

Review

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[Matheus A. Chaves](#)[†], [Leticia S. Ferreira](#)[†], [Lucia Baldino](#)^{*}, [Samantha C. Pinho](#), [Ernesto Reverchon](#)

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Review

Current Applications of Liposomes for the Delivery of Bioactives: A Review on the Encapsulation of Vitamins

Matheus A. Chaves ^{1,2,†}, Letícia S. Ferreira ^{1,†}, Lucia Baldino ^{3,*}, Samantha C. Pinho ¹ and Ernesto Reverchon ³

¹ Laboratory of Encapsulation and Functional Foods (LEnAlis), Department of Food Engineering, School of Animal Science and Food Engineering, University of São Paulo, Av. Duque de Caxias Norte, 225, Pirassununga, SP, Brazil; matheus.chaves@usp.br (M.A.C.); leticia2.ferreira@usp.br (L.S.F.); samantha@usp.br (S.C.P.)

² Laboratory of Molecular Morphophysiology and Development (LMMD), Department of Veterinary Medicine, School of Animal Science and Food Engineering, University of São Paulo, Av. Duque de Caxias Norte, 225, Pirassununga, SP, Brazil; matheus.chaves@usp.br (M.A.C.)

³ Department of Industrial Engineering, University of Salerno, Via Giovanni Paolo II, 132, 84084, Fisciano, Italy; ereverchon@unisa.it (E.R.)

† Authors contributed equally to this work.

* Correspondence: lbaldino@unisa.it; Tel.: +39089964356

Abstract: Liposomes have been used for several decades for the encapsulation of drugs and bioactives in cosmetics and cosmeceuticals. On the other hand, the use of these phospholipid vesicles in food applications is more recent and has been increasing significantly in the last ten years. Although in different stages of technological maturity - in the case of cosmetics, many products are on the market - processes to obtain liposomes suitable for the encapsulation and delivery of bioactives are highly expensive, especially aiming at scaling-up. Among the bioactives proposed for cosmetics and food applications, vitamins are the most frequently used. Despite the differences between the administration routes (oral for food and mainly dermal for cosmetics), some challenges are very similar (e.g., stability, bioactive load, average size, increase of drug bioaccessibility and bioavailability). In the present work, a systematic review of the technological advancements in the nanoencapsulation of vitamins using liposomes and related processes was performed; challenges and future perspectives were also discussed in order to underline the advantages of these drug loaded biocompatible nanocarriers for cosmetics and food applications.

Keywords: vitamins; nanoencapsulation; nanodispersions; phospholipid vesicles; liposomes; cosmetics; food application

1. Why Encapsulating Vitamins for Food and Cosmetics Application?

Vitamins are organic compounds required for metabolic processes, and not produced by the human body in sufficient amounts. Each vitamin has specific functions in the body and cannot be replaced by any other substance [1]. Having different chemical structures, vitamins can be classified in two main groups: fat-soluble and water-soluble compounds. Thirteen main vitamins have been identified; they are characterized by different ways of action and different beneficial roles in the body. In addition, vitamins play a key role in transforming energy and regulating the metabolism pathways of the human body [2].

Regarding food products, the incorporation of vitamins is generally performed: (i) to fortify; (ii) to replace vitamin losses during processing; and (iii) to use them as antioxidants and/or natural colorants. On the other hand, in cosmetics, vitamins are present in products for skin care, hair, and oral health [2].

Vitamins A, D, E, and K are fat-soluble compounds; therefore, they require a certain amount of fat in the diet to be efficiently absorbed. On the other hand, group B vitamins and vitamin C are

water-soluble compounds and cannot be stored in the body, requiring daily consumption or supply. All these vitamins (A, D, E, K, B, and C) can be used as ingredients in cosmetics according to European Union legislation, with the exception of vitamin D₃ (cholecalciferol); whereas, in USA and Japan, there are no restrictions on the use of vitamin D₃ in cosmetics [2]. Chemical structure, ways of obtainment and food sources of some main vitamins are summarized in Table 1.

1.1. *Hydrophobic Vitamins*

Vitamin A can be found as retinoids (retinol, retinal, and retinoic acid) and provitamin A carotenoids (mainly β -carotene) [3]. It is important to emphasize that vitamin A is not produced endogenously. The retinol is the first precursor of two vital active metabolites: retinal, which plays an important role in the development of vision, and retinoic acid that acts as an intracellular signal that alters the transcription of a range of genes. Vitamin A is not found directly in plants; however, plants can contain carotenoids, like β -carotene, which is transformed in vitamin A in the intestine and other body tissues. Therefore, vitamin A supply is necessarily obtained through ingestion (dietary sources or vitamin supplements) [4]. Foods such as fish, meat (mainly liver), eggs, and whole milk are animal sources of retinol [2,3]. Fruits and vegetables such as carrots, spinach, and mango are sources of carotenoids. In terms of biological activity, vitamin A is important for the growth and development of children and for the maintenance of the immune system [5]. In adults, vitamin A deficiency can lead to adverse effects on the immune system, reproductive function, and eyes, such as night blindness [3,4,6]. On the other hand, excessive exposures to vitamin A are associated with adverse health effects, such as teratogenicity [7]. In the cosmetic industry, vitamin A is widely used due to its ability to delay photoaging effects. Being the main bioactive for skin treatment, it promotes the regeneration of the skin aged by UV radiation, reduces wrinkles and improves skin elasticity [2]. Vitamin A is not degraded by heat; but it is easily oxidized, and care is required during its processing. To reduce this undesired effect, antioxidants are added to vitamin A products as, for example, vitamin E [2].

