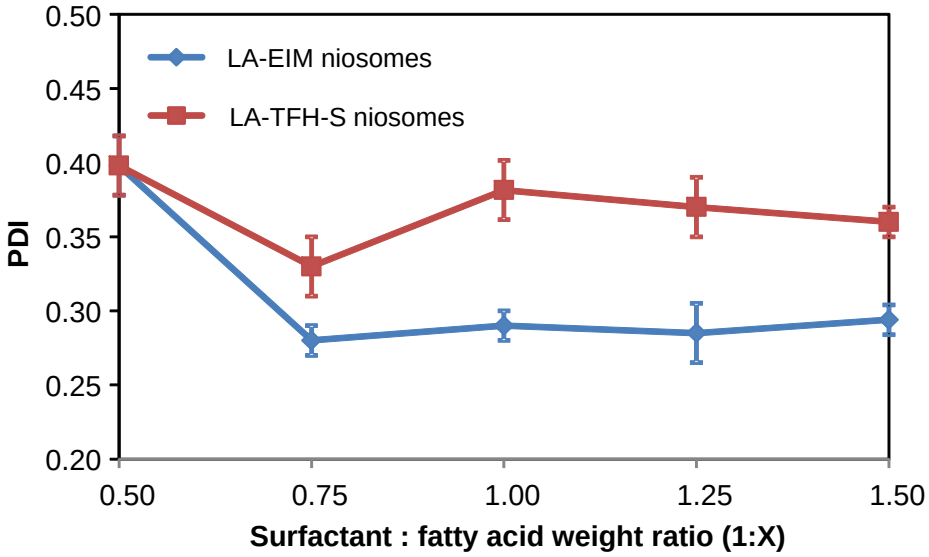
As shown in Figure 1(b) an opposite trend was observed when niosome size was plotted versus the G64 : LA weight ratio for both preparation methods. Niosomes prepared by the EIM method exhibited large variations ($p < 0.05$), while no significant differences were found with the TFH-S method for all the G64 : LA weight ratios used.

These results show that the selection of LA or OA as penetration enhancer involves significant differences in niosomes size, and are highly dependent on both the surfactant : fatty acid weight ratio and the preparation method.

PDI values of niosomes formulated with G64 as surfactant, and OA or LA as penetration enhancer, at different weight ratios are shown in Figure 2.



(a)



(b)

Figure 2. Effect of surfactant (G64) to fatty acid weight ratio on PDI of niosomes prepared using EIM and TFH-S methods. (a) Oleic acid (OA); (b) Linoleic acid (LA)

For niosomes made of G64 and OA as penetration enhancer, Figure 2(a), there was significant relation between G64 : OA weight ratio and sample PDI ($p < 0.05$) for both preparation methods. The same trend was observed regarding the best PDI values at a

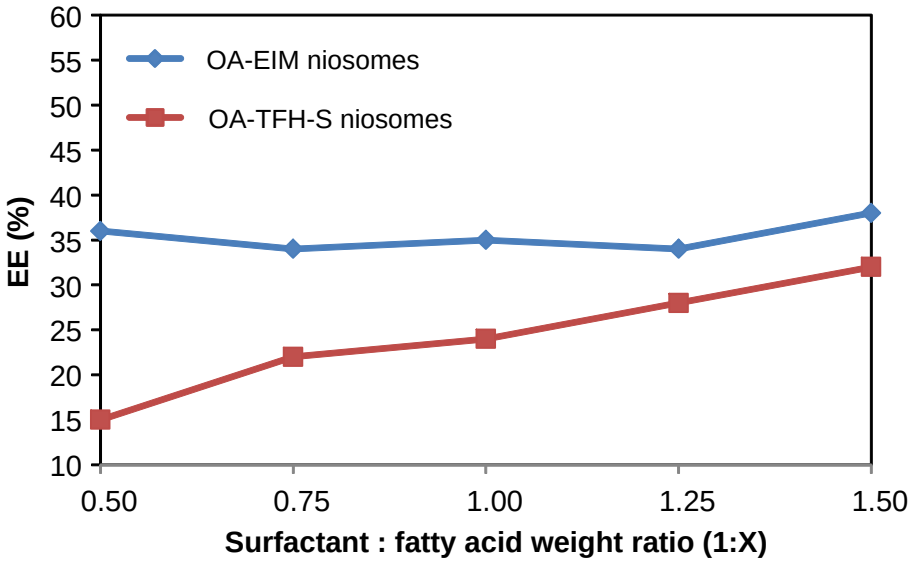
weight ratio G64 : OA of 1:0.75 for both methods. For the G64 : OA weight ratio range studied, the EIM method yielded lower PDI values.

There was also a significant relation between G64 : LA weight ratio and PDI of the sample ($p < 0.05$) for niosomes made with G64 and LA as penetration enhancer, Figure 2(b). The best PDI values corresponded to the same weight ratio (1:0.75) for both methods. G64–LA niosomes prepared by the EIM method showed lower PDI values than G64–LA niosomes prepared by the TFH-S method.

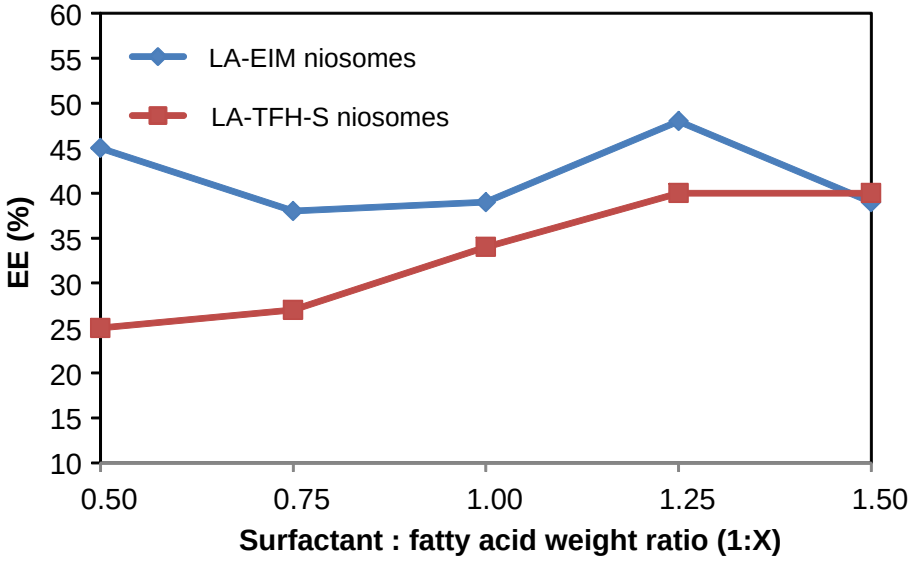
These results prove that the G64 : fatty acid weight ratio is significantly relevant in order to reach low PDI value, which involves a monodisperse distribution. The best PDI value is obtained for weight ratio 1: 0.75 independently of the niosomes formulation and method of preparation.

3.2. Niosomes entrapment efficiency (EE)

Figure 3 shows the EE values obtained as a function of G64 : fatty acid weight ratio.



(a)



(b)

Figure 3. Effect of surfactant (G64) to fatty acid weight ratio on EE of niosomes prepared using EIM and TFH-S methods. (a) Oleic acid (OA); (b) Linoleic acid (LA)

EE of RSV showed a clear trend with the amount of OA used as penetration enhancer, Figure 3(a), for niosomes prepared with the TFH-S method ($p < 0.05$), the higher G64 : OA weight ratio, the higher EE. However, slight variations were obtained on EE for

G64-OA niosomes prepared with the EIM method, which were in all cases higher than those obtained by TFH-S method.

For G64-LA niosomes, there was a relationship between EE and amount of LA used, slightly more evident for niosomes prepared by TFH-S method ($p < 0.05$), since EE increased with G64 : LA weight ratio. Also in this case, niosomes prepared by EIM method showed higher EE than niosomes prepared by TFH-S method, except for G64 : LA weight relation of 1:1.5, where similar EE values were obtained with both methods.

These results show a clear dependence of EE on the preparation method ($p < 0.05$), higher values being obtained for niosomes prepared with the EIM method. Only the niosomes formulated at G64 : LA weight ratio of 1:1.5 have similar EE values for both methods.

It had been previously reported that the presence of the active compound did not change the average size of vesicles (Manca, *et al*. 2013). Moreover, some authors found relation between vesicle size and EE, being the largest vesicles the ones with the highest EE (Maestrelli, *et al*. 2006; Srisuk, *et al*. 2012; Cadena, *et al*. 2013). However, in the present work it was not observed a clear relationship between EE and niosomes mean size.

