Effect of drug molecular weight on niosomes size and encapsulation efficiency

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Abstract

Encapsulation into nanocarriers, such as niosomes, is a promising way to protect them from degradation, and allow controll and target delivery of bioactive compounds. For biotechnological applications, a tight control of particle size with acceptable encapsulation efficiencies (EE) is a technological challenge, especially for hydrophilic compounds due to its capability to diffuse across biological barriers. Niosomes formulated with mixture of surfactants represent promising nanocarriers due to the advantages of non-ionic surfactants, such as low cost, versatility and enhanced physico-chemical properties. In this work, the effect of both composition of the hydrating solution and molecular weight of the loaded compound on the particle size and EE of niosomes prepared by using the thin film hydration method was studied. Particularly, mili-Q water, glycerol solution and PEG-400 solution were tested for niosomes formulated with Span®80-Tween® 80 with/without dodecanol as membrane stabilizer. It was found that particle size highly depends on hydration media compositions and an interaction with compound MW could exist. Larger vesicles results in an increase in EE, which could be purely related with physical aspects such as vesicle loading volume capacity. The effect of hydration solution composition could be related with their ability to change bilayer packing and physical properties as observed by differential scanning calorimetry. Finally, it was possible to compare the suitability of dialysis and gel filtration as purification methods, demonstrating that gel filtration is not an adequate purification method when viscous solutions are used, since they could affect the particle vesicles retention and hence EE measurements would be misrepresentative.

Keywords

Niosomes, hydrophilic drugs carrier, encapsulation efficiency, particle size control, thin-film hydration

1. Introduction

Vesicles are commonly used as drug delivery systems for different active compounds. Vesicles are promising drug carries due to their unique properties such as nanometric size, high surface-volumen ratio, and ease of drug-release modulation [1]. Niosomes are a specific type of vesicles

formed by the self-assembly of non-ionic surfactants in aqueous media that leads to closed bilayers [2]. This structure enables them to encapsulate aqueous solutions leading either the encapsulation of hydrophilic and hydrophobic compounds [3].

Niosomes offer some advantages over other encapsulation technologies such as their low cost, chemical stability, biocompatibility, among others [4]. Furthermore, non-ionic surfactants self-assemblies are easily derivatized, which provides functional versatility to their structure [5].

In recent years, niosomes have been used for encapsulating drugs [6], nutraceuticals [7], antioxidants [8,9], micronutrients [10], etc. There are still many challenges in the development of delivery systems that could encapsulate hydrophilic compounds effectively. There are colors, nutraceuticals and vitamins of industrial interest that need to be protected from chemical degradation, to inhibit adverse interaction with other components, to mask off-flavors, or to obtain a particular release profile. For example, some water soluble colors are susceptible to chemical degradation under certain conditions, e.g., pH, light or temperature [11]. Water soluble vitamins are not stored in the body, and could be not properly absorbed during food processing making necessary to replenish them daily [12].

In this work, three bioactive hydrophilic compounds with different molecular weight (MW) and industrial interest were encapsulated (**Figure 1**): ascorbic acid (Vitamin C), rhodamine B (Fluorescent organic dye) and cobalamin (Vitamin B_{12}). Ascorbic acid, found in citrus fruits, berries and vegetables, acts mainly as antioxidant, but also promotes the production of noradrenaline, collagen, bile acids, and increases the intestinal absorption of non-heme iron [12,13]. This water soluble vitamin is highly unstable under exposition to high temperature, light and oxygen and can be degraded by several mechanisms, thus the encapsulation could help to overcome these drawbacks [14,15]. Rhodamine B is a synthetic and highly soluble molecule used as pigment in drug and cosmetic formulations due to its absorption and emission properties [16,17]. However, there are some negative effects on human health related with the exposure to this organic dye since it could cause skin, eye and respiratory tract irritation. In this sense, the encapsulation could reduce the side effects associated with this hydrophilic compound [17,18]. Finally, cobalamin belongs to the B-complex vitamins and can be found in cheese, fish, milk or eggs. It functions as coenzyme and as an

important intermediate in the metabolism of folic acid, a compound really important to prevent congenital disorder during the first stage of pregnancy [12]. The encapsulation of this nutrient via vesicles would help to improve its sensitive to heat, light, and low skin permeability [19,20].

On the other hand, it is important to consider the effect of the composition of aqueous solution used to hydrate the film during the preparation, over particles morphology (size and PDI) and functional characteristics such as encapsulation efficiency (EE), with special focus on hydrophilic compounds encapsulation. The use of different co-solvents in the self-assembly process of the niosomes, expands their application through the possibilities to tune particle size and increase EE values, depending on selected applications. For example, glycerol has been used to enhance the solubility of bioactive compounds [21], but also as cryoprotector agent for liophilization [22], or to enhance the drug carriers penetration for transdermal administration [21,23] In addition, polyethylene glycol (PEG), a water soluble polymer with several MW versions, has been used to prepare highly stable niosomes [24], and as sterical stabilizer of liposomes [25], allowing longer circulation times in blood [26].

In this work, niosomes with a formulation based on an equimolar mixture of surfactants Tween® 80 and Span® 80 were prepared [27]. Moreover, in some formulations dodecanol, was used as membrane additive, and using aqueous solutions of water, glycerol and PEG solutions as hydration media. In addition, the effect of drug MW on final niosomal suspension size and EE was studied. Two different purification methods, dialysis and Size Exclusion Chromatography (SEC Chromatography) such as gel filtration were studied in order to compare their effectivity at different hydration media for several encapsulated drugs.

Figure 1

2. Materials and methods

2.1 Materials

Niosomes were formulated by the use of non-ionic surfactants Tween® 80 (Tw80, MW 1310 g/mol, HLB 15.0) and Span®80 (Sp80, MW 428.60 g/mol, HLB 4.3), from Sigma Aldrich (USA) and

Fluka Analytical (Romania) respectively. 1-Dodecanol 98 % (Dc, Mw 186.34 g/mol) from Sigma Aldrich was used as optional membrane additive. L-(+)-ascorbic acid (AA, MW 176.12 g/mol) was obtained from J. T. Baker (USA), while Rhodamine B purity ≥ 95 % (RB, MW 479.02 g/mol), and Vitamin B_{12}purity ≥ 98.5 %(B12, MW 1355.38 g/mol) were also purchased from Sigma Aldrich.

For the film hydration solution, polyethylene glycol 400 (PEG-400, MW 380-420 g/mol, density 1.128 g/cm^3, VWR International LLC, BDH PROLABO), glycerol bidistilled 99.5 % (GLY, MW 92.09 g/mol, density 1.261 g/cm^3, VWR International LLC, BDH PROLABO) and, ultrapure water (MQ) were used.

Absolute ethanol from J.T. Baker was used for bilayer components stock solutions. Methanol HPLC grade from VWR International LLC, BDH PROLABO), and acetic acid solution (49-51 %, HPLC grade) from Sigma Aldrich were used for high performance liquid chromatography (HPLC).

