Lyophilised nanovesicles loaded with vitamin B_{12}

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ABSTRACT

The use of nanomaterials in recent years has shown many advantages for the production of pharmaceuticals, cosmetics, food, and food packaging. Nanovesicles are emerging as carriers of bio-compounds and drugs at a wide variety of applications due to the hydrophilic and lipophilic character of the structure: an aqueous core surrounded by a lipid layer. In this work, vitamin B_{12} (cobalamin) was encapsulated, due to its synergic activity with antibiotics. Moreover, this bio compound is present in several food products and pharmaceuticals products. A freeze-drying process is proposed for nanocarriers developments, due to its advantages for storage and transportation. Three types of vesicles formulations were tested: liposomes, niosomes and positively charged niosomes. Maltodextrin at 20% concentration (w/w) was added to the formulation to protect the vesicles during the lyophilisation process. The nanovesicles particle size and morphology was characterized by Dynamic Light Scattering and Transmission Electron Microscopy, and the encapsulation efficiency by HPLC with UV-Vis's detection. The lyophilised powder was resuspended in three aqueous phases: pure water, glycerol and PEG400 aqueous solutions. An increase in the mean vesicle size was observed as a general trend. It was also studied the effect of adding vitamin B_{12} before or after the lyophilisation step on mean vesicles size, results indicated that vesicles were less affected in size when vitamin B_{12} was added before the lyophilisation step. Moreover, encapsulation efficiency (EE) of vitamin B_{12} in lyophilised vesicles arise values up to 70% while the loading capacity (LC) of these systems were 100 mg/g, values obtained for liposomes when PEG400 solution was used as hydrating media, showing that the method developed for loaded lyophilised nanocarriers do not alter EE and LC.

Keywords: nanovesicles, lyophilisation, encapsulation, vitamin B_{12}, hydration media

INTRODUCTION

Vitamin B_{12} (cobalamin) is an organometallic molecule with essential roles in prokaryotes and eukaryotes. A large variety of enzymes uses the major biological forms of vitamin B_{12} as cofactor, such as methyltransferases, dehydratases, deaminases, and ribonucleotide reductases [1]. Vitamin B_{12} cannot be synthesized, and it needs to be incorporated in the diet, thus making it a good candidate as a drug carrier. In addition, the vitamin B_{12} uptake mechanisms in bacteria may be used to improve the efficacy of antibiotics and to design novel carrier systems for industrial or biomedical applications. Some works reported a synergistic antimicrobial activity of vitamin B_{12} with antibiotic in resistant bacterial cells [2], and the good efficiency of vitamin B_{12}-antibiotic conjugates[3] . Thus,

this biomolecule, together with alternative strategies to overcome antimicrobial resistance, showed great potential as a delivery vehicle [4] . Moreover, the use of antisense oligomers-vitamin B_{12} conjugates may be used to target undesirable biofilms and inhibit bacterial growth, and their internalization by bacterial cells may increase by improving B_{12} bioavailability [5]. In this sense, the encapsulation of vitamin B_{12} in vesicles and its slow release enhances the absorption and bioavailability, in addition to protecting the vitamin from degradation induced by heat, light, air and bad storage.

Vesicles are nanocarriers able to encapsulate both hydrophilic and hydrophobic drugs since these colloidal systems have an aqueous core surrounded by a lipid layer. Vesicles can be used for controlled drug release, in pharmaceutical, cosmetics and food applications. Liposomes are vesicles formed by phospholipids that show several disadvantages such as instability due to oxidation or lipid hydrolysis, drug leakage and aggregation or fusion of vesicles. Niosomes are vesicles whose membrane bilayer is formed by non-ionic surfactants showing some advantages respect traditional liposomes. Lower cost and higher biodisponibility due to its easier functionalization are among the most interesting advantages [6]. However, niosomes have also stability problems such as sedimentation, and aggregation as in the case of liposomes [7].

Lyophilisation is one of the possible strategies to ensure the colloidal stability of the nanovesicles and extend the useful life avoiding problems in their storage and transportation. It is a complex process requiring four stages: freezing, sublimation, desorption and storage in which the resulting material is a dried powder. Thus, it is important to choose excipients to protect the membrane integrity from the stresses during freeze-drying to reduce drug leakage [8].

The excipients commonly used are sugars, especially disaccharides like maltodextrin or dextran. Several authors have tried to explain the protection mechanism of these type of molecules, however, at present there is not a clear established mechanism [9,10]. Authors suggest the mechanism is based on interactions (hydrogen bonds) between phospholipids and sugars. In fact, it was demonstrated that sugars interact not only with the phospholipids phosphate groups but also with the hydrophobic part of the molecule. [11]. In any case, during the freezing stage sugars form an amorphous matrix with high viscosity and low mobility, in this way vesicles are enabled to keep their distance.

Proniosomes are vesicles-based formulation that reduce fusion, leak and aggregation during storage. Proniosomes are dried formulations of the surfactant which are rehydrated by short stirring in hot water at a temperature higher than the surfactant mean transition phase temperature (Tc) [12]. They can be synthesized using methods such as the slurry method, the slow spray drying coating method [13] or lyophilisation after dissolving first the membrane components in organic solvent and evaporating the solvent later [14]. In case that those proniosomes were required to be used as a nanocarriers the bio compound was added on the hydration step, making frequently necessary the storage of the hydration solution containing the bio compound in specific conditions.

Maltodextrin is a hydrophilic and biocompatible molecule used especially for the formulation of proniosomes, in fact it attaches to the niosomes surface and increases their stability and bioavailability [15].

In this work, the encapsulation of vitamin B_{12} in lyophilised nanovesicles has been studied. This is a compound with high hydrophilicity, and its affinity for the external phase enhances its release during synthesis and storage but makes its encapsulation more difficult. Moreover, its high molecular weight (1355.38 g/mol) and sterically hindering offers additional difficulties for encapsulation (Figure 1).