3.3. Ex-vivo skin penetration and permeation studies

The release of RSV entrapped niosomes across a series of barriers and anatomical structures of the skin, as function of different formulations and preparation methods, was studied. Skin penetration occurs by diffusion of the active compound across the skin layers into the receptor phase, *i.e.* subcutaneous fluids and blood vessels (Pando, *et al*. 2013b).

Release experiments were carried out using skin of newborn pig, as it is known that pig skin is a good substitute in ex-vivo permeation experiments due to its similarity with the SC of human skin in terms of lipid composition. Although it presents a marked difference in thickness, newborn pig SC is considerably thinner than that of adult pigs, and more similar to the human skin, even if the number of hair follicles is higher (Pando, *et al*. 2013b). Several studies have been carried out with newborn pig skin, confirming its suitability for skin permeation screenings (Manconi, *et al*. 2011).

RSV effects on the skin only appear when RSV penetrates to the deeper layers of skin (EDD), being necessary to cross the SC. Due to the difficulty to precisely separate epidermis and dermis, in this study three parts were distinguished: SC, EDD and receptor fluid (RC).

The amount of RSV accumulated into these parts (SC, EDD and RC) was analysed by RP-HPLC. These measurements enabled to make a mass balance of RSV in the system to reinforce the method applied, since total RSV lost was less than 10% in all cases.

RSV penetration into the deeper layers of skin (EDD), using different formulations and niosomes preparation methods, are shown in Figure 4.

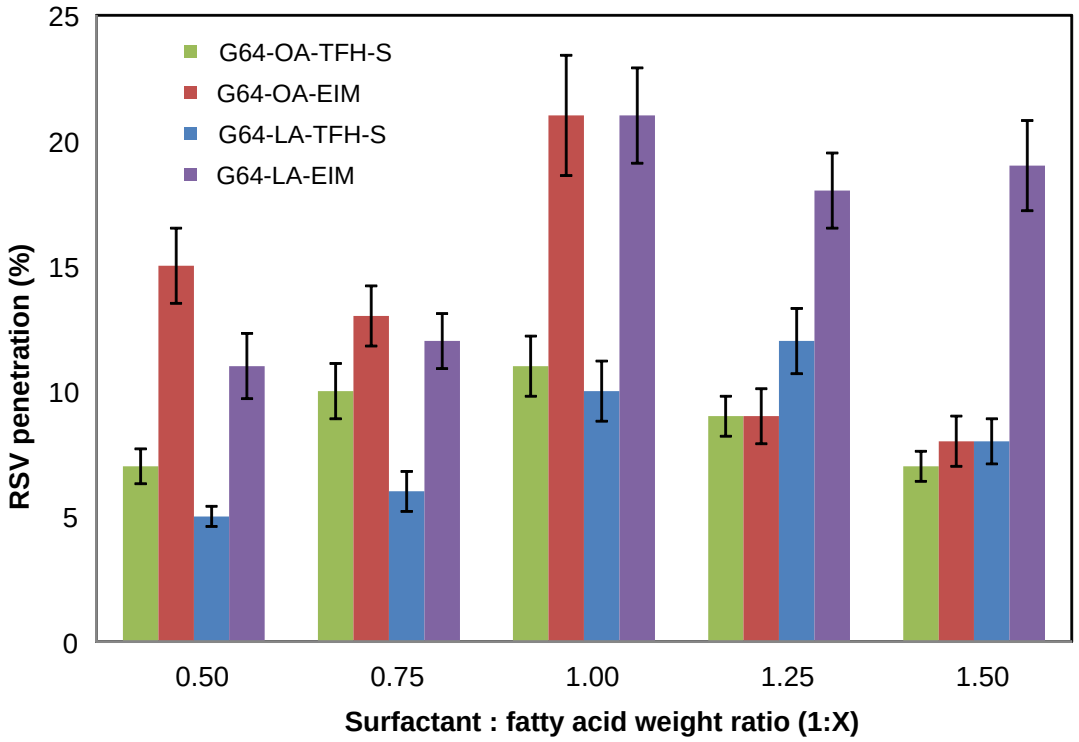


Figure 4. RSV penetration in epidermis and dermis (EDD): influence of formulation and niosomes preparation method

A close correlation between the amount of fatty acid used and RSV penetration ($p < 0.05$) was observed, being stronger for niosomes prepared with the EIM method, for both penetration enhancers, OA and LA. Therefore, less RSV accumulation in the SC was found when this method was applied.

It was also clear the dependence between RSV penetration in EDD and the niosomes preparation method ($p < 0.05$), being the EIM more effective for all formulations tested ($p< 0.05$). Niosomes prepared with this method at a weight ratio of 1:1 for both penetration enhancers were the most effective, showing RSV penetration values up to 21%. In these cases, the RSV accumulation in the SC was in the range of 5.4% - 27.7%. In order to compare RSV penetration with niosomes size, PDI and EE values, all these data are summarized in Table 1.

Table 1. Mean size (Z-average), PDI, EE, and RSV penetration into the EDD layer for niosomes formulated with G64 as surfactant and OA or LA as penetration enhancer, using TFH-S or EIM as preparation methods

Preparation method	Fatty acid (FA)	G64 : FA weight ratio	Mean size (nm)	PDI	EE (%)	RSV penetration (%)
TFH-S	OA	1:0.5	379 ± 36	0.34 ± 0.01	15 ± 1	7.0 ± 0.7
		1:0.75	332 ± 25	0.30 ± 0.01	22 ± 2	10.0 ± 1.1
		1:1	451 ± 42	0.36 ± 0.02	24 ± 2	11.0 ± 1.2
		1:1.25	465 ± 32	0.40 ± 0.02	28 ± 2	9.0 ± 0.8
		1:1.5	496 ± 39	0.35 ± 0.02	32 ± 2	7.0 ± 0.6
	LA	1:0.5	299 ± 19	0.40 ± 0.02	25 ± 2	5.0 ± 0.4
		1:0.75	293 ± 21	0.33 ± 0.02	27 ± 2	6.0 ± 0.8
		1:1	308 ± 28	0.38 ± 0.02	34 ± 2	+10.0 ± 1.2
		1:1.25	312 ± 30	0.37 ± 0.02	40 ± 3	12.0 ± 1.3
		1:1.5	301 ± 30	0.36 ± 0.01	40 ± 3	8.0 ± 0.9
EIM	OA	1:0.5	378 ± 36	0.31 ± 0.01	36 ± 2	15.0 ± 1.5
		1:0.75	340 ± 33	0.29 ± 0.01	34 ± 3	13.0 ± 1.2
		1:1	326 ± 33	0.32 ± 0.01	35 ± 2	21.0 ± 2.4
		1:1.25	361 ± 30	0.36 ± 0.02	34 ± 2	9.0 ± 1.1
		1:1.5	402 ± 36	0.30 ± 0.02	38 ± 2	8.0 ± 1.0
	LA	1:0.5	370 ± 26	0.40 ± 0.02	45 ± 3	11.0 ± 1.3
		1:0.75	338 ± 25	0.28 ± 0.01	38 ± 3	12.0 ± 1.1
		1:1	285 ± 26	0.29 ± 0.01	39 ± 3	21.0 ± 1.9
		1:1.25	299 ± 28	0.28 ± 0.02	48 ± 3	18.0 ± 1.5
		1:1.5	284 ± 28	0.29 ± 0.01	39 ± 2	19.0 ± 1.8

As a general trend, higher RSV penetration corresponded to niosomes with smaller mean sizes. A similar behaviour had been reported by other authors who attributed this effect to the size of the small vesicles that led to an increase vesicle/skin interface enhancing interactions with skin lipids, and increasing transdermal fluxes (Verma, *et al*. 2003; Maestrelli, *et al*. 2006; Srisuk, *et al*. 2012; Manca, *et al.* 2013).

However, it was observed that, not in all cases, formulations with the best EE were not the most suitable regarding RSV penetration, as it had been previously reported by other authors (Maestrelli, *et al*. 2006; Srisuk, *et al*. 2012; Kong, *et al*. 2013).

3.4. Characterization of the optimum niosomes

Stability with time was examined for the best formulations to compare the effect of both OA and LA as penetration enhancers, as well as niosomes preparation method. Hence, a G64 : fatty acid weight ratio of 1:1 was selected, since this ratio showed successful results with respect to EE and RSV transdermal delivery. Morphology of these niosomes was also confirmed by TEM.