Vitamin D group is composed of ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃). Vitamin D₃, or cholecalciferol, is a fat-soluble compound synthesized by the human epidermis by the irradiation of UV light on 7-dehydrocholesterol [8]. The precursor molecule of vitamin D is ergosterol (or 7-dehydrocholesterol), a rigid structure that is inserted by the body when absorbed by the lipid layer of the plasmatic membrane. The production of provitamin D occurs after solar incidence on the aromatic ring of ergosterol. The structure, then, becomes less rigid, promoting an increase in its permeability and, thus, allowing the incorporation of numerous ions into its interior, including calcium. It can be synthesized by the human epidermis or consumed in the form of supplements or fortified foods. A few foods naturally contain vitamin D in their composition: fish, such as salmon and sardines, butter, and eggs being the main sources. Some benefits conferred to its consumption are the improvement of calcium absorption by the intestine and the homeostasis of this mineral in the bloodstream, preventing diseases such as osteoporosis [9]. Currently, a large part of the urban population is deficient in vitamin D₃ mainly due to the lack of sun exposure and loss of 7-hydrocholesterol reserves in the epidermis with aging [10]. Vitamin D deficiency is responsible for bone diseases; but also for other diseases such as cancer, asthma, arthritis, hypertension, osteoporosis and cardiovascular problems. Signs and symptoms of its deficiency may include bone pain and muscle weakness [11]. Vitamin D is used in the cosmetic industry as it prevents photo-damage, wrinkles and other morphological skin changes [2]. Some studies indicated its topical application for the treatment of psoriasis [12]. However, it has an adverse effect on calcium metabolism and limits its use for topical applications [13].

Vitamin E refers to different compounds, tocopherols and tocotrienols, and they are identified by the prefixes: α , β , γ , and δ . α -tocopherol is the most common occurring form of vitamin E and has much higher bioavailability than the other vitamin E forms [14]. Vegetable oils of peanut, soya, palm, corn, safflower, sunflower, and wheat germ are the most important sources of vitamin E. Vitamin E has relevant physiological functions, including antioxidant, immunity regulation, anti-inflammatory, and neuroprotective capacities [15]. This vitamin also plays the role of protecting body tissues from

oxidation by metabolic processes and exogenous agents, in addition to assisting the body in the synthesis of vitamin A [2]. However, its incorporation in food can be challenging, as it is extremely sensitive to high temperatures, light, oxygen, and alkaline conditions, and has low solubility in water [15]. Encapsulation techniques can help to overcome these obstacles allowing the application of vitamin E in food, cosmetics and nutraceuticals. In the cosmetic industry, vitamin E is generally used as an antioxidant for the skin, aiding in softening and promoting hydration [2].

1.2. *Hydrophilic Vitamins*

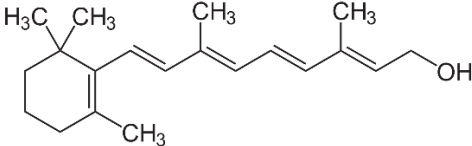
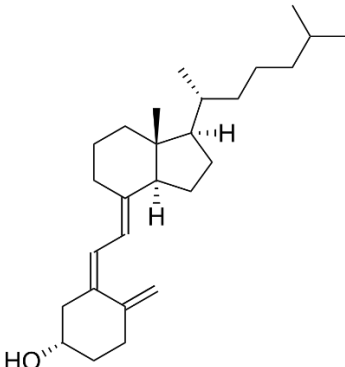
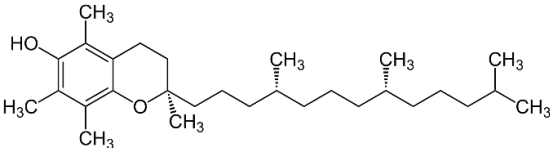
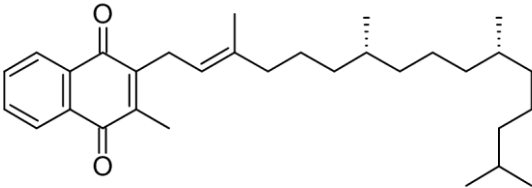
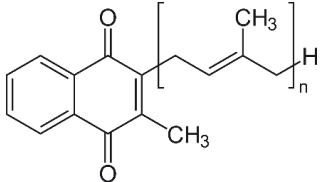
Vitamin K occurs in three forms: vitamin K₁ (phyloquinone, phytonadione, phytomenadione), vitamin K₂ (menaquinone), and vitamin K₃ (menadione). Vitamin K₁ is found in plants; K₂ is synthesized by bacteria in the human and animal intestines; and K₃ is a synthetic compound that is converted to K₂ in the intestinal tract [2,16,17]. Green leafy vegetables (such as spinach, kale, broccoli, and cauliflower) are good dietary sources of this vitamin that is also found in smaller amounts in liver, lean meat, cow's milk, egg yolks, and whole wheat products [2]. Although differing in structure, the vitamin K₁ and K₂ act as cofactor for the enzyme gamma-glutamylcarboxylase, encompassing both hepatic and extrahepatic activity. In addition, vitamin K₂ has been shown to be necessary in regulating osteoporosis, atherosclerosis, cancer and inflammatory diseases without risk of negative side effects or overdosing [16,17]. Also, vitamin K is effective in treating dark circles and bruises on the face, and its application to reduce the effects of bruising after certain dermatological procedures has been also studied [18].