Three formulations were tested: Liposomes, niosomes formed by Span 60 and cholesterol, and positive charged niosomes with Span 60, Cholesterol and CTAB (cetyltrimethylammonium bromide), due to the positive effect of positivity charged particles on biofilms treatment. In order to obtain large multilamellar vesicles the thin film hydration method was the preparation method selected (frequently used for hydrophilic molecules encapsulation in vesicles) [16]. All the formulations were lyophilised after the addition of maltodextrin and restored using three different aqueous media: pure water, glycerol and PEG400 water solutions. In a previous study [16], glycerol and PEG400 solutions seemed good hydration media obtaining large stable vesicles. The effect of adding the encapsulated bio compound (vitamin B12) before lyophilisation was studied in terms of vesicles size, encapsulation efficiency (EE) and loading capacity (LC).

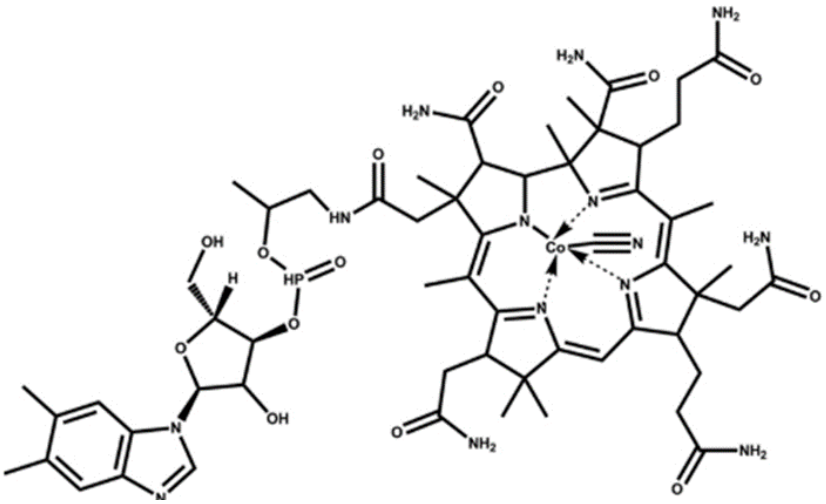

Figure 1. Chemical Structure of vitamin B_{12}

MATERIALS

Liposomes were formulated using phosphatidylcholine (PC), from soybean (Phospholipon 90G) purchased from Lipoid GmbH (Ludwigshafen am Rhein, Germany). For niosomes preparation, cholesterol, Span 60 and CTAB were purchased from Sigma Aldrich (USA). Vitamin B12, purity ≥ 98.5 % (B12, MW 1355.38 g/mol) was also purchased from Sigma Aldrich. The hydration phases used to restore vesicles after freeze-drying were prepared using polyethylene glycol 400 (PEG-400, MW 380−420 g/mol, density 1.128 g/cm3, VWR International LLC, BDH PROLABO), glycerol bidistilled 99.5 % (GLY, MW 92.09 g/mol, density 1.261 g/cm3, VWR International LLC, BDH PROLABO) and ultrapure water (MQ). Maltodextrin (lot no.219425, MD, Pral, Barcelona) was added as cryoprotectant, before the lyophilisation process.

METHODS

Preparation of vesicles

Thin film hydration method was carried out dissolving the surfactants that will form the membrane in a round flask containing the organic phase (Absolute Ethanol). The solvent was then removed under vacuum using a rotary evaporator until a thin film was formed on the wall of the flask. The vitamin B_{12} (hydrophilic) was dissolved in an aqueous phase and added to the flask. The suspension was then incubated at constant temperature above the surfactant transition temperature [17].

A 20 mL solution of 50g/L of surfactants and lipophilic compounds to form the membrane was prepared in absolute ethanol (as organic solvent). Solvent was removed under vacuum in a rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland) at a bath temperature of 45°C and 150 rpm of rotation speed to form a thin film around the wall of the flask. The dry film was hydrated adding 50 mL of an aqueous solution 0.2 mM of vitamin B_{12}. The hydration was performed for 2 h at 150 rpm and a bath temperature of 60°C. The vesicles suspension was homogenized by sonication (Branson Ultrasonics Sonifier SFX150, Tamaulipas, Mexico) for 15 min using an amplitude of 55% continuously, 500 W power and 20kHz frequency [18]. Thin film hydration procedures for loaded vesicles after and before lyophilisation with vitamin B_{12} are summarised in Figure 2. The three different vesicle formulations (membranes) tested are reported in Table 1.

Table 1. Composition of the vesicles synthesized

	Membrane components	**Molar ratio**
I	Span 60, Cholesterol	2:1
II	Phospholipon 90	1:0
III	Span 60, Cholesterol, CTAB	1:1:1