Figure 5 shows four negative stain micrographs of RSV entrapped niosomes obtained with the best aforementioned formulations.

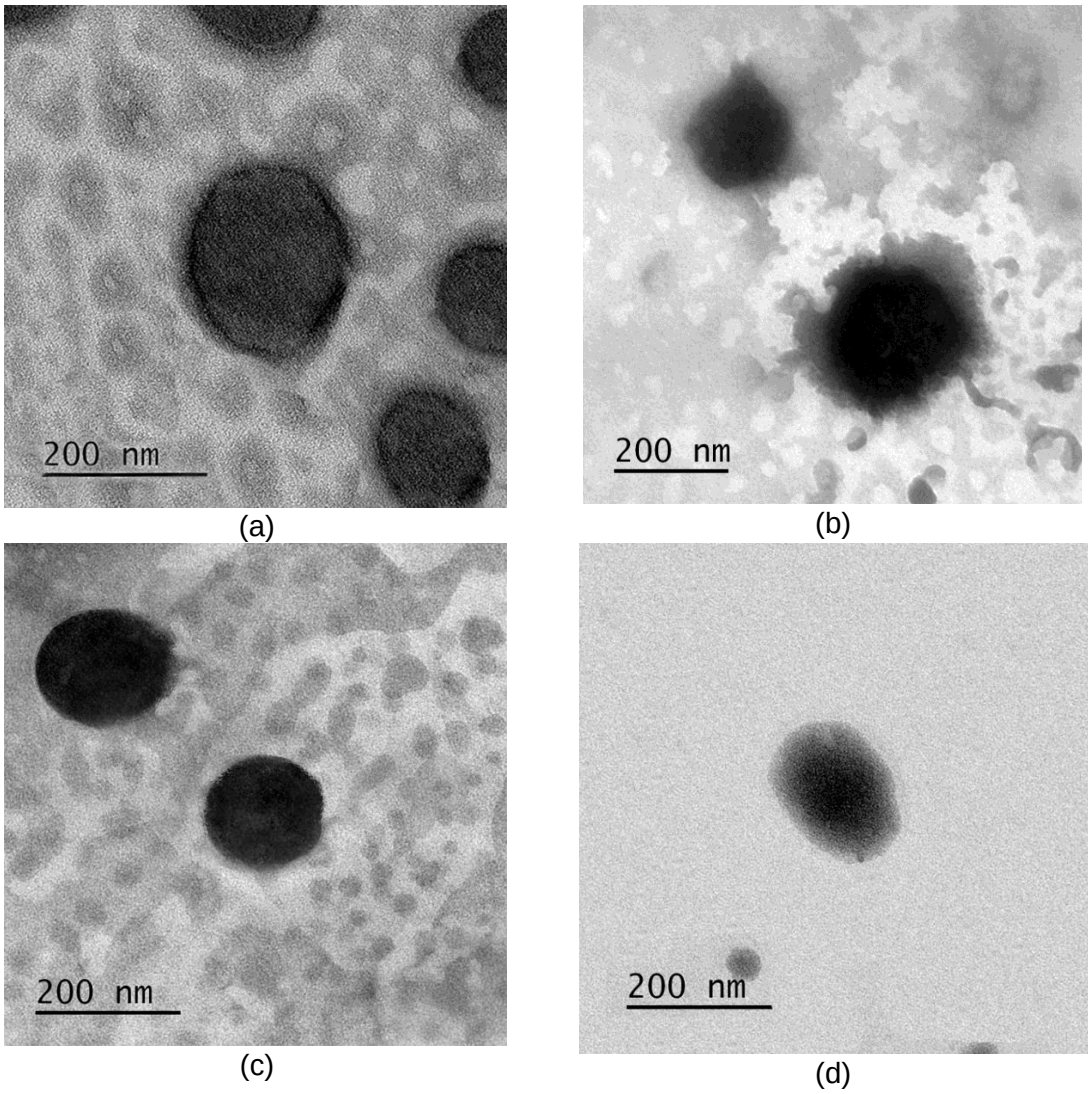


Figure 5. TEM micrographs of different niosomes formulated with a surfactant : fatty acid weight ratio of 1:1. (a) G64-OA-TFH-S; (b) G64-OA–EIM; (c) G64- LA-TFH-S; (d) G64-LA-EIM

Dark-stained niosomes were obtained as a result of the strong interactions between surfactant and phosphotungstic acid, allowing a selective electrons deposit in the sample, which enhanced structural details. Micrographs showed circular and dark structures corresponding to spherical niosomes of approximately 300-400 nm, according to DLS measurements. It can be clearly observed that mean size of niosomes prepared by EIM method, Figures 5(b) and 5(d), were smaller than those prepared by TFH-S method, Figures 5(a) and 5(c). Niosomes formulated with OA, Figures 5(a) and 5(b) also presented larger values.

Figure 6 shows the BS profiles of different niosomal samples examined for 15 days every 3 hours.

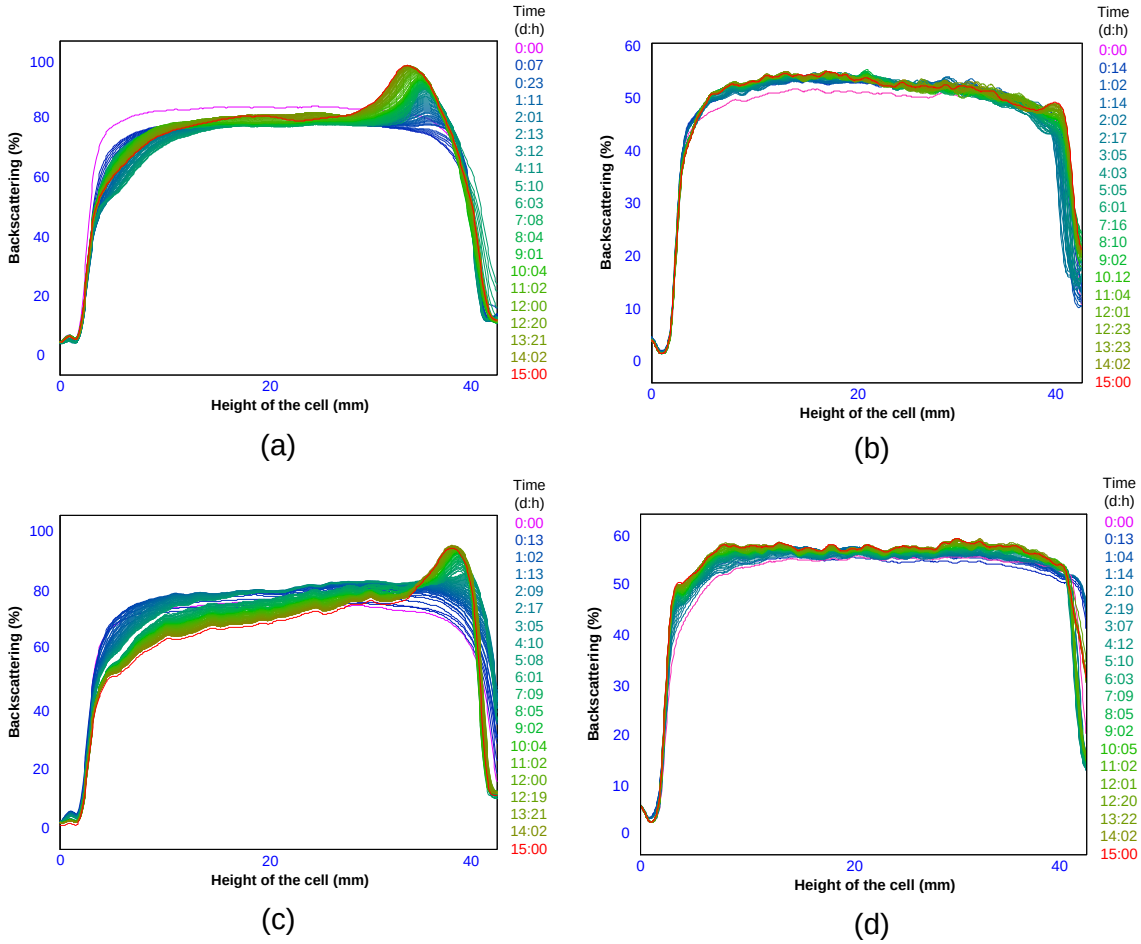


Figure 6. BS profiles of different niosomes formulated with a surfactant : fatty acid weight ratio of 1:1. (a) G64-OA-TFH-S; (b) G64-OA –EIM; (c)G64- LA-TFH-S; (d) G64-LA-EIM

Figures 6(b) and 6(d) indicate the higher stability of niosomes prepared by EIM method, using either OA or LA. In both cases, BS variation (ΔBS) was lower than 10% being homogenous along the cell, which means that there were no significant changes in niosomes size, remaining the sample stable with no destabilization phenomena, such as aggregation or coalescence. Moreover, not significant creaming or sedimentation phenomena were observed in these samples during the monitoring time.