2.2 Niosomes preparation

Niosomes were prepared by a modified Thin Film Hydration method (TFH) without sonication as we previously reported in literature [27].The corresponding amount of surfactants and membrane additives were placed into a 100 mL round bottom flask in an equimolar ratio (from ethanolic stock solutions). The organic solvent was removed using a rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland), until a homogeneous dried film was achieved. This film was then hydrated using different aqueous-based solutions at 60 °C, and agitated at 100 rpm during 30 minutes. Suspension of vesicles were left to acquire room temperature prior to purification and/or characterization.

Niosomes containing the active compound inside, were prepared by the hydration of the thin films with the corresponding aqueous solutions: ultrapure water (or MQ), water:glycerol (60:40, v/v) or GLY, and water:PEG-400 (55.3:44.7, v/v) or PEG. Both solutions have the same density.

Two different formulations of niosomes were studied. The first one contained an equimolar ratio of Tw80 and Sp80, while the second was a mixture of Tw80, Sp80, and Dc as bilayer stabilizer in a 1:1:1molarratio. The total concentration of membrane components was kept constant at 10 mM

(final concentration in vesicles suspension). The molar ratio between both surfactants were selected to yield an HLB value of 10**.**

2.3 Niosomes purification

The purification of niosomes suspension was carried out by using two different methods: dialysis and gel permeation chromatography.

2.3.1 Dialysis

Loaded niosomes suspensions were placed in a dialysis bag (dialysis tubing, 10 K MWCO, Thermo Scientific, Waltham, MA, USA), and let floating in the corresponding hydration media in a 1:100 v/v ratio. Dialysis time was adjusted depending on the encapsulated compound (3h, 4h and 8h for AA, RB and B12, respectively).

Dialysis times were optimized by using a control solution containing the encapsulated compound at the same concentration used for encapsulation experiments. Samples were collected from the external phase, once the concentration on the external media was more than the 99.8% of the original concentration, it was considered that the dialysis time was enough for the purification.

Reverse phase-HPLC (RP-HPLC) was used to determine the concentration of free compound in the collected samples.

2.3.2 Gel permeation chromatography

AA and RB loaded niosomes (with the exception of RB-niosomes in PEG) were purified using a Sephadex G-25 Superfine column (HiTrap™ desalting columns, GE Healthcare Life Sciences, UK); while B12 (in all media) and RB loaded niosomes in PEG were purified using a gravity elution PD Column (V_0 = 2.5 mL) packed with Sepharose CL-4B (both from, GE Healthcare Life Sciences). Sepharose CL-4B was used since in optimization steps, we realized that B12 and RB in PEG solution were not properly retained in Sephadex G25. **Table 1** summarizes the gel filtration unit used in each case.

Control solutions of the corresponding encapsulated compounds in the selected media were used to assess the efficiency of the applied method.

Table 1

RP-HPLC was used to determine the absence of free compound in the first collected fractions from the column, where niosomes were eluted, in order to assess the suitability of the SEC column.

2.4 Niosomes size and distribution analysis

Mean diameter (z-average, nm) and Polydispersity Index (PDI, a.u.) for the prepared niosomes were measured by Dynamic Light Scattering (DLS) on a Zetasizer NanoZS Series (Malvern Instruments Ltd., Malvern, UK). Measurements were performed by triplicate at 25 °C. Sometimes, dilution was required to improve the signal quality. Low volume plastic disposable cuvettes were used during size characterization (Malvern Instruments Ltd., Malvern, UK).

2.5 Niosomes encapsulation efficiency (EE)

Encapsulation efficiency was calculated as the ratio between the quantity of encapsulated compound (after proper purification), and the total amount in the unpurified suspension according to **equation 1**.

$$EE = \frac{[compound]_{encapsulated}}{[compound]_{initial}} \times 100 \qquad \textbf{Eq. 1}$$

Purified niosomes were diluted 1:10 (v/v) using methanol in order to break the niosomal bilayers and release the encapsulated compounds. The quantification of the cargo molecules was carried out by RP-HPLC (HP series 1100 chromatograph, Hewlett Packard, Agilent Technologies), with a Zorbax Eclipse Plus C18 column (4.6 mm x 150 mm, 5 µm, Agilent Technologies, Santa Clara, California, USA).UV/vis (HP G1315Adetector, Agilent Technologies) and fluorescence (1260 Infinity A detector, from Agilent Technologies), were used as detection coupled to the chromatographic separation. The following HPLC programs were used:

Ascorbic Acid

A linear gradient was performed with 0.1 % (v/v) acetic acid in MQ (mobile phase A) and methanol (mobile phase B). The gradient started with 95 % of A, reaching 20 % of A at min 15, and kept constant for 5 min. The flow rate was 0.9 mL/min. Retention time for AA was 2.28 min at λ = 278 nm.

Rhodamine B

A linear gradient was used with MQ (mobile phase A) and methanol (mobile phase B). The gradient started with 2 % of B, running 100 % of B at min 21, and kept constant for 5 min. The flow rate was 1 mL/min. Retention time for RB was 19 min at λ= 554 nm.

Vitamin B_{12} (B12)

A linear gradient was used with MQ (mobile phase A) and methanol (mobile phase B). The gradient started with 20 % of B, obtaining 100 % of B at 5 min and kept constant for 10 min. The flow rate was 0.8 mL/min. Retention time for B12 was 4.35 min at λ = 361 nm.

2.6 Differential Scanning Calorimetry (DSC)

Liquid hydrated samples (10 mg) were analyzed by Differential Scanning Calorimetry (DSC). Measurements were conducted in aluminum sealed pans, heating mode (5 °C/min), from – 40 °C to 25 °C, under N_2 atmosphere, in a DSC Mettler Toledo model 821e (Mettler Toledo International Inc., Barcelona, Spain).

2.7 Particle concentration

Particle concentration was measured by *nanoparticle tracking analysis* (NTA) with a NanoSight LM10 equipment (Malvern Instruments). All samples were diluted 1:10000 on their hydration solution.

Three independent measurements were acquired and averaged for each sample. These measurements were performed by Nanovex Biotechnologies (Asturias, Spain).

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 and t-Student) at the 95 % confidence

198 level. Tukey's test ($p < 0.05$) was used to calculate the least significance difference (LSD) using
199 statistical software (Minitab® 17.1.0).
200

201 **3. Results and discussion**

202 **3.1. Niosomes size *vs* hydration solution composition and cargo molecular weight**

203 **Figure 2** presents the vesicle size for all vesicles formulated without (A) and with (B)
204 encapsulated drug.

205 Hydration solution composition seems to have an important role in final vesicles size. In
206 particular, larger vesicles were obtained when PEG solution was used being the smaller vesicles the
207 ones obtained when MQ was used as hydration medium ($p < 0.05$ for all formulations, see Figure
208 S1, Supplementary Material). It is important to point out that GLY and PEG compounds can be
209 attached to the membrane compounds producing higher stability to the system but also could
210 increase the particle size [21,28].

211 The influence of Dc over vesicle size had a discrete effect on final vesicle size. According to
212 results presented in **figure 2A**, where size of non-loaded vesicles is presented, just a slight increase
213 on vesicle size is observed ($p < 0.05$ in all media, see Table S1, Supplementary Material). Dc could
214 be located at the membrane layer and reduces the surfactant curvature in the presence of alcohol
215 [7]. Previously, Vankayala et al. reported that fatty alcohols can rearrange parallel to the alkyl
216 portion of surfactants with their polar heads towards the aqueous surface [29].