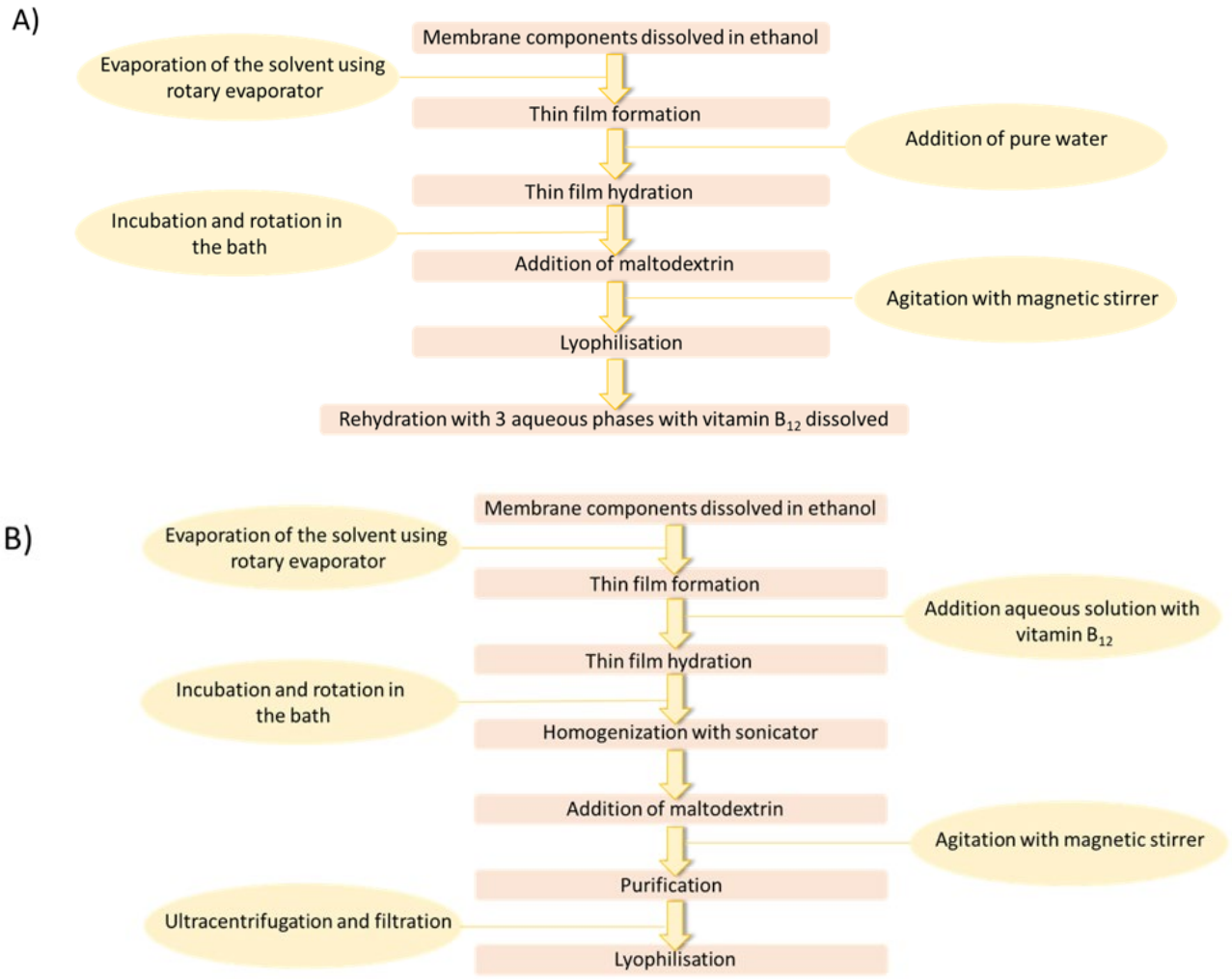

Figure 2. Steps used in the Thin film hydration: A) Loaded vesicles after lyophilisation B) Loaded vesicles before lyophilsation

Vesicles purification

Purification was carried out to remove the non-encapsulated material by ultracentrifugation using ultra 0.5 mL centrifugal filter devices in a microcentrifuge (Espresso centrifuge, Thermo electron corporation) for 15 min at a 14,500 rpm. The supernatant was filtered using 0.22 µm pore diameter PES syringe filters for further analysis by Reverse phase High Performance Liquid Chromatography (RP-HPLC).

Preparation of lyophilised vesicles

Experiments using procedures described in Figure 2 were carried out for nanovesicles lyophilisation. For the loaded vesicles after lyophilisation procedure each suspension was maintained at -80°C for 24 hours. Lyophilisation was performed under vacuum conditions (0,1 mbar) using a Telstar Cryodos Lyophiliser (Terrassa, Spain) Each powder was rehydrated using a 0.2 mM of vitamin B_{12} aqueous solution. The concentration used was choosen consindering that this amount it is enough to have a synergistic antimicrobial activity [3,4] Three different aqueous phases were used: (i) MilliQ water,

(ii) a (60:40) MilliQ water and glycerol solution and (iii) a (55.7:44.3) MilliQ water and PEG400 solution respectively.

For the loaded vesicles before lyophilisation procedure each suspension was maintained at -80°C for 24 hours. Lyophilisation was carried out using the same instrument described for method A but in this case the vitamin B_{12} was added before lyophilisation. The powders obtained for each formulation were resuspended in the three different aqueous solutions aforementioned using the sonicator (Branson Ultrasonics Sonifier SFX150, Tamaulipas, Mexico) with an amplitude of 55% continuously for 10 min, 500 W power and 20kHz frequency.

CHARACTERIZATION

Determination of size distribution and morphology

Vesicles size were determined using Dynamic Light Scattering on a Zetasizer NanoZS series (Malvern Instruments Ltd.,Malvern, UK. The number-based size distribution was used for vesicles characterization. The analysis was made for vesicles not lyophilised before and after the addition of maltodextrin, and for vesicles restored with the three different aqueous media after lyophilisation.

Morphology was studied using negative staining transmission electron microscopy (NS-TEM), with a JEOL-2000 Ex II transmission electron microscope (Tokyo, Japan). A drop of the sample was placed into the carbon-coated copper grill and a 2% (w/w) phosphotungstic acid solution was added to the grill as dye to have a negative staining of the samples.

ENCAPSULATION EFFICIENCY (EE%) AND LOADING CAPACITY (LC)

In order to evaluate the encapsulation efficiency and loading capacity, purified and non-purified vesicles were treated using methanol 1:10 (v/v). This step breaks the membrane bilayer and releases the encapsulated vitamin B_{12}. RP-HPLC (HP series 1100 chromatograph, Hewlett Packard, Agilent Technologies), using a Zorbax Eclipse Plus C18 column (4.6 mm x 150 mm, 5 μm, Agilent Technologies, Santa Clara, California, USA) was used for the quantification of vitamin B_{12}. The chromatographic method used included a linear gradient 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 Vitamin B_{12} was 4.35 min at λ =361 nm. [16]

Encapsulation efficiency (EE) was calculated according to equation 1:

$$EE\% = \frac{[Concentration\ of\ vitamin\ B12\ in\ purified\ vesicles\ after\ methanol\ treatment]}{[Concentration\ of vitamin\ B12\ at\ no\ purified\ vesicles\ after\ methanol\ treatment]}\ x\ 100 \quad (1)$$

Calibration curves for each hydration phase were performed in the range of 0.03-0.2mM of vitamin B_{12}. In order to simulate the vesicles samples measured all samples used for the calibration were diluted 1:10 with water: methanol. A linear regression was obtained for the three aqueous phases and from the equation the concentration of vitamin B_{12} presents in the samples was calculated (Table 4).

In addition to the EE of the system, it is important to quantify the mass of Vitamin B_{12} present, this will indicate the load capacity of the vesicles [19]. An indirect method was used to determine the amount of bio compound in the vesicles based on the ratio between the total mass of the initial vitamin

added and the mass of the membrane components forming the membrane bilayer plus the grams of vitamin obtained from the calculation of the concentration through the calibration curves.

Equation 2 was used to calculate the loading capacity (LC):

$$LC = \frac{W_b}{W_T} \quad (2)$$

Were W_b corresponds to the weight of the bio compound and W_T corresponds to the total weight of bio compounds and vesicle membrane compounds used.