However, niosomes prepared using TFH-S method, Figures 6(a) and 6(c), showed creaming phenomena evidenced by an increase of BS at the top of the cell, and a simultaneous decrease at the bottom, with ΔBS up to 20%. For niosomes formulated with OA this effect could be attributed to their larger sizes, and hence migration phenomena could easily take place due to differences between densities of niosomes and aqueous external phase. Similar behaviour has been found in other colloidal systems (Gutiérrez, *et al*. 2014).

4. Conclusions

The present work indicates that the EIM method produces niosomes with smaller mean sizes, narrower size distributions, higher EE and stability than those prepared with the TFH-S method. Furthermore, the EIM method is more suitable for scaling up.

A clear dependence was observed between RSV penetration in EDD and niosomes preparation method, being the EIM method more effective for all formulations tested, which can be related to the smaller niosomes mean size obtained with this method.

An optimum dosage of penetration enhancer is needed to obtain high RSV penetration values, although no significant differences were observed between both, OA and LA, enhancers tested.

No relationship between EE and niosomes mean size, as well as with RSV penetration was found. Further research is needed to have a better understanding of the mechanisms involved in RSV skin delivery through niosomes.

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Figure captions

Figure 1. Effect of surfactant (G64) to fatty acid weight ratio on niosomes size prepared by EIM and TFH-S methods. (a) Oleic acid (OA); (b) Linoleic acid (LA)

Figure 2. Effect of surfactant (G64) to fatty acid weight ratio on PDI of niosomes prepared using EIM and TFH-S methods. (a) Oleic acid (OA); (b) Linoleic acid (LA)

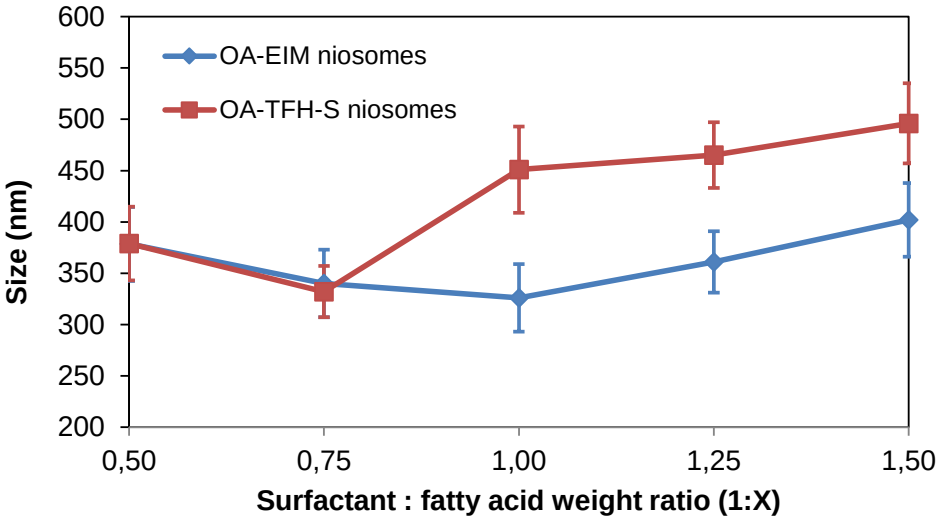
Figure 3. Effect of surfactant (G64) to fatty acid weight ratio on EE of niosomes prepared using EIM and TFH-S methods. (a) Oleic acid (OA); (b) Linoleic acid (LA)

Figure 4. RSV penetration in epidermis and dermis (EDD): influence of formulation and niosomes preparation method

Figure 5. TEM micrographs of different niosomes formulated with a surfactant : fatty acid weight ratio of 1:1. (a) G64-OA-TFH-S; (b) G64-OA–EIM; (c) G64- LA-TFH-S; (d) G64-LA-EIM

Figure 6. BS profiles of different niosomes formulated with a surfactant : fatty acid weight ratio of 1:1. (a) G64-OA-TFH-S; (b) G64-OA –EIM; (c)G64- LA-TFH-S; (d) G64-LA-EIM

Figure 1 Colour



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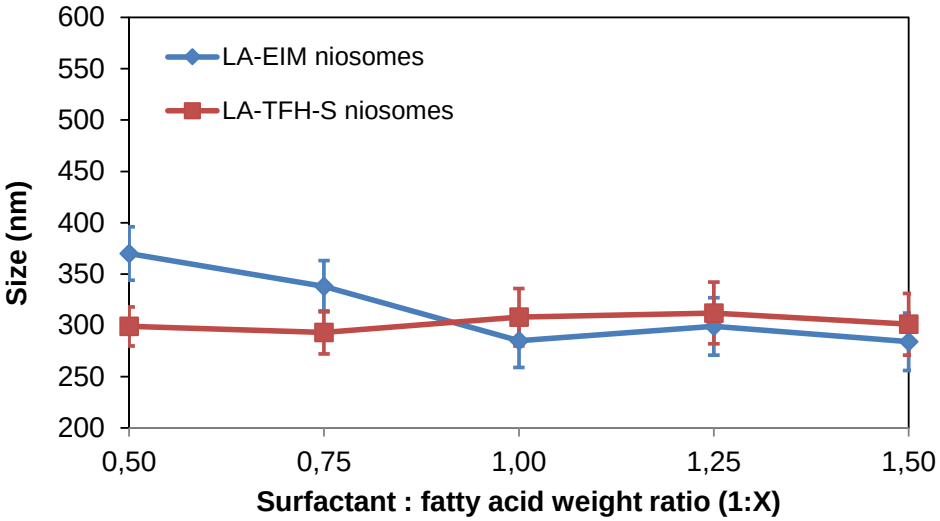
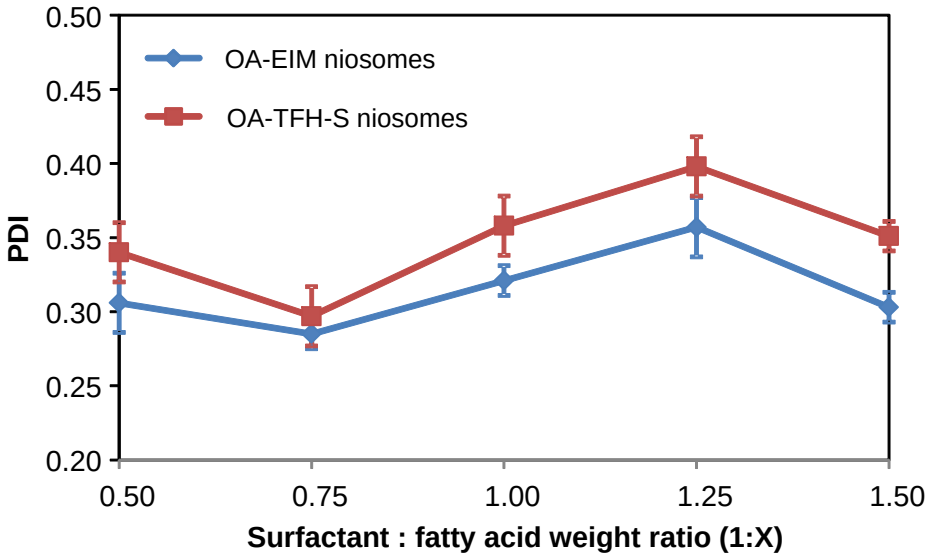