217 Drug MW has influence on vesicle size when the niosomes were formulated with Tw80:Sp80, in
218 general, larger drug MW produced larger vesicle size. However, this effect seems to be less
219 significant that the effect produced by the hydration media used.

220 Surprisingly, when Dc was used as membrane stabilizer the effect of drug MW seemed to have
221 an opposite effect. Larger MW lead to smaller vesicle. In **figure 2B** it can be clearly noticed that
222 vesicles with Dc carrying RB or B12 have lower size than the ones carrying AA and the same
223 membrane compounds.

224 Results indicate that, even all encapsulated drug molecules are hydrophilics, some of them have
225 interactions with the vesicles membrane compounds. RB and B12 could interact with Dc, GLY and
226 PEG and hence being encapsulated at the membrane layer, the incorporation of these drugs to this
227 membrane layer could reduce surfactant curvature leading to a reduction in a vesicle size.

228 **Figure 2**

229 PEG of different MW in aqueous solution for vesicles preparation (mainly niosomes) has been
230 also studied [24,30,31]. PEG-400 and PEG-6000 are the most popular options, frequently used for
231 surfactant mixture based formulations, where Tween® and Span® (60 and 80) are widely used. The
232 concentration of PEG-400 in aqueous solutions was reported to influence the niosomal formation
233 in a formulation [32]**¡Error! Marcador no definido.** based on Tween® 80. Similarly, PEG-6000
234 concentration influenced the particle size of niosomes formulated with Tw80:Sp80 1:0.3 mass ratio
235 [33]. Similar niosomes size values to the ones reported in this work have been found by other
236 authors when Span® 60 is combined with PEG-400 [28].

237 These glycol compounds alter the packing of the bilayer and increase their curvature, which is
238 manifested as an increment in particle radius. For example, glycerol has the property to change the
239 dielectric constant of the inner bilayer, and bilayer components can re-arrange with different
240 interactions [34]. PEG molecules can interact by H bonds with Tween® 80 molecules, so at low
241 concentration can be part of the bilayer, which is less rigid. However, at high concentration it can
242 decrease the stability of the membrane until the disruption [23]**¡Error! Marcador no definido.**.

243 Other authors [21] have checked the influence of chemical composition of the film hydration
244 solution for niosomal formulation, with special focus on the presence of poly-ol compounds
245 (alcohols). These authors reported the influence of alcohol type and concentration over particle size,
246 and they found differences. Particularly, they reported that glycerol concentration in hydration
247 solution has positive effect: as glycerol concentration increases (up to 40%, similar to the percentage
248 used in the present study), bigger particles were obtained without any impact over niosomes
249 monodispersity.

Some other works using aqueous-glycerol solutions for vesicles preparation by direct hydration with sonication, have reported that empty and loaded vesicles did not differ in terms of size, however hydration solutions such as propylene glycol (1:1, v/v) yields bigger particles compared to MQ or GLY solutions. These authors have attributed this phenomenon to the interaction of this compound with bilayer components [23,30].

Regarding the monodispersity of the suspensions, in all the cases, both empty and loaded vesicles showed PDI values from 0.2 to 0.8 for all vesicles formulated (Table S2, supplementary material). As a general trend, it can be appreciated that the use of GLY and PEG solutions try to reduce PDI values, effect especially noticeable for empty vesicles and low molecular weight carrier vesicles.

All samples presented a low zeta potential as it was expected since all surfactants used had non-ionic character (Table S2, supplementary material), values below -25 mV were recorded in all cases. Samples in which MQ was used as hydration medium higher values were obtained (-25 mV) while when PEG or GLY solutions were used the values were close to zero. The presence of the encapsulated drug did not affect the zeta potential value of samples hydrated with PEG or GLY solutions. However, a reduction of zeta potential values was observed when MQ was used reaching values from -25 mV to values lower than -10 mV.

instead of pure water present even smaller values. The presences of encapsulated drug do not affect the zeta potential value in any case.

Differential Scanning Calorimetry (DSC) is a useful technique carried out to study the phase behavior of lipids and surfactant based bilayers, and gives information about molecular interactions in the structure, which allows getting information about stability and fluidity of the vesicle bilayer [35]. Also, this technique has been applied to measure the EE of hydrophobic compounds [32], since they are loaded into the structure and then, alter the cohesion of the bilayer.

Figure 3 represents the obtained DSC curves for non-loaded vesicles prepared into the three different hydration solutions. It is clear that, the incorporation of PEG and GLY changes the stability of the bilayer, since transition temperature decreased and the morphology of the peaks showed a

277 less ordered structure, evidenced by a broad peak transition and loss of symmetry [36]. GLY and
278 PEG transitions from gel-to-liquid seem to be in two steps, indicating heterogeneity of the bilayer.
279 As consequence, bilayer becomes more fluid, and bigger particles could be formed.

280 **Figure 3**

281

282 **3.2. Encapsulation Efficiency *vs* hydration solution composition and cargo molecular weight**

283 Regarding the EE of the compounds, differences were observed related to the MW of the
284 encapsulated drug for both formulations tested. The best EE values were obtained for AA in all the
285 hydration media used (**figure 4**). In most of the cases, the presence of Dc at the interface seems not
286 significantly affect the EE ($p < 0.05$, see Table S3, Supplementary material). The greater difference
287 in the EE of AA in relation with B12 and RB could be due to the hydrogen bond interactions between
288 hydroxyl groups of AA and hydrophilic portion of surfactants in the niosomes at the aqueous
289 interface. Li and Hao attributed the higher EE and slower release of *p*-hydroxil benzoic acid,
290 comparing with salicylic acid, to the intermolecular interactions between vesicle membrane and
291 these compounds [37].

292 **Figure 4**

293 It seems that PEG greatly improves RB and B12 encapsulation. However, RB seems to have an
294 important dependence with hydration solution composition, since a great difference in EE values
295 can be observed depending on the medium ($p < 0.05$, see Figure S2 and S3, Supplementary material).
296 In the case of RB, EE ranged between 8 % (GLY) and 60% (PEG) when dialysis was used as purification
297 method with and without the presence of Dc.

298 Muzzalupo et al. [21]**¡Error! Marcador no definido.** reported that 40% of GLY yields the
299 higher EE compared to other alcohols for sulfadiazine, a hydrophilic drug. They attributed this effect
300 to the presence of multiple –OH groups in the alcohol that could help the drug to be totally
301 solubilized, as consequence of H bonds between both molecules. A similar effect could be related
302 to B12 EE when no Dc is used as membrane stabilizer since EE increases from 10 to 40% when the

hydration media change from MQ to GLY. However, contrary effect is found in RB EE, since it decreases from 30 to 10% when the hydration media change from MQ to GLY. The presence of PEG in the hydration media increases EE of both (RB and B12), in both formulations used. PEG has been tested to enhance the solubility of amphiphilic compounds such as ellagic acid [23], quercetin [33], paclitaxel [28], however no data about hydrophilic compounds have been found.