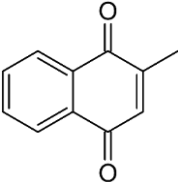
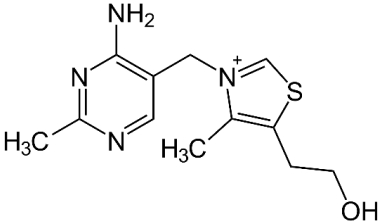
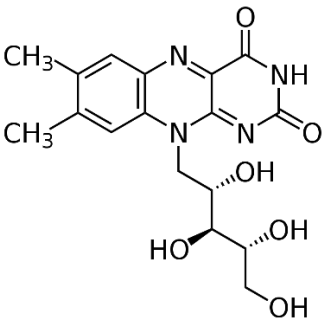
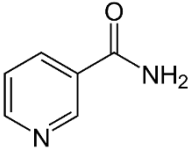
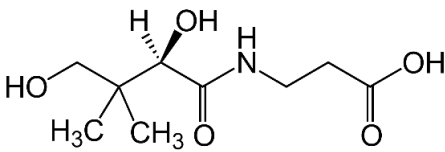
Group B vitamins comprises B₁ (thiamine), B₂ (riboflavin), B₃ (niacin), B₅ (pantothenic acid), B₆ (pyridoxine), B₈ (D-biotin), B₉ (folic acid), and B₁₂ (cobalamin) vitamin. Vitamin B₁ has been found in brewer's dried yeast and pork, lamb and beef, poultry, whole grains, nuts, vegetables, and legumes, but in small amounts. It is essential for the breakdown of food, especially carbohydrates, to release energy, and for healthy nerve and muscle function. Deficiency causes growth retardation and disorders of the nervous and cardiac systems, such as 'beri-beri' disease. In the cosmetic industry, it acts as a coenzyme in the metabolism of aminoacids and keeps the skin healthy [2]. Vitamin B₂ (riboflavin) is found in all plants and animal cells; but these are poor sources. The commonest dietary sources are milk and dairy products, meat, eggs and leafy green vegetables; however, animal sources of riboflavin are better absorbed than vegetable sources. Riboflavin is vital for the release of energy from foods and for healthy skin, eyes and growth. It plays a major role in oxidation and reduction processes in cells. Deficiency is rare, and generally occurs in combination with deficiencies of other water-soluble vitamins. Riboflavin is not used in skin care cosmetics; but, due to its bright deep yellow color, it can be used as a colorant in cosmetic products [2]. Vitamin B₃ (nicotinic acid or niacin) is found in yeast, liver, poultry, lean meats, nuts, and legumes. Niacin is used for the treatment of lipid disorders and cardiovascular diseases, essential for growth and hormone synthesis [19]. Together with vitamins B₁ and B₂, it maintains a healthy nervous and digestive system. Pantothenic acid is necessary for the release of energy from food, for the production of antibodies and healthy growth. Vitamin B₅ requires vitamin A, vitamin B₃, vitamin B₆, vitamin B₉, and vitamin B₁₂ in order to act properly. Vitamin B₅ is used in many cosmetic skin and hair care products. Its main functions can improve wound healing, anti-inflammatory, and moisturizing activities [2]. Pantothenic acid is present in almost every type of food; but it is particularly abundant in yeast and organs of animals (liver, kidney, heart, brain). However, eggs, milk, vegetables, legumes, and whole-grain cereals are probably the most common sources of this vitamin. Vitamin B₁₂ is essential for DNA synthesis and red blood cell production. Its deficiency results in megaloblastic macrocytic anemia (characterized by tachycardia, palpitations, generalized weakness, fatigue, and pallor), cognitive impairment, peripheral neuropathies, sensory and neurological abnormalities [20].

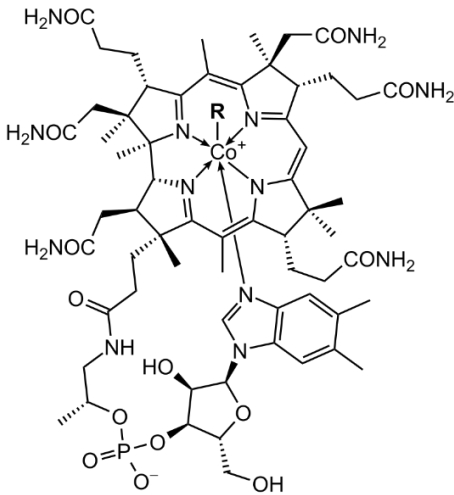
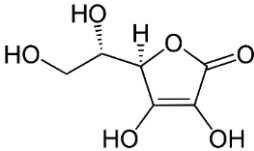
Vitamin C or ascorbic acid is largely used in cosmetic and pharmaceutical products due to its antioxidant activity. However, the biggest challenge in the incorporation of vitamin C is to maintain its stability and improve its delivery to the active site [21]. It is naturally found in citrus fruits, currants, peppers, parsley, cauliflower, potatoes, sweet potatoes, broccoli, brussels sprouts, strawberries, guava, and mangoes. Vitamin C is also frequently used as a natural antioxidant in foods

and beverages spoilage. This molecule is also important for the production of collagen, connective tissue and protein fibers that give strength to teeth, gums, muscles, blood vessels, and skin. In the immune system, vitamin C helps the white blood cells to fight infection. It also helps the body to absorb iron [2,21].