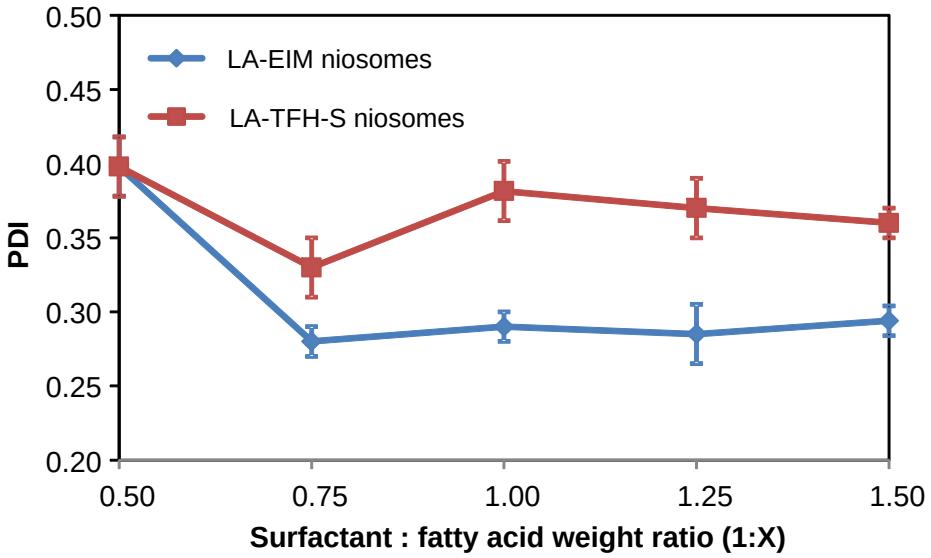
(b)

Figure 1

Figure 2 Colour



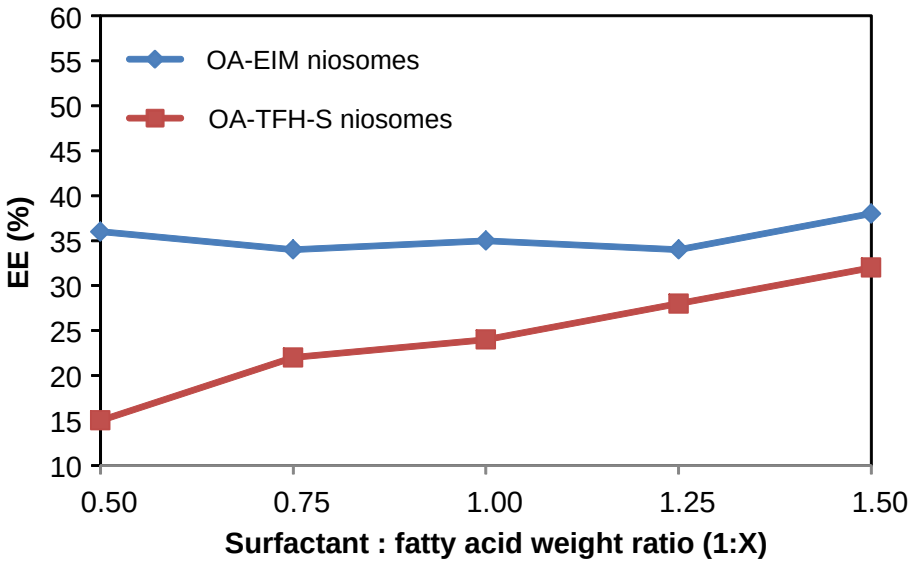
(a)



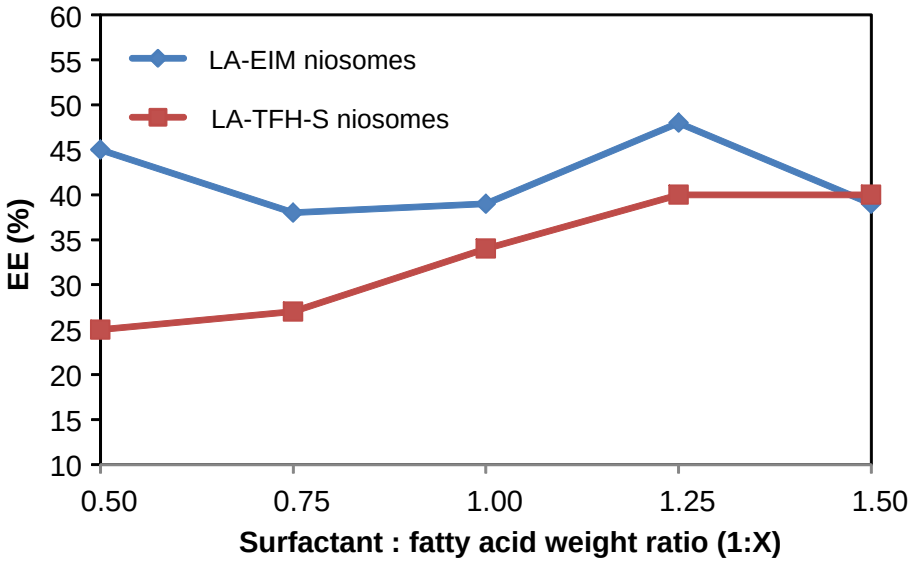
(b)

Figure 2

Figure 3 Colour



(a)



(b)