Interestingly, the formulation presented in this article offers better EE for AA than liposomes found in the literature, where only 10% was reached [38]. Regarding vitamin B12, PEG niosomes with/without Dc, offer better encapsulation than liposomes reported elsewhere [39], however there is a great difference in size due to the preparation method (2 µm for niosomes presented in this study *vs* less than 100 nm for liposomes reported in previous works). EE values for RB are rarely studied since reported RB encapsulated works do not focus on the final EE values obtained [40, 41].

These results evidence the interaction between the drug and hydration solution composition. The composition of hydration solution could be a key parameter for the EE of some compounds, especially for those with large MW.

Besides these good results, when gel filtration was used as purification method, EE for RB remained with similar values for all hydration media used, offering lower values than dialysis purified vesicles for both types of formulation.

A similar pattern of results was described for B12 encapsulation. Again, the use of PEG as hydration solution yielded the higher EE values (60-67%) for dialysis purified vesicles for both formulations, but a not clear effect of medium composition can be observed when gel-filtrated method was used.

Regarding the purification strategies, dialysis is the most popular option chosen to purify vesicles encapsulating hydrophilic and amphiphilic compounds [22,24,28,35]. However, if the permeation through the bilayer is not clear understood for a specific formulation, release and purification can be overlapped, and EE could be underestimated. To assess this SEC chromatography was selected as alternative method [42]. In the present work both techniques were used to compare their suitability in the formulated systems.

As described previously, important statistical significant differences in EE values were obtained when the two purification methods were compared (see Table S4, Supplementary material), and those discrepancies seem to be magnified when GLY and PEG were used as hydration media for the encapsulation of RB and B12.

To assess if this result relies on physical forces involved in the purification methods, empty niosomes prepared in the three tested different aqueous solutions were subjected to gel filtration. Particle concentration was measured by Nanoparticle Tracking Analysis (NTA) before and after the process (**figure 5**).

Figure 5

The results showed a reduction in mean size, and particle concentration for PEG based niosomes suspension. It seems that retention of vesicles for mechanical disruption of particles may occur during their flow through the column. During the process, the bed of the column experiments a visible compaction due to the high viscosity of the fluid even at a low flow rate as recommended the manufacturer for viscous solutions. This phenomenon could yield to mechanical stress of the vesicles due to shear forces as they pass through the compacted gel. Subsequently, loaded vesicles could collapse and their content could be release to the medium where is trapped by the effect of the gel even when is compacted. A reduction in EE value could be then observed for this case as reported.

Interestingly, particles in GLY solutions seem to keep their integrity, since no changes in measured parameters have been reported. And also curiously, MQ suspended niosomes experimented a slight reduction in size and concentration, what could be related to entrapment phenomena of the smallest and highest niosomes by capture and clogging with the stationary phase, respectively.

Besides of being a popular choice for vesicles purification, SEC has some drawbacks that must be taken into consideration. For example, it has been reported that vesicles can be retained by stationary phase [43], and this retention is dependent of particles pores and not by particles size itself. Sepharose CL-40 contains particles with 20 nm pore size, which is enough to allow the flow of

small vesicles through them. However, large flexible particles like PEG-hydrated may clog in the pores and disturb the process, leading to underestimation of EE by vesicles loss and vesicles disruption into the columns. This fact could be potentiated by viscous solution, such as 45% PEG in water.

Dialysis seemed to be as an appropriate method for EE determination. However, the effect of the release through the membrane vesicle layer should be taken into account. Empty vesicles with the non-encapsulated drug should be used as a pattern in order stablish the suitable dialyzing time for each encapsulated drug and vesicle membrane composition.

Conclusions

The preparation of niosomes encapsulating hydrophilic compounds with distinct in MW by thin film hydration method performed with different aqueous based solutions, allowed us to check the influence of these parameters over particle size and encapsulation efficiency, two important and related characteristics for potential used in bioapplications.

Hydration solution composition has been found to have a clear influence in particle size for a niosomal formulation based on surfactant mixture with and without Dc as membrane stabilizer. DSC curves showed a change in bilayer structure with the use of glycerol and polyethylene glycol 400, that may interact with surfactants and create a less organized structure.

A possible interaction with cargo could exist, since when Dc was not used as membrane stabilizer, particles became slightly bigger as cargo MW increases. However, when Dc was used as membrane stabilizer the effect of drug MW seemed to have an opposite effect. Larger MW lead to smaller vesicles indicating a possible interaction of drug with the Dc what could led to a variation of surfactant curvature radio.

The composition of hydration solution could be a key parameter for the EE of some compounds, especially for those with large MW.

Large discrepancies of EE using dialysis and gel filtration were found, especially for samples where PEG solutions were used a hydration media. This effect was more noticeable for systems with

large MW cargos. Mechanical stress of particles during separation could lead to unsatisfactory results in terms of particle integrity and subsequent EE values. Dialyzing time should be optimized for each system in order to avoid interactions of the dialysis of the real free drug with the dialysis of the encapsulation drug through the vesicle membrane layer.

Disclosure

The authors declare no conflicts of interest in this work.

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507

Highlights

- Vesicle size depended on the composition of the hydrating solution.
- PEG-400 aqueous-based solution led the formation of larger vesicles than glycerol and aqueous based media.
- Encapsulation efficiency (EE) increased when poly-ol based solution were used, probably because the bigger vesicle size
- Vesicle size was affected by the molecular weight of the compounds incorporated.
- The composition of the hydrating solution might enhance the EE especially in particular cases

Figure 1. Chemical structures of the encapsulated compounds.

Figure 2. (A) Size values of empty vesicles. Tw80: tween® 80; Sp80: span® 80; Dc: dodecanol. (B) Effect of hydration solution and cargo molecular weight over particle size, for niosomal formulations Tw80:Sp80 (1:1 molar ratio) and Tw80:Sp80:Dc (1:1:1 molar ratio) prepared by Thin Film Hydration method (TFH).

Figure 3. DSC curves acquired in heating mode for the formulation without dodecanol in the three different hydration solutions: ultrapure water (MQ), water:glycerol 60:40 v/v (GLY), and water:PEG-400 55:45 v/v (PEG).

Figure 4. Effect of hydration solution and cargo molecular weight over encapsulation efficiency (EE) for niosomal formulations Tw80:Sp80 (1:1 molar ratio) and Tw80:Sp80:Dc (1:1:1 molar ratio) prepared by Thin Film Hydration method (TFH). Tw80: tween® 80; Sp80: span® 80; Dc: dodecanol. Dialysis (10 MWCO membranes) and gel filtration (Sepahdex G25 or Seharose CL-4B, depending on the compound) were used as purification methods.

Figure 5. Nanoparticle Tracking Analysis (NTA) of the three different hydration solution based niosomes after and before SEC purification. MQ and GLY vesicles were purified using Sephadex G25 and PEG vesicles by Sepharose CL-4B.

Table 1. Chromatography mediums used for gel filtration based purification of loaded niosomes.