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.

RESULTS AND DISCUSSION

The results obtained in the present work will be divided in two sections. Those obtained with method A, when vitamin B_{12} were added on the hydration step, procedure similar to the one followed in the literature for proniosomes preparation [20]and method B, in which vitamin B_{12} was added before the lyophilisation step.

Method A. Bio compound addition on the hydration step

The effect of maltodextrin concentration on the resulting loaded vesicles after lyophilisation size and size distribution was studied. Maltodextrin was added in the range 10-40% w/w. The size of vesicles prepared was not affected up to 20% w/w of maltodextrin concentration, with no significant difference on the mean particle size. Results of size and PDI (polydispersity index) values for the three formulations used before and after the addition of maltodextrin 20% are summarized in Table 2. However, higher maltodextrin concentration lead to lower size reproducibility results due to the large agglomerates formation, most likely due to the excessive addition of maltodextrin which saturates the solution. The addition of a sugar as cryoprotectant is important to improve the stability of the formulations and protect them during the lyophilisation process, this is confirmed by the water replacement theory in which sugar molecules replace the hydrogen bonds of water [21]and interacting with phospholipids or surfactant maintaining the space between the phospholipid head and reducing the van der Walls forces of the tails. In this way the contact among membranes in proximity is less and prevents the aggregation and damage of liposomes caused by ice crystals during the freezing process [22].

The results obtained from DLS before lyophilisation process and after restored with different hydration media in which vitamin B_{12} was added are also shown in table 2. Particle size distributions obtained are presented in Figure 3.

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235 **Table 2.** Vesicles size and PDI before and after the lyophilisation process adding vitamin B_{12} on the
236 rehydration step.

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FORMULATION	BEFORE MALTODEXTRIN		BEFORE LYOPHILISATION		AFTER LYOPHILISATION		
	Size (nm)	PDI	Size (nm)	PDI	Hydration media	Size (nm)	PDI
Liposomes	72±35[a]	0.25±0.01	77±65[a,w]	0.45±0.01	MQ water	679±73[x]	0.43±0.04
					MQ:Glycerol	718±105[x]	0.62±0.03
					MQ:PEG400	1888±530[y]	0.83±0.30
Niosomes	89±29[a]	0.35±0.03	96±72[a,w]	0.50±0.09	MQ water	243±135[w]	0.60±0.03
					MQ:Glycerol	2522±185[x]	0.37±0.15
					MQ:PEG400	8023±389[y]	1.01±0.05
Niosomes+CTAB	57±18[a]	0.32±0.01	43±13[a,w]	0.41±0.01	MQ water	216±118[x]	0.91±0.06
					MQ:Glycerol	2131±12[y]	0.36±0.10
					MQ:PEG400	9000±357[z]	0.61±0.27

238 Letter a indicates no significant differences between samples before lyophilisation with and without
239 maltodextrin, letters (w- z) indicate significant differences between samples before and after lyophilisation.

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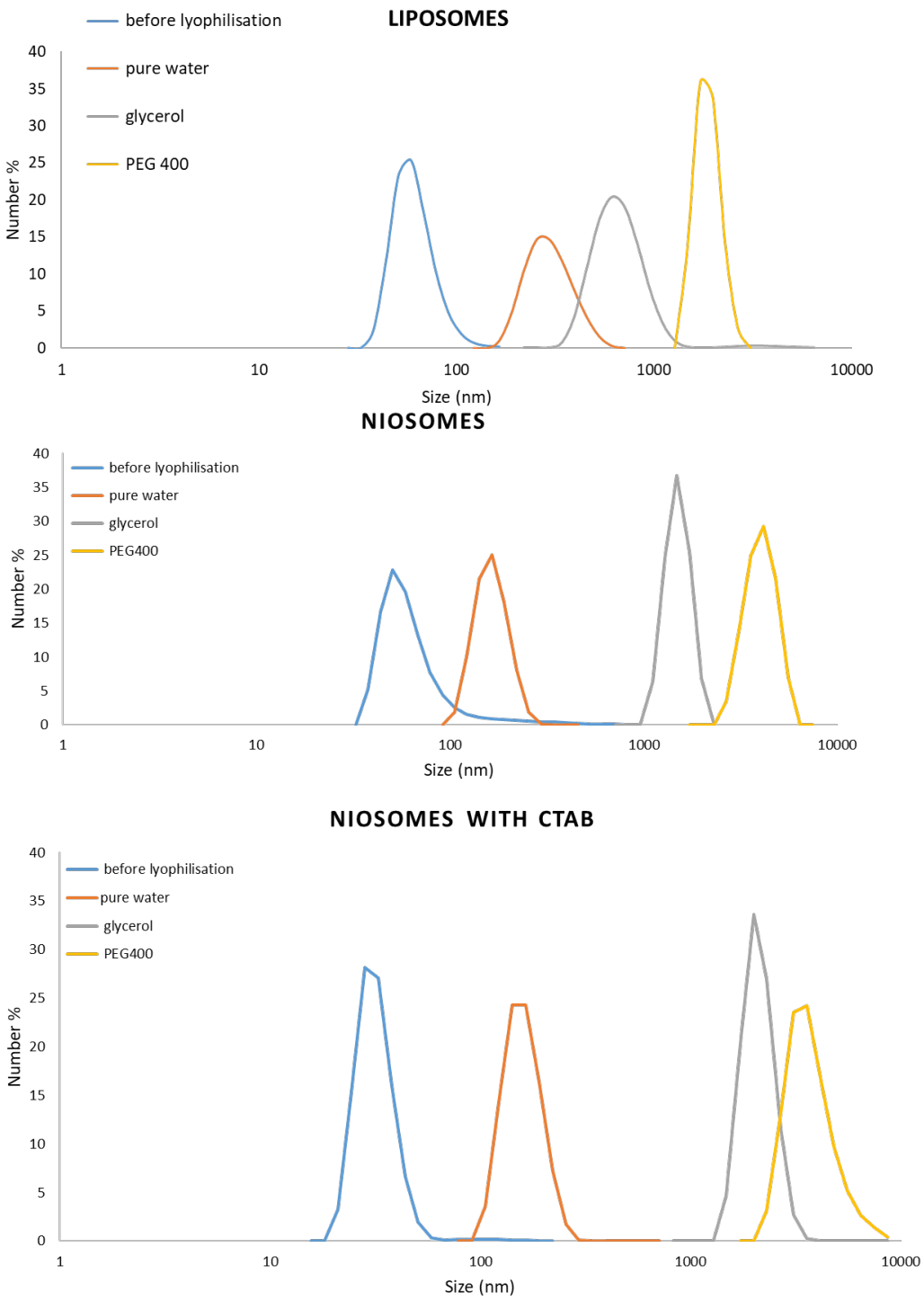

Figure 3. Size distribution curves of the vesicles before and after the lyophilisation process and rehydration with 0.2 mM of vitamin B_{12} dissolved in the three aqueous phases.