Table 1. Chemical structure,ways of obtainment and main sources of vitamins in foods.

Vitamin	Chemical structure	Way of obtainment	Sources
A (retinol)		Intake of dietary sources or vitamin supplements	Fish, meat, eggs, whole milk, carrots, spinach, and mango
D ₃ (cholecalciferol)		Synthesized by the human epidermis or consumed in the form of supplements or fortified foods	Fish, as salmon and sardines, butter and eggs
E (α-tocopherol)		Intake of dietary sources or vitamin supplements	Vegetable oils of peanut, soya, palm, corn, safflower, sunflower, and wheat germ
K ₁ (phylloquinone)		Plants	Green leafy vegetables, liver, lean meat, cow's milk, egg yolks, and whole wheat products
K ₂ (menaquinone)		Synthesized by bacteria in the human and animal intestines	-

K ₃ (menadione)		Converted to K ₂ in the intestinal tract	-
B ₁ (thiamine)		Intake of dietary sources or vitamin supplements	Brewer's dried yeast and pork, lamb and beef, poultry, whole grains, nuts, vegetables, and legumes
B ₂ (riboflavin)		Plants and animal cells	Milk and milk products, meat, eggs, and leafy green vegetables
B ₃ (niacin)		Intake of dietary sources or vitamin supplements	Yeast, liver, poultry, lean meats, nuts, and legumes
B ₅ (pantothenic acid)		Intake of dietary sources or cosmetic products	Organ of animals, eggs, milk, vegetables, legumes, and whole-grain cereals

B ₁₂ (cobalamin)	 <p>R = 5'-deoxyadenosyl, CH₃, OH, CN</p>	Intake of dietary sources or vitamin supplements	Milk, and dairy products, eggs, salmon
C (ascorbic acid)		Intake of dietary sources or vitamin supplements	Citric fruits, currants, peppers, parsley, cauliflower, potatoes, sweet potatoes, broccoli, brussels sprouts, strawberries, guava, and mango

2. Formation and Classification of Liposomes

In 1965, it was reported, for the first time, that phospholipid molecules were able to instantaneously form closed bilayer vesicles in aqueous media due to the amphiphilic nature of phospholipids [22]. Liposomes are vesicular structures formed by one or more phospholipid bilayers that encapsulate part of the aqueous medium in which they are dispersed [23]. Their average diameters range from 20 nm to several microns. Phospholipids are the main constituents of vesicles, being an amphiphilic molecule in which the hydrophilic polar head groups are oriented towards the aqueous phase and the hydrophobic non-polar hydrocarbon tails are oriented towards each other in an ordered bilayer structure. A wide variety of phospholipids can be used for the production of liposomes, such as eggs, soy, milk that are natural and safe sources. The most used phospholipid for the production of liposomes in the food and cosmetic industry is phosphatidylcholine (PC). In addition to PC, phospholipids such as lysophosphatidylcholine (LPC), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) are also used. Phospholipids contain different amounts of unsaturated fatty acids that are prone to oxidative reactions, and the resulting products can cause changes in the permeability of the lipid bilayers. In addition, interactions between phospholipids and bioactives can alter the overall chemical stability of the system.

Phospholipids, when added to an aqueous medium, due to their low solubility in water, associate with each other resulting in lipid bilayers, and by adding a sufficient amount of energy to the system (which can be provided by external methods such as sonication, heating or homogenization), the unfavorable interaction between molecules of fatty acids and water is eliminated and the bilayers arrange themselves in their organized form. In this process, liposomes


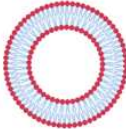
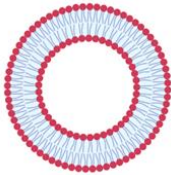
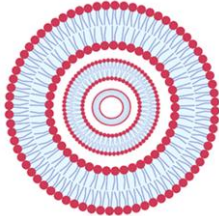
are capable of incorporating hydrophilic compounds in their aqueous core, in addition to hydrophobic compounds in the internal regions of the lipid bilayer [24].

Liposomes are among the current commercialized lipid nanocarriers most used for various nutraceutical purposes and are increasingly studied for incorporation into foods for functional applications [25,26].

During formation, liposomes acquire structural characteristics (number of layers) and various sizes [27,28,29]. Based on their size and number of bilayers, liposomes can be classified in two main categories: unilamellar vesicles and multilamellar vesicles (Table 2). Liposomes that contain only a single bilayer membrane are called small unilamellar vesicles (SUV) with a size of 20 to 200 nm; large unilamellar vesicles (LUV), with size above 200 nm, and giant unilamellar vesicles (GUV), with size higher than 1 μm . Liposomes that contain more than one bilayer membrane are called multilamellar vesicles (MLV) and are composed of several concentrically arranged vesicles with a size of 0.5 to 5 μm , or multivesicular vesicles (MVV), where there are smaller vesicles within a vesicle of larger size [12,24]. Such size characteristics directly depend on the chosen production method and the type(s) of phospholipid(s) used.