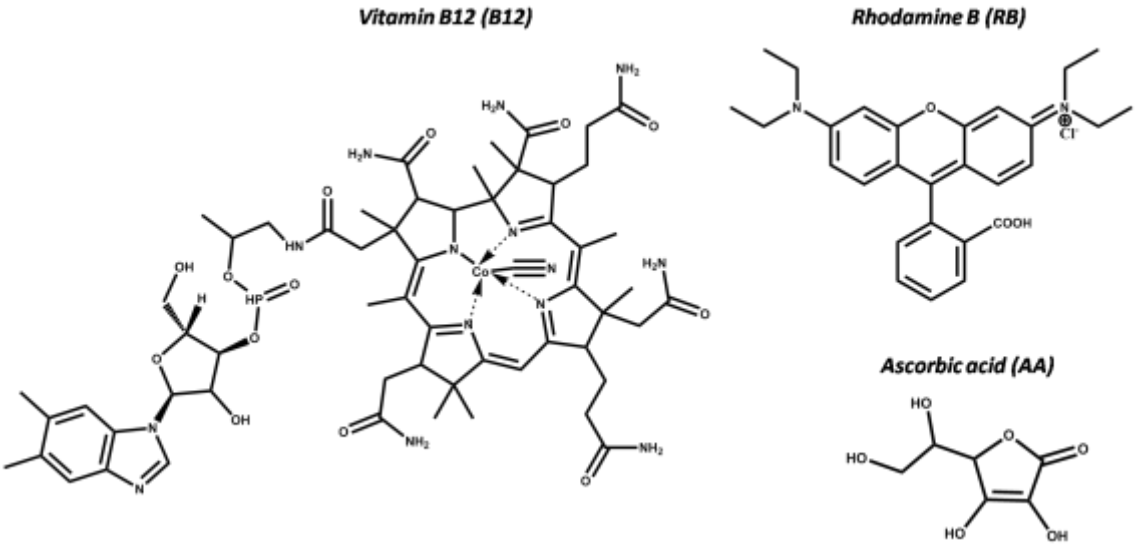

Figure 1

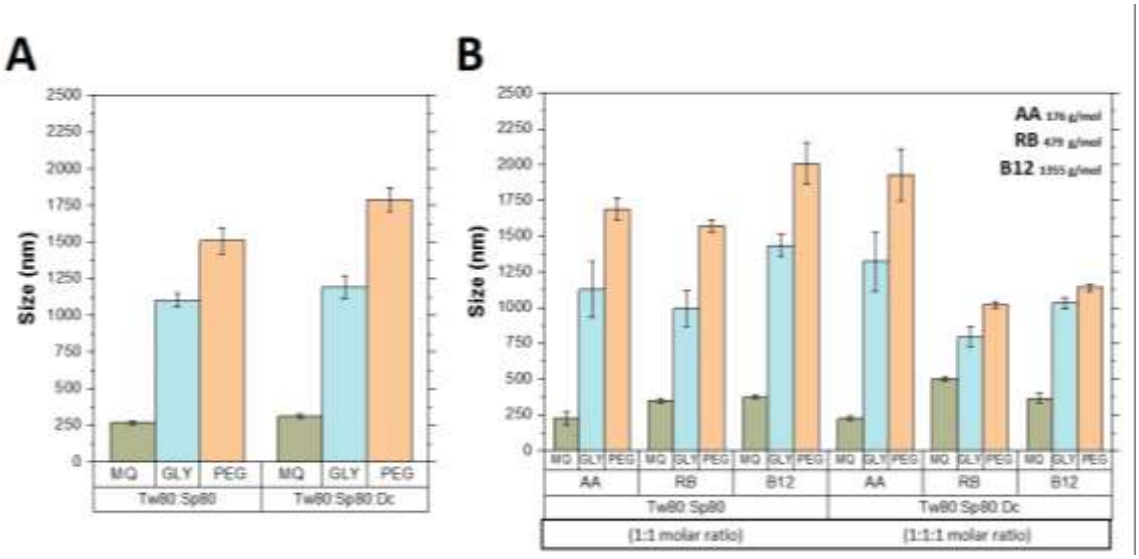
A
Size (nm)
2500
2250
2000
1750
1500
1250
1000
750
500
250
0
MQ
GLY
PEG
MQ
GLY
PEG
Tw80 Sp80
Tw80 Sp80 Dc
B
Size (nm)
AA 176 g/mol
RB 479 g/mol
B12 1355 g/mol
MQ GLY PEG MQ GLY PEG MQ GLY PEG MQ GLY PEG MQ GLY PEG MQ GLY PEG
AA
RB
B12
AA
RB
B12
Tw80 Sp80
Tw80 Sp80 Dc
(1:1 molar ratio)
(1:1:1 molar ratio)