For the three formulations prepared no significant differences on mean particle size was observed. For these three formulations used it was observed that the hydration media used has a large significant influence on the final vesicle size. All vesicles increases in size after resuspension, being the pure water the hydration media that allows to obtain smaller vesicles. Moreover, the use of pure water on liposomes resuspensions offered larger size increase compared to the resuspension of niosomes, specially for those without CTAB on its formulation.

However, the use of glycerol and PEG400 solutions as hydration media presented larger influence on niosomes than in liposomes. For all formulations tested, larger values were obtained with those hydration media, being the effect even larger in the case of PEG400 solutions. As a general trend, the PDI value of all vesicles increased after lyophilisation and resuspension processes for all formulations and hydration media used. Similar values were obtained by other authors when glycerol was used for vesicles formulation [23] what was found to make vesicle membrane more fluid enhancing molecules penetration.

Method B. Bio compound addition before lyophilisation step

Size and morphology

Loaded vitamin B_{12} vesicles were analyzed with the same criteria evaluating the effect of maltodextrin and the lyophilisation process on the mean sizes and PDI values shown in table 3. Maltodextrin did not have large influence on vesicle size. A slight increase was observed for liposomes increasing from 31 to 42 nm while not significant differences were observed for any type of niosomes tested. Figure 4 presents TEM images of liposomes, niosomes and niosomes with CTAB after the maltodextrin addition. It can corroborate the small size of liposomes compared the two niosomal systems prepared.

Table 3. Size and PDI values before and after maltodextrin addition, after lyophilisation process and the further rehydration with different hydration medias, with addition of the bio compound before lyophilisation step.

	BEFORE MALTODEXTRIN		AFTER MALTODEXTRIN		AFTER LYOPHILISATION		
	Size (nm)	PDI	Size (nm)	PDI	Hydration media	Size (nm)	PDI
Liposomes	31± 3^{a}	0.31±0.02	42±5^{b,x}	0.32±0.06	MQ water	123±28^{y}	0.47±0.03
					MQ:Glycerol	225±99^{y}	0.58±0.10
					MQ:PEG400	4326±208^{z}	0.46±0.05
Niosomes	125±6^{c}	0.32±0.12	112±17^{c,x}	0.45±0.05	MQ water	268±64^{y}	0.94±0.11
					MQ:Glycerol	468±71^{z}	0.62±0.18
					MQ:PEG400	504±85^{z}	0.76±0.16
Niosomes+ CTAB	123±26^{c}	0.96±0.04	110±29^{c,x}	0.95±0.04	MQ water	105±26^{x}	0.82±0.04
					MQ:Glycerol	121±15^{x}	0.90±2.03
					MQ:PEG400	1412±139^{y}	0.40±0310

Letters (a-c) indicate significant differences between samples before lyophilisation with and without maltodextrin, letters (x- z) indicate significant differences between samples before and after lyophilisation.

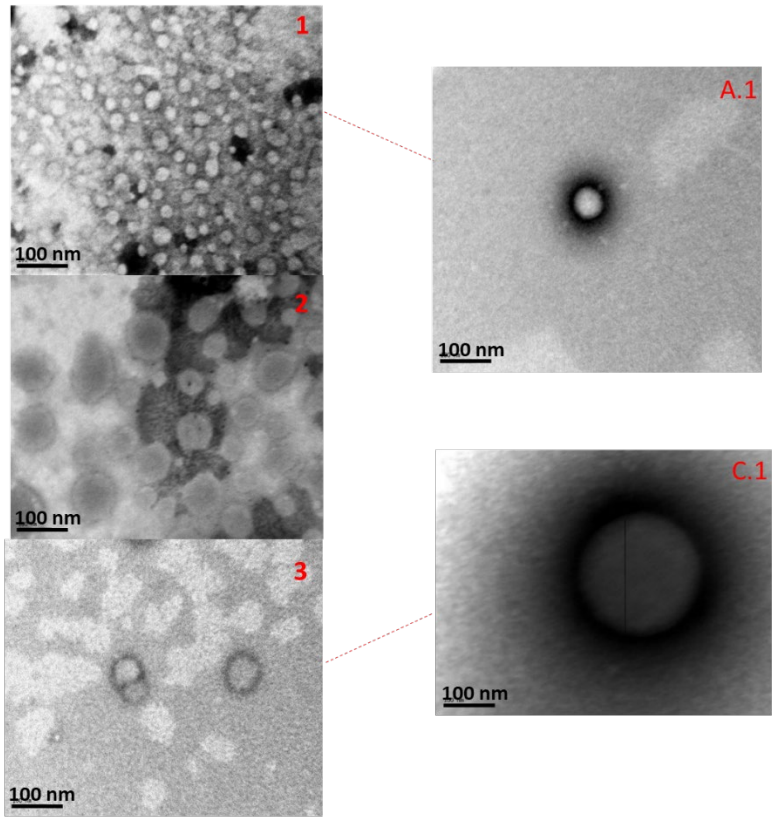

Figure 4. A) Liposomes formulated after maltodextrin addition-A.1) magnification of liposomes. B) Niosomes with maltodextrin. C) Niosomes+ CTAB with maltodextrin-C.1) magnification of niosomes+CTAB.

After the lyophilisation and further powders hydration processes, it was observed a common trend in size increase for all formulations passing from water, glycerol and PEG400 as it was also observed in vesicles were vitamin B_{12} was added at the hydration step. This behaviour is common, not only for the size but there is also an increase of PDI according to the amount of cryoprotectant and polymer used in the hydration step[24].The liposomes before lyophilisation presented a small size (40 nm) followed by niosomes with a mean size around 110 nm with a PDI between 0.4 and 0.9 that indicates a relatively broad size distribution.