Despite their versatility, liposomes are physicochemically unstable. This is due to the fact that the lipids present in their structure can undergo to natural degradation through oxidation or hydrolysis, or even because the particles can form agglomerates. Liposomes originally show repulsive forces between their particles that provide a certain physical stability; but external factors, such as high temperatures or pH changes, can affect their structure and change the permeability of the bilayer, causing the release of the encapsulated compound or the formation of agglomerates. In order to reduce these undesired effects, a possible solution is the coating of liposomes with biopolymers that can increase the physical stability of these particles through steric and electrostatic factors, thus creating a hybrid system. Among the biopolymers used for the coating of liposomes, starches, gelatin, proteins, cellulose, pectin and chitosan can be mentioned.

Table 2. Classification of liposomes based on size and lamellarity.

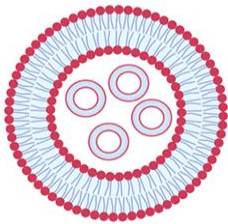
Type of liposome	Abbreviation	Characteristic	Diameter	Schematic representation
Small unilamellar vesicles	SUV	Small unilamellar vesicles	20 to 200 nm	
Large unilamellar vesicles	LUV	Unilamellar liposomes with average diameters higher than SUV	Above 200 nm	
Giant unilamellar vesicles	GUV	Unilamellar liposomes with average diameters higher than LUV	Higher than 1 μm	
Multilamellar vesicles	MLV	Several concentrically arranged vesicles	0.5 to 5 μm	

Multivesicular vesicles

MVV

Smaller vesicles within a vesicle of large size

Higher than 1 μm



3. Methods for the Production of Vitamin-Loaded Liposomes

Generally, the successful encapsulation of bioactive molecules in liposomes, with desired and specific size and structure, depends on several factors such as the correct choice of the main lipid, the affinity between the liposome and the bioactive of interest, and, most of all, the production method. The latter is considered as extremely important when encapsulating vitamins for several reasons that may include: (i) the efficiency of encapsulation, as some methods may result in higher encapsulation efficiencies than others; (ii) the stability of the vitamin, as certain methods may be less harmful to them, which is particularly important for sensitive vitamins that can be easily degraded by heat, light, or exposure to oxygen; (iii) the control of particle size and distribution, which are directly related to the release rate of the vitamin from the encapsulating material and also to its bioaccessibility and bioavailability; and (iv) the process scalability, to ensure both consistent quality and efficient production.

Conventional methods of liposome production for vitamin encapsulation are still on the spotlight of many researchers due to their simplicity, recognition and wide range of applications. On the other hand, novel methods have arisen thanks to their advantages over the conventional ones, such as higher encapsulation efficiencies, reduced toxicity, versatility, and scalability. Overall, the production method used for encapsulating vitamins plays a critical role in ensuring the stability, efficacy, and quality of the final product. The main methods currently found in the literature for liposome production are discussed below.

3.1. Conventional Methods

In the conventional methods, five main steps are basically followed: (i) lipids are dissolved in an organic solvent (ethanol, ether, chloroform, dichloromethane); (ii) the organic solvent is removed using evaporation, rotary evaporation, or distillation; (iii) the resultant lipid layer is hydrated using an aqueous medium (distilled water, buffer solutions, serum-containing media, or physiological saline solutions) and agitated; (iv) vesicles are analyzed and eventually treated by downsizing steps (see Supplementary Material for the detailed description of these methods), depending on the liposome final use; and (v) post-formation processing (purification or sterilization) is carried out to increase the stability of the liposomes [30]. Vitamins must to be dissolved in the liquid medium in which they are easily solubilized; i.e., in the organic solvent if lipophilic (A, D, E, and K) or in the hydration medium if hydrophilic (complex B vitamins and C) vitamins are selected.

3.1.1. Thin-Film Hydration (TFH) Method (Bangham Method)

The thin-film hydration technique, also referred as the Bangham method due to the British biochemist that first described it in 1965, is the simplest method used for the preparation of MLVs. It is mostly used for the encapsulation of lipophilic molecules and it is based on the production of a thin lipid film by the evaporation at 45 - 60 °C of an organic solvent from a lipid-solvent solution during flask rotation under vacuum and its further hydration using an aqueous media. When small volumes of liposomes are desired, the organic solvent can be dried by using argon steam or dry nitrogen in a fume hood [31]. Hydration processes should be performed above the phase-transition temperature of the lipids (e.g., 60 - 70 °C) for a duration of at least 1 - 2 h [30]. Besides the ability to encapsulate both hydrophilic and hydrophobic molecules, the TFH method presents some limitations

such as low encapsulation efficiencies and batch-to-batch variability. The production of liposomes using TFH method is usually followed by a downsizing process to obtain SUVs.