Figure 3

Figure 4 Colour

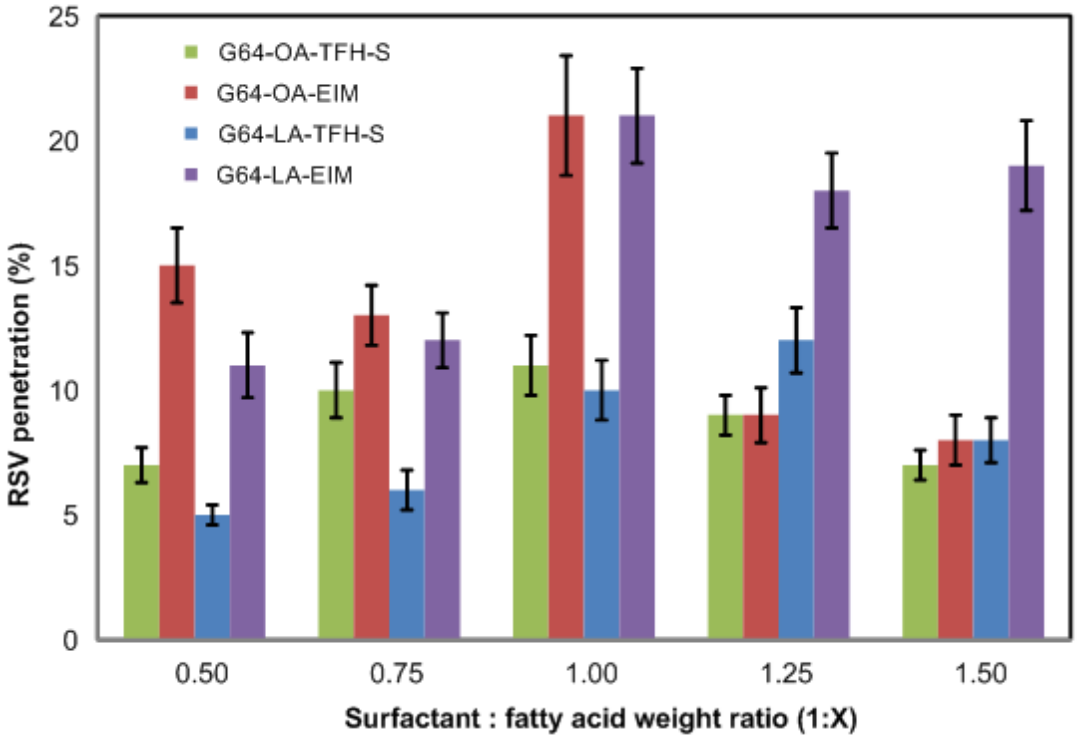


Figure 4

Figure 5

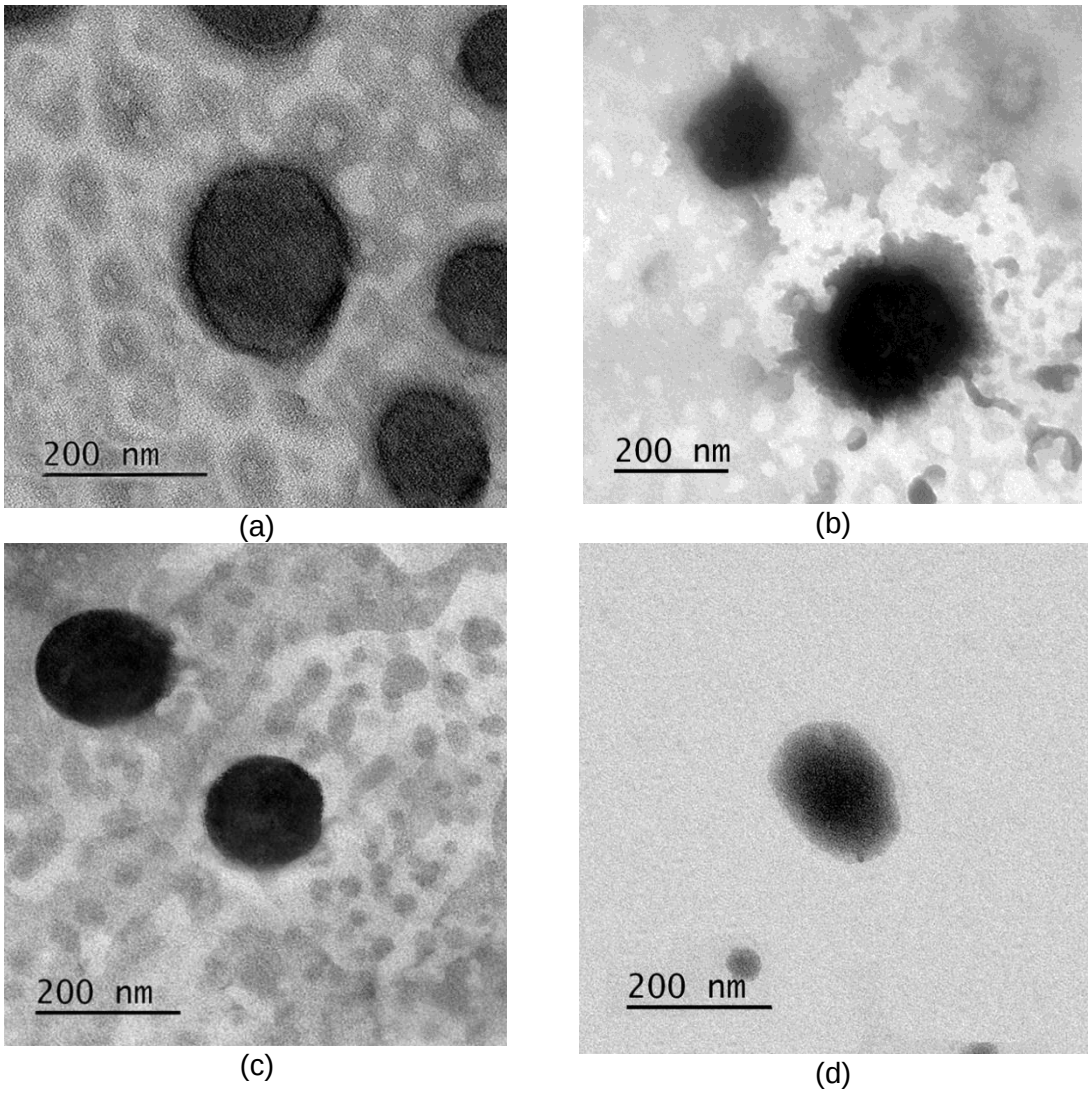


Figure 5

Figure 6 Colour

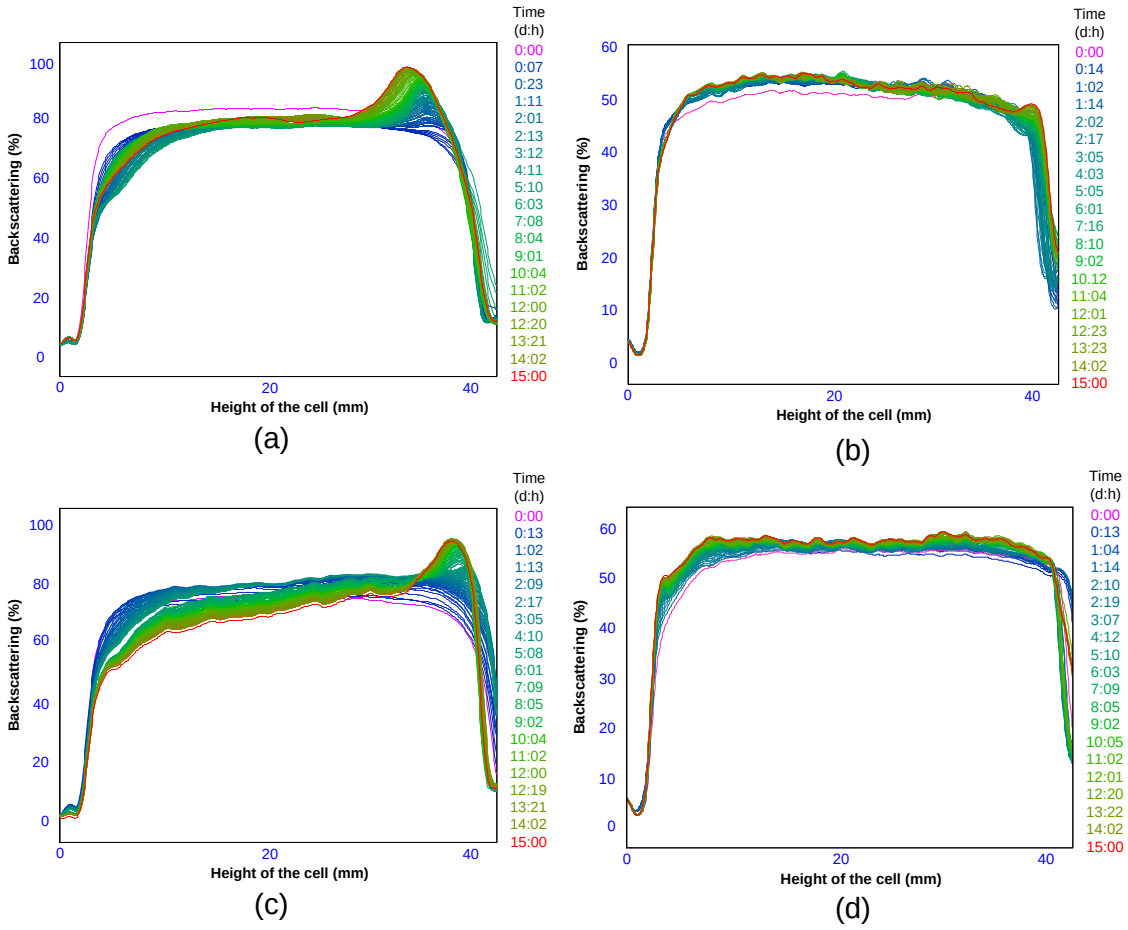


Figure 6

Table 1

Table 1. Mean size (Z-average), PDI, EE, and RSV penetration into the EDD layer for niosomes formulated with G64 as surfactant and OA or LA as penetration enhancer, using TFH-S or EIM as preparation methods

Preparation method	Fatty acid (FA)	G64 : FA weight ratio	Mean size (nm)	PDI	EE (%)	RSV penetration (%)
TFH-S	OA	1:0.5	379 ± 36	0.34 ± 0.01	15 ± 1	7.0 ± 0.7
		1:0.75	332 ± 25	0.30 ± 0.01	22 ± 2	10.0 ± 1.1
		1:1	451 ± 42	0.36 ± 0.02	24 ± 2	11.0 ± 1.2
		1:1.25	465 ± 32	0.40 ± 0.02	28 ± 2	9.0 ± 0.8
		1:1.5	496 ± 39	0.35 ± 0.02	32 ± 2	7.0 ± 0.6
	LA	1:0.5	299 ± 19	0.40 ± 0.02	25 ± 2	5.0 ± 0.4
		1:0.75	293 ± 21	0.33 ± 0.02	27 ± 2	6.0 ± 0.8
		1:1	308 ± 28	0.38 ± 0.02	34 ± 2	10.0 ± 1.2
		1:1.25	312 ± 30	0.37 ± 0.02	40 ± 3	12.0 ± 1.3
		1:1.5	301 ± 30	0.36 ± 0.01	40 ± 3	8.0 ± 0.9
EIM	OA	1:0.5	378 ± 36	0.31 ± 0.01	36 ± 2	15.0 ± 1.5
		1:0.75	340 ± 33	0.29 ± 0.01	34 ± 3	13.0 ± 1.2
		1:1	326 ± 33	0.32 ± 0.01	35 ± 2	21.0 ± 2.4
		1:1.25	361 ± 30	0.36 ± 0.02	34 ± 2	9.0 ± 1.1
		1:1.5	402 ± 36	0.30 ± 0.02	38 ± 2	8.0 ± 1.0
	LA	1:0.5	370 ± 26	0.40 ± 0.02	45 ± 3	11.0 ± 1.3
		1:0.75	338 ± 25	0.28 ± 0.01	38 ± 3	12.0 ± 1.1
		1:1	285 ± 26	0.29 ± 0.01	39 ± 3	21.0 ± 1.9
		1:1.25	299 ± 28	0.28 ± 0.02	48 ± 3	18.0 ± 1.5
		1:1.5	284 ± 28	0.29 ± 0.01	39 ± 2	19.0 ± 1.8