Figure 2

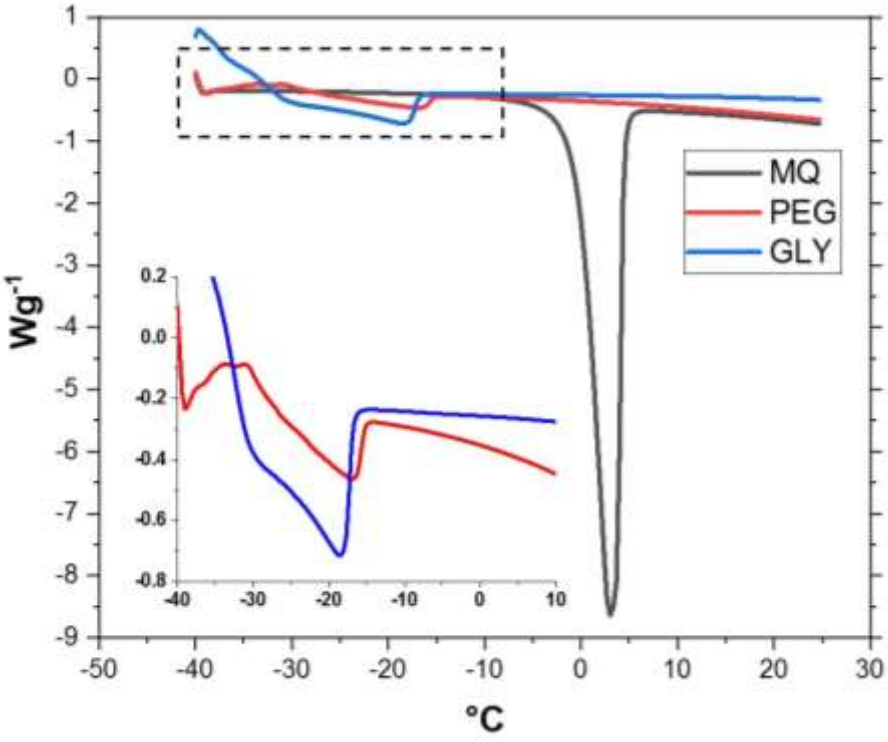

Figure 3

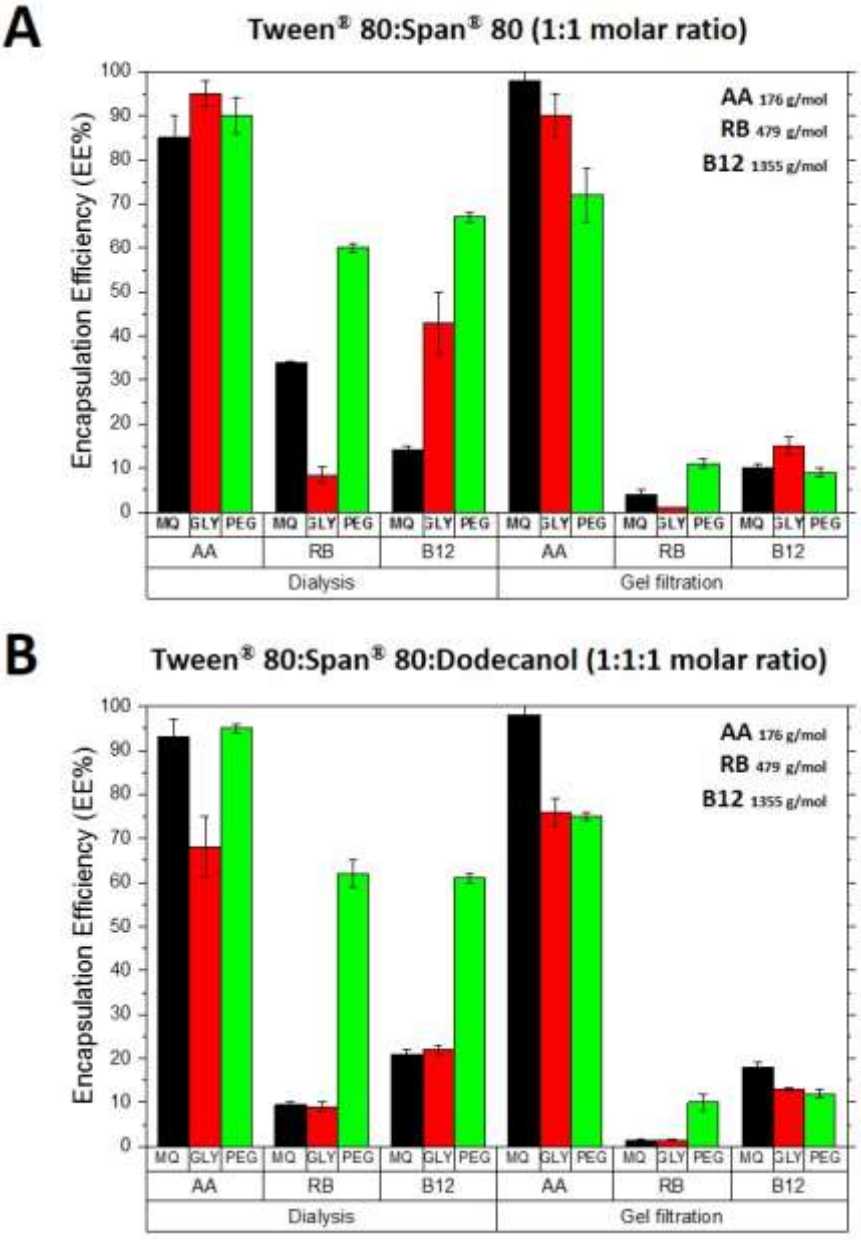
A
Tween® 80:Span® 80 (1:1 molar ratio)
Encapsulation Efficiency (EE%)
AA 176 g/mol
RB 479 g/mol
B12 1355 g/mol
MQ GLY PEG
AA RB B12 AA RB B12
Dialysis Gel filtration
B
Tween® 80:Span® 80:Dodecanol (1:1:1 molar ratio)
Encapsulation Efficiency (EE%)
AA 176 g/mol
RB 479 g/mol
B12 1355 g/mol
MQ GLY PEG
AA RB B12 AA RB B12
Dialysis Gel filtration

Figure 4

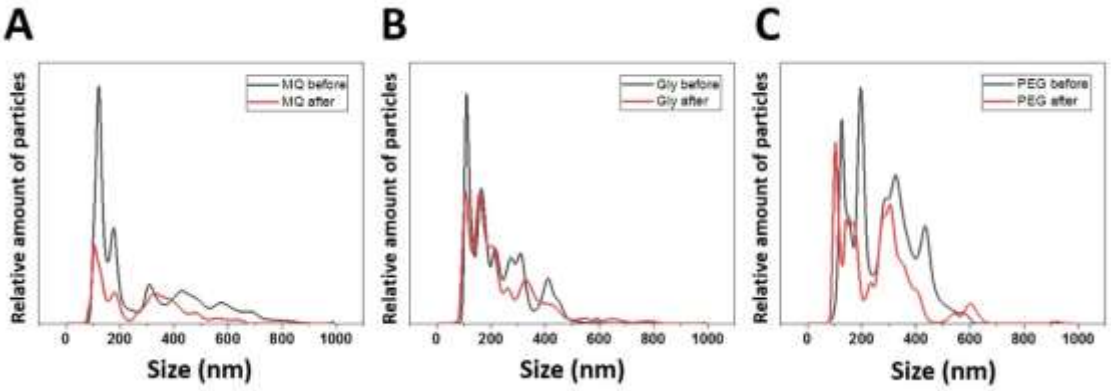
A
B
C
Relative amount of particles
MQ before
MQ after
Gly before
Gly after
PEG before
PEG after
0
200
400
600
800
1000
Size (nm)

Figure 5

Table 1

Component Encapsulated	Hydration media	Gel filtration medium used
Ascorbic acid	MilliQ	Sephadex G25
	MilliQ/GLY	Sephadex G25
	MilliQ/PEG	Sephadex G25
Rodamine	MilliQ	Sephadex G25
	MilliQ/GLY	Sephadex G25
	MilliQ/PEG	Sepharose CL-4B 25
Vitamin B12	MilliQ	Sephadex G25
	MilliQ/GLY	Sephadex G25
	MilliQ/PEG	Sepharose CL-4B 25

Supplementary material

Effect of drug molecular weight on niosomes size and encapsulation efficiency

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Table S1. Comparison on size between empty Tw80:Sp80 and Tw80:Sp80:Dc niosomes in all media using *t*-Student test.

Media	T value	P value
MQ	-3.99	0.001
MilliQ/GLY	-2.21	0.040
MilliQ/PEG	-7.95	<0.0001

Table S2. Particle size, polydispersity index and zeta potential of all formulations used for empty and loaded vesicles

Formulation	Hydration media	Encapsulated compound	Size (nm)	PDI	Zeta Potential (mV)
Tw80:Sp80	MilliQ	Empty	267±10	0.47±0.05	-24.2±2.1
		Ascorbic acid	226±42	0.60±0.10	-3.40±0.8
		Rodamine	349±13	0.50±0.07	-3.78±1.1
		Vitamin B12	373±11	0.63±0.02	-7.35±1.2
	MilliQ/GLY	Empty	1104±48	0.50±0.12	-0.26±0.6
		Ascorbic acid	1127±194	0.57±0.09	-0.62±0.2
		Rodamine	996±128	0.26±0.04	-0.63±0.4
		Vitamin B12	1434±74	0.33±0.01	-2.45±1.2
	MilliQ/PEG	Empty	1509±89	0.32±0.11	-3.51±1.1
		Ascorbic acid	1688±77	0.30±0.08	0.27±0.3
		Rodamine	1571±41	0.30±0.10	0.22±0.7
		Vitamin B12	2009±148	0.46±0.04	-0.39±0.9
Tw80:Sp80:Dc	MilliQ	Empty	312±16	0.50±0.10	-24.8±3.1
		Ascorbic acid	224±14	0.50±0.10	-2.86±1.2
		Rodamine	503±17	0.33±0.01	-4.24±3.2
		Vitamin B12	366±34	0.44±0.09	-12.1±1.2
	MilliQ/GLY	Empty	1192±78	0.30±0.10	-3.2±0.5
		Ascorbic acid	1322±208	0.30±0.20	-0.44±0.7
		Rodamine	796±67	0.30±0.10	-1.44±0.7
		Vitamin B12	1030±46	0.35±0.01	-3.37±0.6
	MilliQ/PEG	Empty	1189±81	0.28±0.08	-0.41±0.1
		Ascorbic acid	1930±183	0.70±0.10	-0.41±0.7
		Rodamine	1017±24	0.60±0.10	-0.32±0.4
		Vitamin B12	1143±23	0.54±0.01	-0.51±0.2