Bi et al. [32] used egg-PC, cholesterol (Chol), and vitamin D₃ (3:1:1 w/w) to produce vitamin D₃-loaded MLVs by the TFH method followed by high-pressure homogenization. Obtained liposomes presented a particle size of 169 nm and an encapsulation efficiency (EE%) of 62%. Vitamin B₁₂-loaded liposomes were produced by Andrade et al. [33] prepared liposomes by the TFH technique followed by ultrasonication, using 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), Chol, polyethylene glycol 2000 (PEG200) and amine 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2000) in a 52:45:3:0.06 molar ratio. LUVs showed sizes of 116 nm and an EE% of 14%. The authors stated that the the low EE% was due to the vitamin B₁₂ that is an extremely water-soluble molecule. Campani et al. [34] produced a liposome-based formulation loaded with vitamin K₁ by the TFH technique followed by extrusion through polycarbonate membranes with decreasing porosity (400, 200, and 100 nm). Lipid films were prepared using soy phosphatidylcholine (SPC) and were rehydrated by PBS at pH 7.4 or aqueous solution at 0.01% w/v of benzalkonium chloride. Samples produced in these conditions were all in the nanometric range (131 - 147 nm) and presented vitamin K₁ encapsulation ratios of 3.4 - 154.0 µg VK₁/mg SPC. Vitamin E-loaded nanoliposomes were efficiently prepared by Qu et al. [35] by using egg lecithin, cholesterol, sodium deoxycholate, and vitamin E, in a 5.8:1:1.1:1.8 mass ratio, by the TFH method followed by sonication and extrusion. The obtained vesicles presented an average hydrodynamic diameter of 231 nm, EE% of 97%, a narrow size distribution (polydispersity index, PDI = 0.217) and high zeta potential (-52.39 mV).

3.1.2. Reverse-Phase Evaporation (RPE) Method

This technique was first described by Szoka & Papahadjopoulos in 1978 [36] and is generally used to encapsulate large amounts of hydrophilic bioactives with high encapsulation efficiencies [37]. Similarly to the TFH method, lipids are first dissolved in an organic solvent, such as diethyl ether, chloroform, isopropyl ether, or a mixture of two solvents, in order to form inverted micelles [38]. After the addition of an amount of aqueous phase, water-in-oil (W/O) microemulsions are formed due to the rearrangement of lipids at the interface between water and oil. During this step, a large amount of the aqueous phase is encapsulated within the microemulsion, as well as the hydrophilic molecules. The organic solvent can be slowly removed using a rotary evaporator under vacuum until the conversion of the micelles to a semi-solid viscous gel-like structure is obtained. The gradual removal of the solvent favors the disruption of the gel and promotes the formation of LUVs [30]. As discussed for the TFH method, a downstream step such as sonication or extrusion is required to reduce the average size of liposomes and to obtain a narrow size distribution [39]. The main drawbacks related to this technique include: (i) the use of a large amount of organic solvents and the presence of residual solvent at the end of the process; (ii) the high complexity and difficulty to industrial scaling-up; (iii) the unsuitability to be employed for the encapsulation of sensitive molecules due to the long-lasting contact with the organic solvent; (iv) the time-consuming process; and (v) the sterile boundary is quite hard to establish [40].

There are a few studies exploring this method for the encapsulation of vitamins, mainly due to their high degradability rates. Favarin et al. [41] employed the RPE method to encapsulate vitamin C into liposomes. In their study, the aqueous phase was composed of polysorbate 80, at pH 3.65, and vitamin C; whereas the organic phase was composed of phospholipid Lipoid® S100, cholesterol and ethanol. The inverted micelles were formed after the addition of the aqueous phase to the organic phase under ultrasonic conditions. The mixture was, then, submitted to a slow evaporation in a rotary evaporator operating at 80 rpm and 35 °C for the removal of the organic solvent, and the formation of the gel-like structure. Liposomes were produced after the addition of the aqueous phase under agitation. To homogenize the vesicles size, samples were extruded using membranes with pores of 0.45 µm and 0.22 µm. Vitamin C-loaded liposomes showed size of 160 nm and PDI of 0.23, besides a slight negative zeta potential of -7.3 mV and an EE% equal to 19%.

3.1.3. Injection Methods

The injection methods are based on the dissolution of lipids into an organic solvent (ethanol or diethyl ether) and the further injection of the resulting solution into an aqueous phase. Among all the liposome production techniques, the injection methods are suitable to operate continuously [42].