Liposomes are found to be more affected by the hydration step than niosomes, even it can be observed that cationic niosomes size were not significantly affected by the hydration media of pure water and glycerol solution. However, a ten-fold increase in size was observed when the hydration of cationic niosomes was made by PEG400 solution. The low effect produced by glycerol solutions could be attributed to the presence of CTAB due to charge interaction between the surfactant CTAB and glycerol that forms a more compact system of vesicles [25].

After lyophilisation, differences can be appreciated between both methods, a comparative Table can be seen on supplementary material Table S1. As a general trend, it can be observed that the use of glycerol as hydration media produced larger size when vitamin B_{12} was added after the lyophilisation process but in case of use of PEG400 solution as hydration medium different behaviour was observed depending on the type of vesicles used, higher size increase was produced when vitamin B_{12} was added to liposomes before the lyophilisation step, while in the case of niosomes the higher increase

in size was observed when vitamin B_{12} was added after the lyophilisation step. However, it is important to point out the large vesicle size obtained in all cases when this PEG400 solution was used, sizes values between 500 and 9000 nm were registered with PDI values between 0.4-1.0.

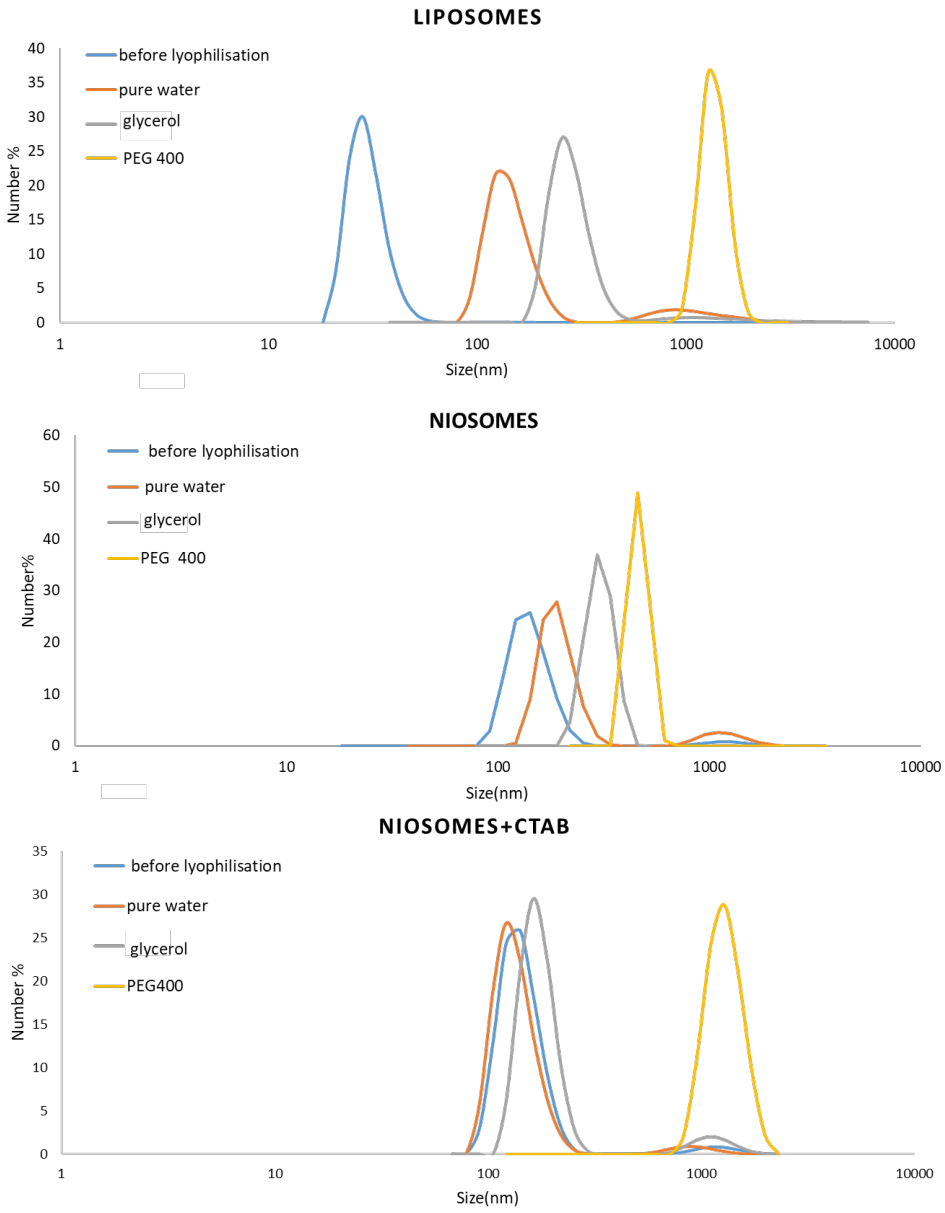

Figure 5- Distribution curves in comparison between vesicles containing 0.2mM of vitamin B_{12} before lyophilisation and restored vesicles after lyophilisation with the three aqueous phases.

From the DLS and TEM analyses (Figure 5 and Figure 6) it can be seen the influence of the hydration media used to restore the suspension in each formulation. The size increased using glycerol and PEG400, in liposomes and niosomes, to values up to 500-1500 nm.

In recent years, glycerol has been used as hydration media cosolvent with water, they called vesicles containing glycerol glycerosomes [26]. Glycerol increases the layer membrane thickness, increasing the particles radius as glycerol concentration increases[16] .