Table S3. Comparison on EE between loaded Tw80:Sp80 and Tw80:Sp80:Dc niosomes in all media using *t*-Student test. (70 % of p values < 0.05)

Gel filtration						
Media	**AA**		**RB**		**B12**	
	T value	**P value**	**T value**	**P value**	**T value**	**P value**
MQ	-0.93	0.382	4.44	0.001	-8.88	<0.0001
MilliQ/GLY	6.84	<0.0001	-10.03	<0.0001	2.91	0.016
MilliQ/PEG	-0.69	0.522	0.93	0.376	-2.61	0.026
Dialysis						
Media	**AA**		**RB**		**B12**	
	T value	**P value**	**T value**	**P value**	**T value**	**P value**
MQ	-2.53	0.039	29.87	<0.0001	-6.79	<0.0001
MilliQ/GLY	11.78	<0.0001	-1.03	0.325	-4.67	0.002
MilliQ/PEG	-2.36	0.050	-1.96	0.079	6.48	<0.0001

Table S4 Comparison on EE obtained by Gel filtration and Dialysis in all media using *t*-Student test. (94 % of p values < 0.05)

Tw80:Sp80						
Media	**AA**		**RB**		**B12**	
	T value	**P value**	**T value**	**P value**	**T value**	**P value**
MQ	4.46	0.001	-30.81	<0.0001	-4.90	0.001
MilliQ/GLY	-3.14	0.011	-97.64	<0.0001	-10.83	<0.0001
MilliQ/PEG	-5.86	<0.0001	-50.66	<0.0001	-61.10	<0.0001
Tw80:Sp80:Dc						
Media	**AA**		**RB**		**B12**	
	T value	**P value**	**T value**	**P value**	**T value**	**P value**
MQ	4.39	0.001	-26.72	<0.0001	0.45	0.665
MilliQ/GLY	3.83	0.003	-12.88	<0.0001	-51.70	<0.0001
MilliQ/PEG	-19.59	<0.0001	-42.50	<0.0001	-45.37	<0.0001

Figure S1, **S2** and **S3** are graphs for the multiple comparison test using the Tukey method. Vertical line indicates the zero value and if an interval does not contain zero, the corresponding means are significantly different.

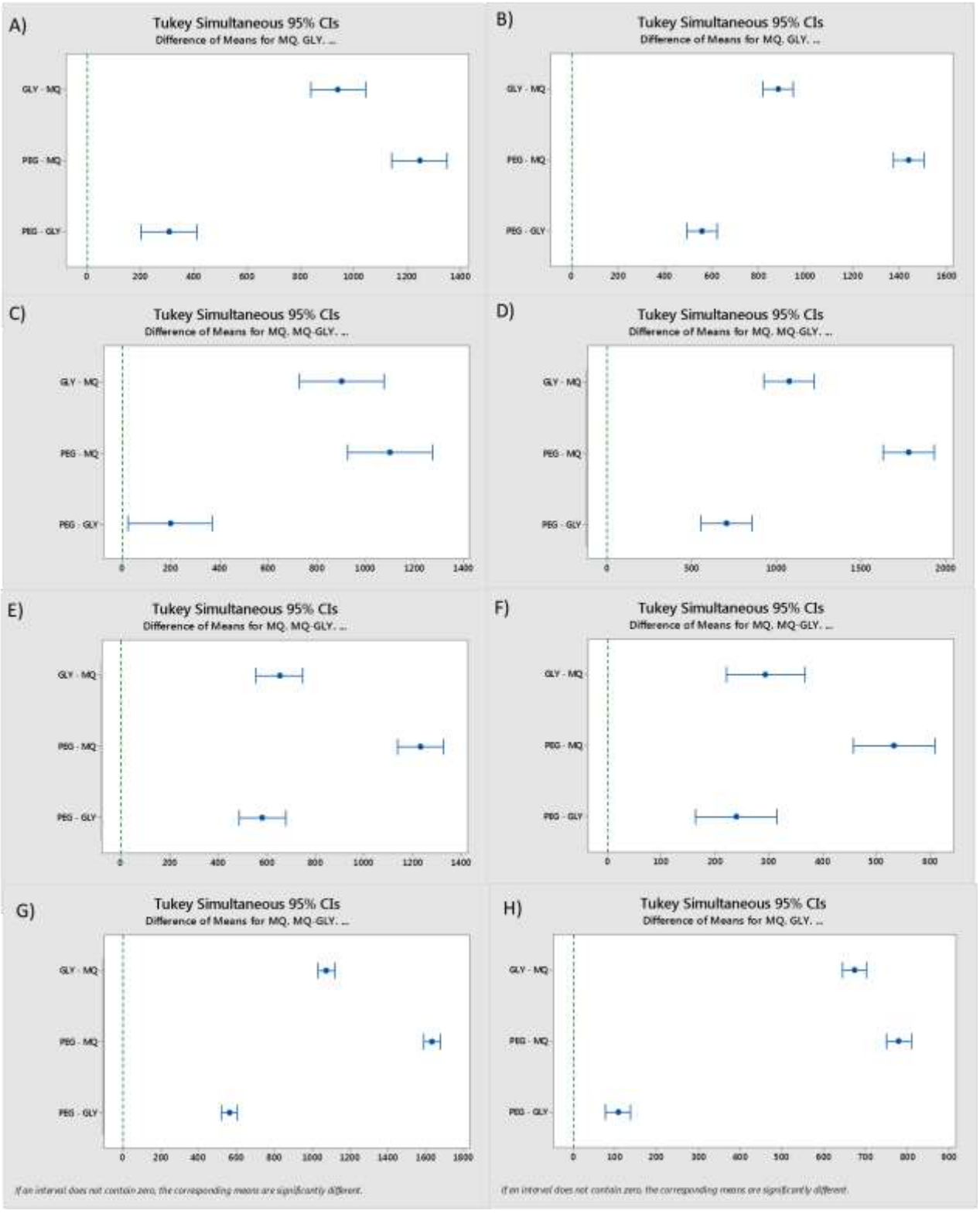

Figure S. 1. Size analysis using Tukey test for niosomes in different media. A), C), E) and G) corresponds to Tw80:Sp80 niosomes, and B), D), F) and H) corresponds to Tw80:Sp80:Dc niosomes. A, B) Empty, C, D) with AA; and E, F) with RB, G, H) with B12.