- Ethanol injection (EI) method: this method was first described by Batzri & Korn [43] and is based on the dissolution of phospholipids in ethanol and, then, the injection of the solution into a rapidly stirred aqueous phase [43]. Part of the ethanol evaporates upon contact with the aqueous phase, producing a lipid film that forms liposomes upon hydration. Vesicles are formed due to the immediate diffusion of the ethanol in the aqueous medium, leading the lipid molecules to precipitate and to form bilayered planar structures that tend to liposomes [44]. A change in the solubility of lipids leads to the spontaneous formation of vesicles that encapsulate a small volume of the aqueous phase. This method is relatively simple and produces liposomes characterized by a high entrapment efficiency of hydrophobic molecules. SUVs can be formed if proper process parameters, such as low lipid concentrations and fast rate of injection, are used. A disadvantage of ethanol injection is that the encapsulation efficiency of hydrophilic compounds is low, and the control of both size and size distribution of the resulting liposomes can be challenging. On the other hand, some advantages of this method include: (i) it is a straightforward method, which makes it a popular choice for liposome production; (ii) its high reproducibility; and (iii) its easy scale-up, making it a practical choice for commercial production [44]. However, this method is limited by the need for subsequent processing to solvent evaporation and the residual content [45]. The use of the EI method to produce liposomes for food or cosmetics is usually hindered by the low encapsulation efficiency of hydrophilic bioactive molecules such as vitamin C. This occurs because hydrophilic bioactives tend to be preferentially retained in the external aqueous phase of vesicles instead of in their small aqueous core [46]. Charcosset et al. [47] developed a continuous process for the ethanol injection method coupled with membrane extrusion, in which vitamin E-loaded MLVs were produced using Lipoid® S100 as the source of phospholipids. Liposomes were stored in a vacuumed double jacketed reactor in which the ethanol residue was constantly removed. Using this method, volumes between 60 mL to 3 L of liposomes with sizes ranging from 89 to 118 nm were produced.

- Ether injection method: this method requires the dissolution of lipids in diethyl ether and, then, a slow injection of the solution into an aqueous phase under high pressure. The ether rapidly evaporates upon contact with the aqueous phase under warming, resulting in a lipid film that forms liposomes upon hydration [48]. This method results in a concentrated liposomal (LUVs) product with a narrow size distribution and high entrapment efficiency [45]. Some differences can be pointed out between this method and EI: (i) ethanol is a polar solvent, unlike ether that is nonpolar; this can affect the lipid solubility and the self-assembly of lipids in liposomes, resulting in differences in size, size distribution, and other properties of liposomes; (ii) ether injection method may be more complex and time-consuming than EI method; and (iii) different effects on the stability of lipids and liposomes as the use of ether may cause a larger lipid degradation or oxidation than the use of ethanol due to the formation of peroxides [30]. However, an advantage of using the ether injection method consists of the more efficient removal of the organic solvent from the final product [30]. For food purposes, it is generally preferable to use ethanol instead of ether. Ether is highly flammable and can pose a fire hazard, which may be a concern in a food production setting. Ethanol, on the other hand, is a safer solvent that is commonly used in food processing and is generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA). Ethanol is also a polar solvent, thus more compatible with the polar environment of aqueous foods. The ether injection method is not commonly used for the encapsulation of vitamins in liposomes, basically because of its high toxicity [49].

3.1.4. Detergent Removal (Depletion) Method

The detergent depletion method is a mild process capable of producing highly homogeneous liposomes. This method is based on the formation of mixed micelles of detergents and lipids and the further removal of the detergent to form LUVs. The size of vesicles is based on the rate at which the detergent is removed from the formulation and the initial detergent to phospholipid ratio [50].

Detergents, such as sodium cholate, sodium deoxycholate, and octyl glycoside, are often used in the initial stages of liposome preparation to solubilize the lipids and to form a homogeneous solution. However, detergents can also destabilize the liposome membrane and lead to aggregation and fusion of the liposomes. To remove the detergent and to obtain stable liposomes, various methods are used. One of the most commonly used method is dialysis [51]. Using this method, the detergent-containing liposome dispersion is submitted to a dialysis step. This process is typically carried out over several hours to several days, depending on the amount of detergent and the desired degree of detergent removal [52]. Another method for detergent removal is the use of adsorbents such as Bio-Beads [53]. The obtained liposomes are, then, separated from the beads containing adsorbed detergent molecules by filtration or centrifugation, resulting in detergent-free liposomes [53].

Vitamin-loaded liposomes can also be produced by the detergent depletion method. In this case, vitamins should be solubilized with the lipids in a detergent-containing solution. Hydrophilic compounds are generally encapsulated more efficiently in liposomes produced by detergent removal than hydrophobic bioactives since these latter may be more prone to being removed along with the detergent during the depletion process. However, in the case of food applications, the choice of detergents used in the liposome production should be carefully considered; some safety concerns or regulatory restrictions may apply. Secondly, this process can be time-consuming and labor-intensive, and may not be easily scalable to commercial production levels. Some detergents such as Tween 20, Tween 80, and Brij-35 are considered safe for food applications, as they are approved by FDA and European Food Safety Authority (EFSA). Triton X-100 cannot longer be used for food applications; but its use in cosmetics is still feasible due to the regulation approved by several agencies. It is important to notice that the choice of the detergent can affect the properties of the resulting liposomes, including their size, stability, and encapsulation efficiency [45]. Therefore, it is necessary to carefully optimize the detergent concentration and preparation method to achieve the desired properties of the liposomes for the intended final application. Additionally, it is important to ensure that any residual detergent is removed from the liposomes during the detergent depletion process to avoid potential health risks [54]. When producing liposomes for cosmetic applications, it is important to choose a detergent that is safe for use on the skin and does not cause irritation or other adverse effects on humans.