Figure 1 Black&White

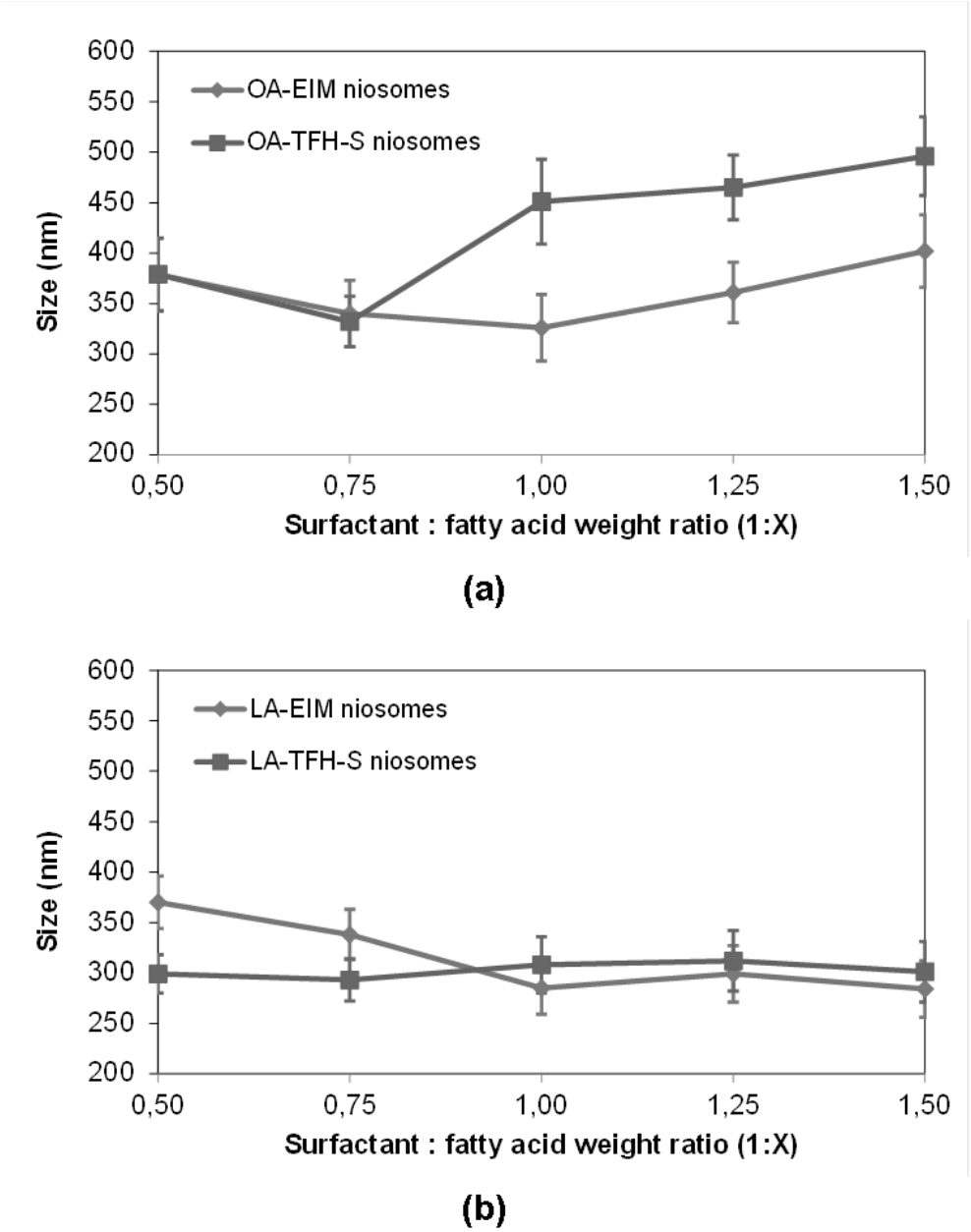
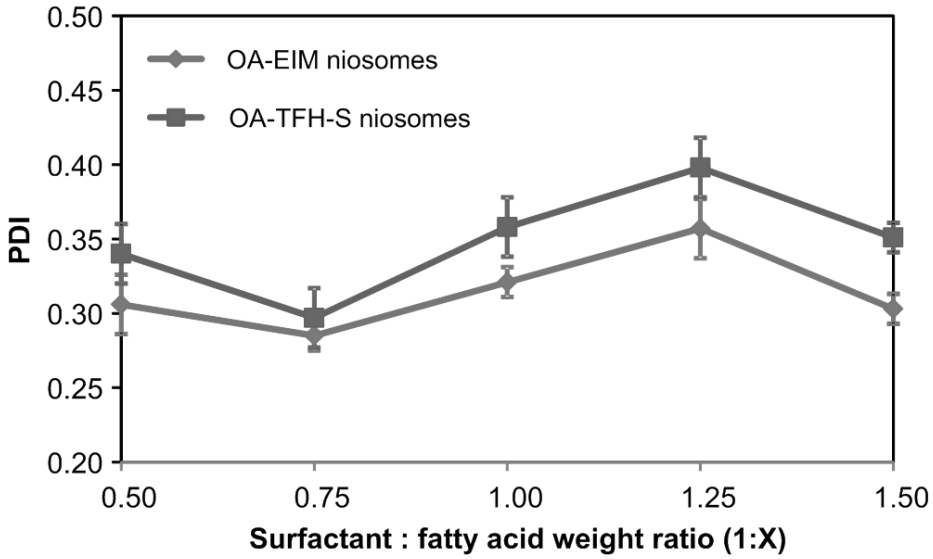
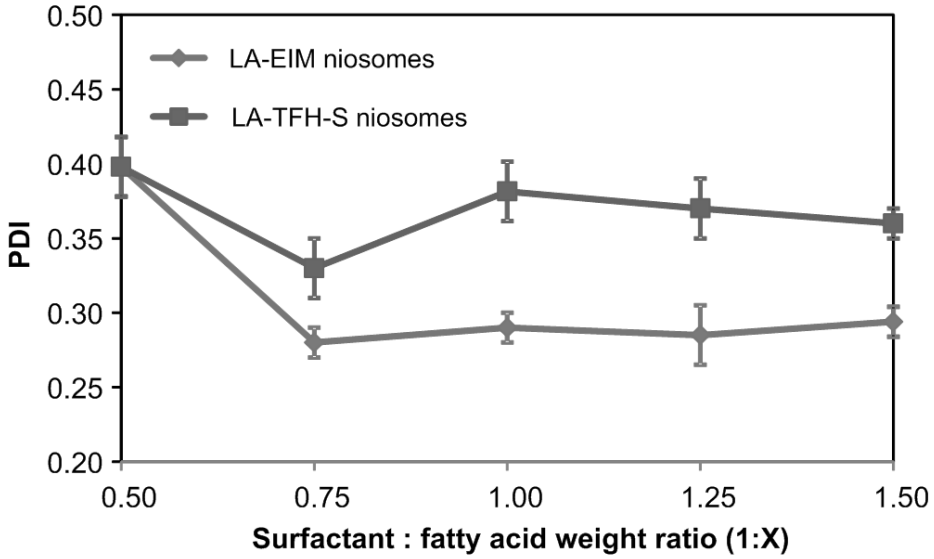


Figure 1

Figure 2 B&W



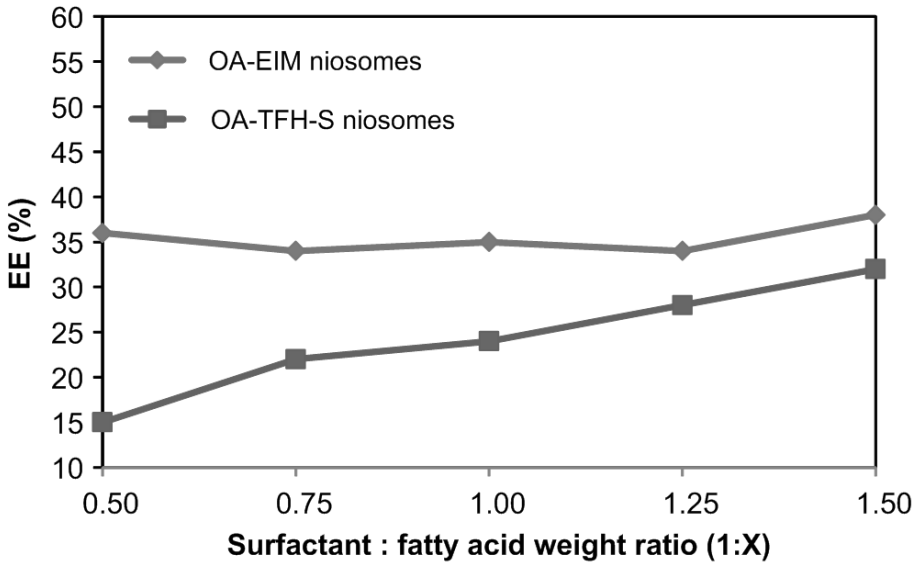
(a)