Glycerol has been used also as cryoprotectants increasing the fluidity of the lipid layer after the hydration and as stabilizer during the process of freezing and drying, providing more integrity of the membrane and reducing the leakage of the compound encapsulated[27].
In TEM picture, figure 6, for each formulation rehydrated by glycerol is significant the presence of large vesicle with a well-defined shape, similar to a sphere, and multilamellar character. Moreover, individual vesicles are distinguished without any aggregation phenomenon detected as in previous studies with similar systems [28].
Largest vesicles have been obtained with PEG400 solution. PEG interacts with bilayer components since it is hydrophilic and can adsorb to the out layer of vesicles and increase steric hindrance which prevents from aggregation and flocculation.
Large vesicle size was attributed also for the use of the two stabilizers: cholesterol, PEG400 and span 60 and CTAB[29] have longer saturated alkyl chain.
As it was reported in previous works, another aspect to have into consideration is the drug to be encapsulated: vitamin B_{12} interacts with glycerol and PEG400 in the membrane layer with interactions that affect the vesicle size as it was reported in previous studies in which vesicles were prepared using PEG400 and glycerol as hydration media[16].
Nevertheless it is important reconstruct the formulation with an appropriate solvent to guarantee not only the stability but also the homogeneity even if lyophilisation influences size, morphology and bilayer structure of liposomes and encapsulation efficiency[30].

From TEM images (Figure 6) it was corroborated that the size measured by DLS corresponds to vesicles and no aggregation was observed in any system being in good agreement with the DLS size distributions measured which reported low values of PDI for most formulations, normally it is observed when thin film hydration is used as a synthesis method, however after lyophilisation the PDI values, depending on the formulation and aqueous phase used, are higher.

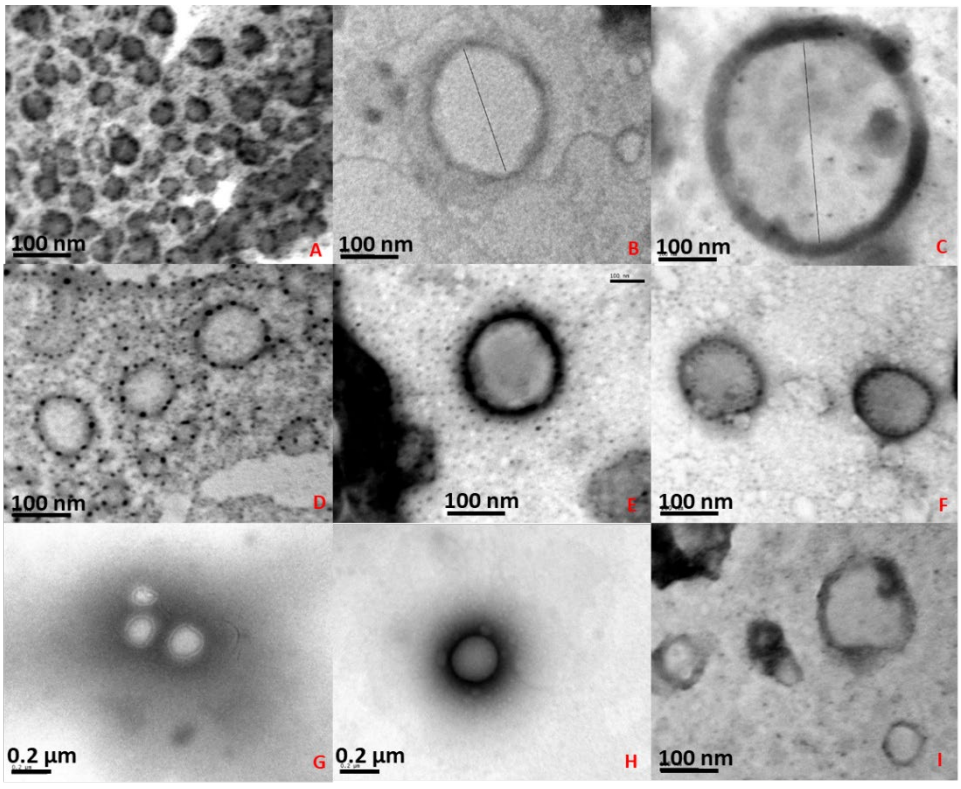

Figure 6. A) Liposomes restored with water MQ. B) Liposomes restored with glycerol. C) Liposomes restored with PEG400. D) Niosomes restored with water MQ. E) Niosomes restored with glycerol. F) Niosomes restored with PEG400. G) Niosomes with CTAB restored with water MQ. H) Niosomes with CTAB restored with glycerol. I) Niosomes with CTAB restored with PEG400.

Encapsulation efficiency and loading capacity

In order to study the effect of the lyophilisation process to the encapsulation of vitamin B_{12} and vesicles LC a liquid chromatography method was developed.

Table 4 shows the concentration of vitamin added (total vesicles) and the encapsulated vitamin B_{12} (purified vesicles). Moreover, the calculated EE was registered for each colloidal system before and after lyophilisation process. The LC of the final systems were also calculated.

Before lyophilisation the EE for the three formulations was less than 50%, the three systems tested presented EE values between 35-45% without significant difference between them.

370 **Table 4.** Encapsulation efficiency (EE%) and loading capacity (LC) in liposomes, niosomes and
371 niosomes with CTAB before and after lyophilisation in which the powder was restored with three
372 aqueous media.

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FORMULATION	C(mM)	EE(%)	LC (mg/g)
	BEFORE LYOPHILISATION		
Total liposomes	0.23 ±0.01	44±6[a]	70±9[x]
Purified liposomes	0.11 ±0.01		
Total niosomes	0.23 ±0.02	35±8[a]	50±11[x]
Purified niosomes	0.08 ±0.01		
Total niosomes+CTAB	0.23 ±0.01	39±4[a]	60±6[x]
Purified niosomes+CTAB	0.09 ±0.01		
	AFTER LYOPHILISATION hydration with MQ		
Total liposomes	0.23±0.07	42±1[a]	70±10[x]
Purified liposomes	0.11±0.01		
Total niosomes	0.23±0.02	18±2[b]	30±3[y]
Purified niosomes	0.04±0.01		
Total niosomes+CTAB	0.23±0.01	32±7[a]	50±10[z]
Purified niosomes+CTAB	0.08±0.04		
	AFTER LYOPHILISATION hydration with MQ:Glycerol		
Total liposomes	0.23±0.01	36±5[a]	60±8[x]
Purified liposomes	0.09±0.01		
Total niosomes	0.23±0.01	38±4[a]	60±7[x]
Purified niosomes	0.09±0.01		
Total niosomes+ CTAB	0.19±0.01	75±14[c]	90±16[z]
Purified niosomes+CTAB	0.14±0.06		
	AFTER LYOPHILISATION hydration with MQ:PEG400		
Total liposomes	0.23±0.02	70±9[c]	100±12[z]
Purified liposomes	0.17±0.05		
Total niosomes	0.22±0.01	34±4[a]	50±6[x]
Purified niosomes	0.07±0.01		
Total niosomes+CTAB	0.18±0.01	48±6[d]	50±7[x]
Purified niosomes+CTAB	0.09±0.01		

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375 Letters (a-d) indicate significant differences EE values, letters (x- z) indicate significant differences between
376 LC values.