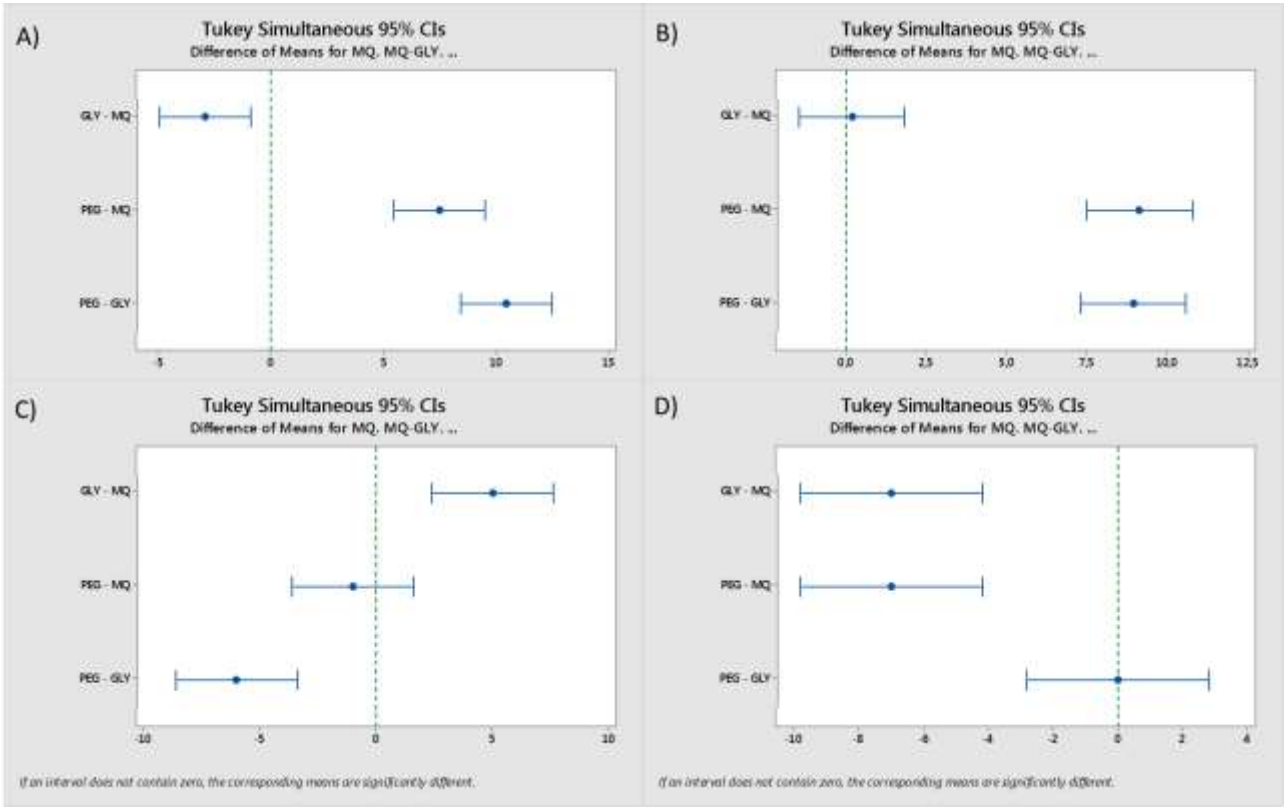

Figure S. 2. EE analysis using Tukey test for niosomes in different media obtained by Gel Cromatography. A, C) corresponds to Tw80:Sp80 niosomes; B, D) corresponds to Tw80:Sp80:Dc niosomes. A, B) with RB; and C, D) with B12.

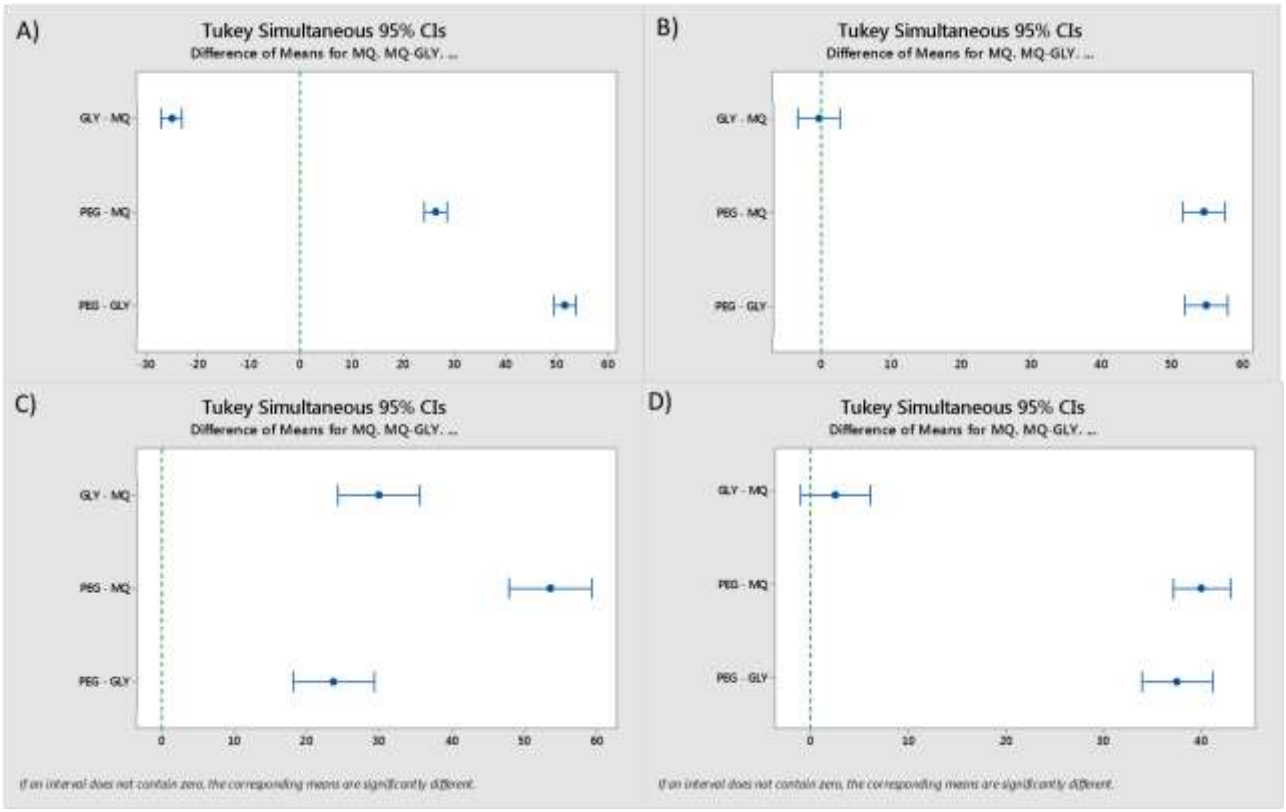

Figure S. 3. EE analysis using Tukey test for niosomes in different media obtained by Dialysis. A, C) corresponds to Tw80:Sp80 niosomes; B, D) corresponds to Tw80:Sp80:Dc niosomes. A, B) with RB; and C, D) with B12.