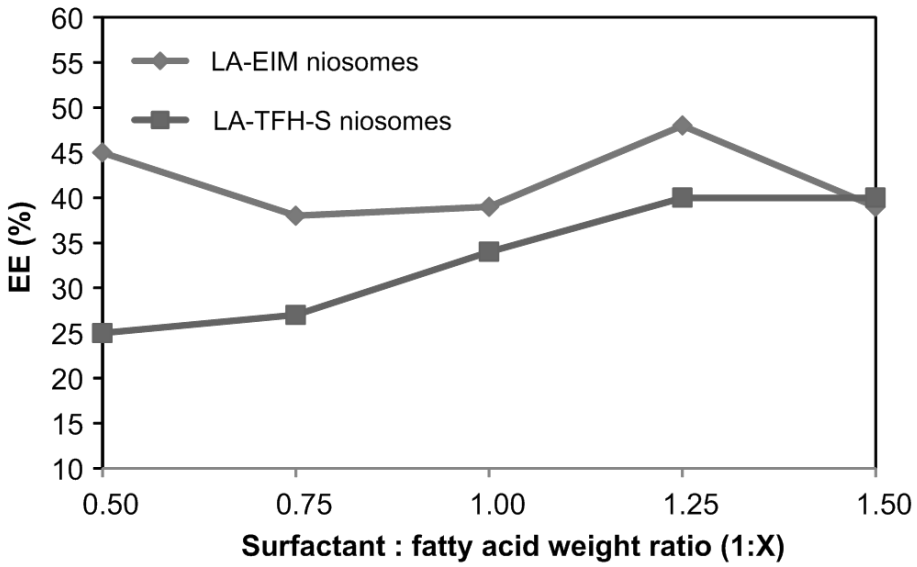
(b)

Figure 2

Figure 3 B&W



(a)



(b)

Figure 3

Figure 4 B&W

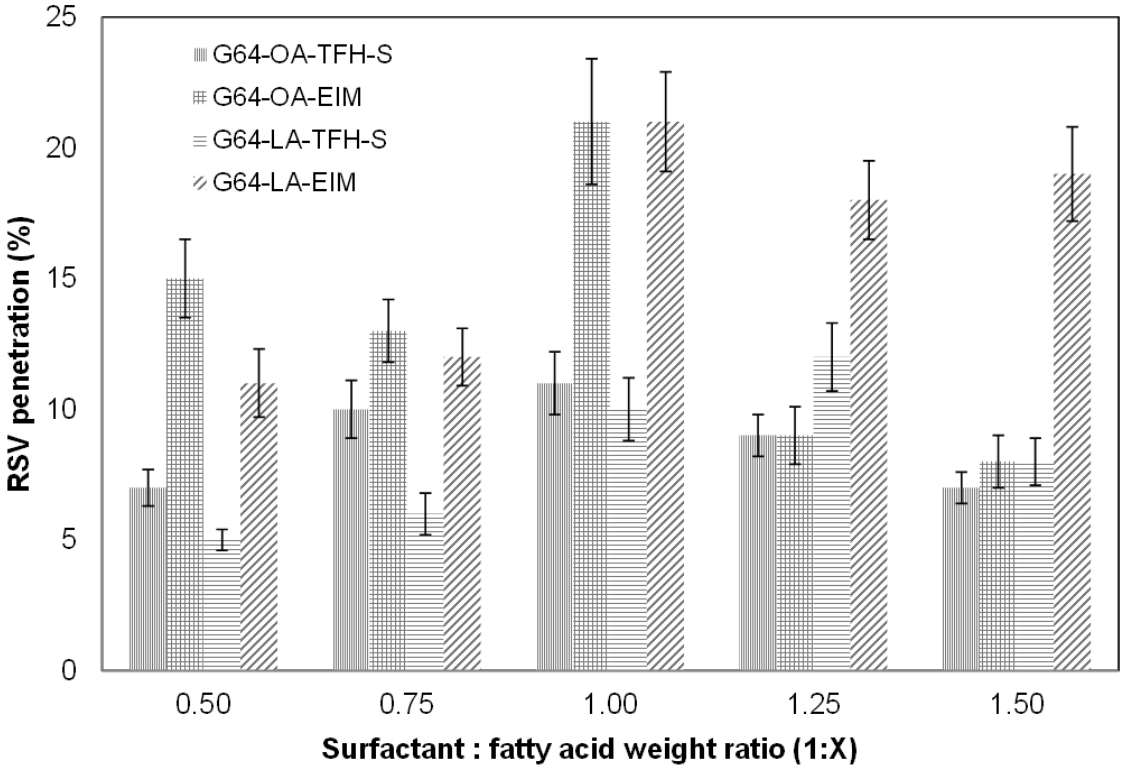


Figure 4

Figure 6 B&W

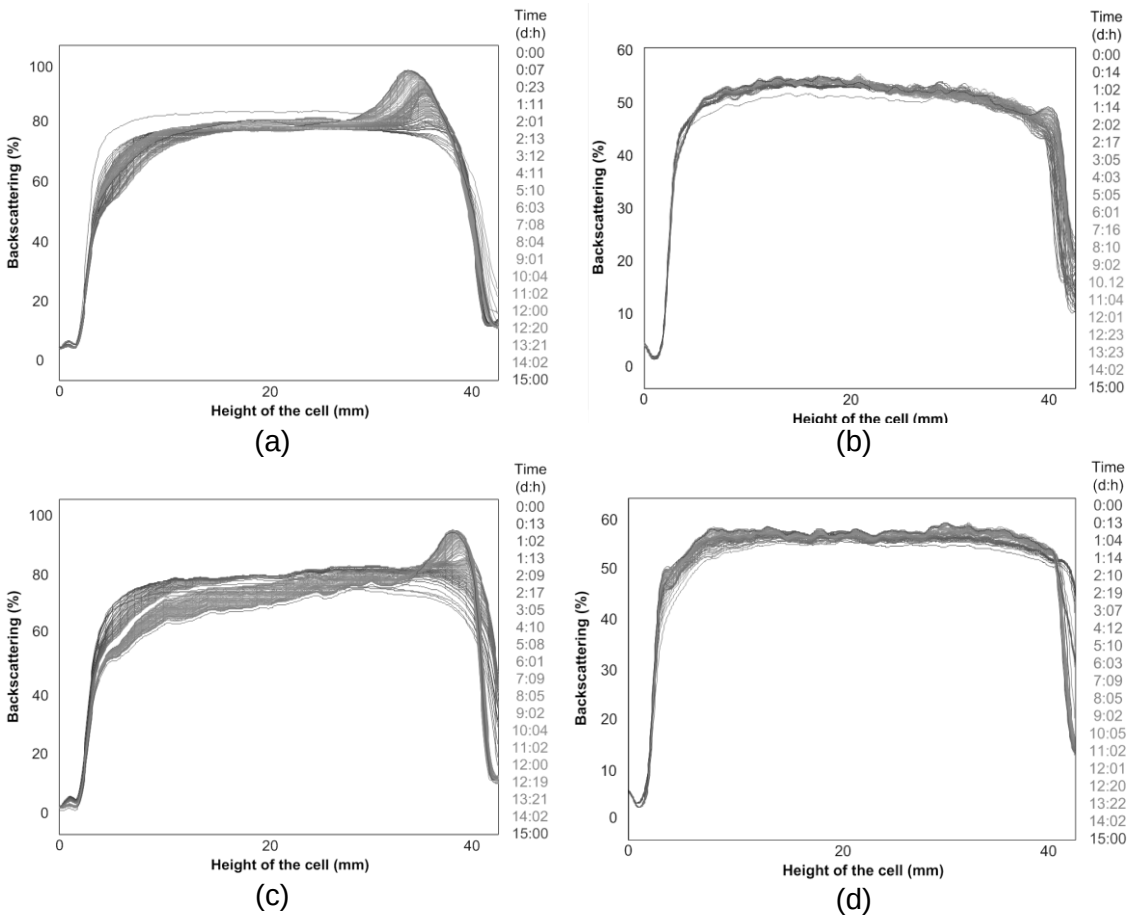


Figure 6