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379 It was observed that after lyophilisation and re-hydration of the powders the EE registered highly
380 depended on the type of hydration media used.

Liposomes were highly affected by the lyophilisation process; EE do not present significant differences when the rehydration process was made with glycerol or pure water, but it increased up to close to double when powder was hydrated with a mixture of water and PEG400.

For the niosomes formulations tested without CTAB a significant decrease of EE was visible when rehydration was made with pure water without being affected by the rehydration of glycerol and PEG400. However, niosomes in the presence of CTAB increase its EE up to close to double values when they were hydrated with glycerol.

The high EE values obtained after lyophilisation in some systems are probably due to the use of glycerol and PEG in vesicles. As confirmed in the previous work for molecules with high molecular weight the interaction with different hydration phases can be a key element to arise large EE.

In previous works [31,32]where PEG solutions were used as hydration media high EE values were observed for hydrophobic drugs, e.g. paclitaxel, an anticancer drug [31] and quercetin a phytosterol [32], since PEG400 increased their solubility in the aqueous core of the vesicles.

In other studies, it was also observed how the use of glycerol improved EE[24] but the increase of glycerol up to 45% produced a EE reduction of hydrophobic biomolecules such as rifampicin, an antibiotic used for lungs disease[26] and lacidipine used for hypertension and atherosclerosis[24] .

During the lyophilisation process the vesicles can also undergo variations, such as rupture of the lipid membrane, with consequent loss of the content, or other aggregation phenomena that could occur during the rehydration step since vesicles can aggregate. Consequently, vitamin B_{12} LC of all systems were evaluated in terms of concentration using a calibration curve performed by RP-HPLC and the individual measurements of all samples.

All data were analysed and calculated for total and purified vesicles in order to calculate the total and the vitamin B_{12} encapsulated concentration in each system.

It can be appreciated that the evaluation system did not present any considerable retention of the vitamin B_{12} since values of non-purified systems were around 0.22±0.3 mM.

Lower vitamin B_{12} concentration were registered for non-purified systems when CTAB was used as one of the vesicle membrane compounds. Therefore the behaviour of this surfactant in presence of organic solvents should be taken into account, CTAB is a surfactant that forms micelles in water and non-ionic solvents that normally stop the micellization process [33] in the presence of alcohols like methanol, isopropanol or ethanol. Shan and coworkers stated that the micellization process depends on the nature and concentration of medium alcohol-water used. The critical micelle concentration (CMC) of CTAB increases with methanol and ethanol content but in mixture of water and ethanol there is a decrease of CMC for this surfactant. Probably it is due to to penetration of alcohol molecule at the core of the micelles[34]. In conclusion more factors can influence the behaviour of niosomes with CTAB due to the nature of this surfactant, the nature of hydration media and the interactions between both elements.

It can be observed that the vitamin B_{12} concentration of the systems increased for liposomes when they were hydrated with PEG solutions, and niosomes with CTAB when they were hydrated with glycerol solutions. While in the case of niosomes hydrated with pure water the vitamin B_{12} concentration was reduced from 0.08 to 0.04 mM.

On the other hand considering the LC, the values for each type of formulation remained between 30-100 mg/g, presenting larger LC liposomes hydrated with PEG400 solutions and niosomes with CTAB hydrated with glycerol. The LC of those systems were not only not affected by the lyophilisation process but also an increase of 30mg/g was observed in both cases. This behaviour indicates that in some particular situations the lyophilisation process and subsequent rehydration could help to encapsulate part of the bio compound that was initially non-encapsualted increasing hence its EE and

LC. Moreover, in any case a significantly reduction of LC was observed as a consequence of the lyophilisation process.

CONCLUSIONS

In this work the lyophilisation process for different formulations of vesicles encapsulating vitamin B_{12} was evaluated since it is known that freeze-drying is an excellent process to give chemical-physical stability, long storage time of the vesicles and easier use of powdered vesicles. It was demonstrated the possibility to obtain a good EE for bio compounds with high molecular weight. The characteristics of different vesicle formulations, before and after lyophilisation, the influence of a cryoprotectant, such as maltodextrin, was compared. Moreover the effect of the bio compound loaded, before and after lyophilisation step, on vesicle size was studied.

Results indicated that an additon of 20% w/w of maltodextrin did not alter the vesicles structure, nor the addition of the bio compound in the mixture of aqueous phases after lyophilisation. The ability of glycerol and PEG400 to produce large vesicles was confirmed in comparison with vesicles formed by just MQ water as aqueous phase.

In contrast, with the traditional methods to produce proniosomes, vesicles contaning vitamin B_{12} were formed before the lyophilisation process through thin film hydration method, being restored after its lyophilisation. The results shown how its morphology and EE were not affected by the lyophilisation process being, in some particular cases, even improved.

The importance of the hydration media used to restore the vesicles was stablished, being the hydroalcoholic mixtures a more appropiate media than pure water with larger EE and LC values. Glycerol and PEG400 allowed to obtain larger vesicles and a higher EE especially in liposomes and in cation niosomes.

Results indicated the suitability to prepare lyophilised cationic niosomes with high potential in some food, cosmetic, pharmaceutical applications incorporating encapsulated vitamin B_{12}, which could be consequently rehydrated to obtain homogeneous systems with high EE. The LC of the lyophilised systems prepared ranged between 30-100 mg/g of bio compound.

Recent scientific advances have demonstrated the beneficial character of vitamins on the transport and penetration of antimicrobial biocompounds in biofilms. For the applicability of the proposed nanoformulations it will probably be necessary to start from a higher initial concentration of the vitamin in order to have the necessary quantity inside the vesicle after purification and lyophilisation and in addition consider the appropriate aqueous phase.

Hence, these systems resuspended in the water and glycerol mixture,considering the known properties of glycerol as an antimicrobial, can be potentially used as carriers of antimicrobial compounds and improve the efficiency of the delivery vehicles.

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