3.1.5. Double-Emulsion Method

The double emulsion method, also known as the water-in-oil-in-water (W/O/W) emulsion method, is a technique for encapsulating both hydrophilic and hydrophobic molecules in liposomes [55]. Hydrophilic bioactives such as proteins are more suitable to be encapsulated using this technique due to the multiple aqueous phases involved in the emulsification process. In this context, the method involves two emulsions, one inside the other one [56]. The first step is to prepare a W/O emulsion. This is performed by mixing a small amount of an aqueous solution containing the hydrophilic compound of interest with an immiscible organic solvent, such as chloroform or toluene, and a lipid solution. The mixture is vigorously shaken to create small droplets of the aqueous solution surrounded by a lipid bilayer. The second step is to create a second emulsion by adding the W/O emulsion to a large amount of the aqueous phase containing a stabilizer, such as a surfactant or polymer. This is typically carried out by sonication or homogenization. The resulting W/O/W emulsion contains small droplets of the aqueous phase plus the hydrophilic compound, surrounded by a lipid bilayer, which in turn is surrounded by a second aqueous phase. Liposomes are formed after the removal of the organic solvent by stripping gas or vacuum pressure, which leads to the direct contact between the external and internal oil-water phases and the formation of the lipid bilayer [57]. The double emulsion method presents some advantages over the other liposome production methods such as high encapsulation efficiency of hydrophilic compounds, drug controlled release, and versatility. However, this process can be time-consuming since it requires several steps to form liposomes, besides the low yield, and the need for specialized equipments such as sonicators or homogenizers [39].

A modification of this method, named “freeze-drying of double emulsions”, has been efficiently used to produce liposomes characterized by improved stability and prolonged shelf-life. This technique involves three main steps: (i) the formation of a double emulsion by the emulsification of an aqueous phase with a lipid phase, followed by a new emulsification with an external aqueous phase; (ii) the freezing of the double emulsion to produce a frozen matrix of liposomes, which helps to stabilize the liposomes and to prevent aggregation or coalescing phenomena; and (iii) the freeze-drying process, in which the frozen matrix is submitted to freeze drying for the removal of water through sublimation under vacuum. This step transforms the frozen matrix of liposomes in a dry powder that can be easily reconstituted with water or other appropriate solvents. Some advantages related to this method include: (i) improved stability, making them suitable for long-term storage and transportation; (ii) preservation of structural integrity, thus maintaining their biological activity; and (iii) flexibility, as liposomes can be produced at different sizes and compositions depending on the final destination. However, this technique shows some drawbacks, such as the requirement for specialized equipments, the potential for loss of encapsulated compounds during the freeze-drying process, and some damage to the freeze-dryer apparatus due to residual solvents [58].

Li et al. [59] produced complex liposomes containing medium-chain fatty acids (MCFAs) and vitamin C using the double emulsion method followed by dynamic high pressure microfluidization. A mass ratio of 100:25:4 soybean phospholipids, cholesterol, and vitamin E, altogether with an ethanolic solution containing MCFAs, was used throughout the production. The procedure was followed by the injection of a small volume of twice-distilled water under vigorous stirring at 50 °C. The primary emulsion was formed after evaporation of part of the solvent under reduced pressure. The aqueous phase composed by twice-distilled water, Tween 80 and vitamin C was, then, incorporated into the organic phase. After agitation, the residual solvent was removed through rotary evaporation under reduced pressure. These authors obtained liposomes with a mean diameter of approximately 93 nm, encapsulation efficiencies of MCFAs and vitamin C of 49% and 64%, respectively, and a good stability over 90 days at 4 °C. In another study, Yang et al. [60] used the same protocol as Li et al. [59] to produce complex liposomes encapsulating MCFAs and vitamin C; but these authors added a freeze-drying step at the end of the process to increase vesicle stability. The reconstituted liposomes presented a mean diameter of 110 nm and an encapsulation efficiencies of MCFAs and vitamin C of 44% and 62%, respectively. Vesicles remained stable for 60 days at 4 °C. In a more recent study, Pattnaik & Mishra [61] produced a multivitamin (A, D, B₉ and B₁₂) liposome using the double emulsion technique and a mix of soy lecithin and vegetable oil blend, besides a polymer solution containing milk protein isolate and trehalose at different concentrations. Liposomes were stable, with size varying from 143 to 396 nm and zeta potential from -20 to -33.5 mV. Interestingly, these authors observed that hydrophilic vitamins showed lower entrapment efficiency than the hydrophobic vitamins (vitamin B₉, EE% = 78-97.57% and vitamin B₁₂, EE% = 96-99.9% vs. vitamin A, EE% > 99.9% and vitamin D, EE% > 98%) and stated that this behavior was probably due to the interaction between the lipophilic vitamins with the hydrophobic tails of phospholipids during freeze-drying, which might have caused a protective effect over the vitamins.

3.1.6. Hydration of Proliposomes

This method of production uses a powder mixture of dry phospholipids and bioactives, called proliposomes. Several techniques can be used to obtain these powders, including fluidized bed, spray drying, freeze-drying, coating of micronized sugars, milling, and supercritical techniques. Proliposomes are defined as dry, free-flowing powders that contain the bioactives to be encapsulated [62]. Upon hydration under appropriate conditions, MLVs are formed. Their solid form confers stability and offers advantages such as improved transport convenience, storage, distribution, and dosage. Proliposomes can be manufactured using industrialized procedures as tablets or capsules, which eliminates the stability problems of liquid liposomes and may increase the oral bioavailability of bioactives [63].

Spray drying is a commonly used technique in the pharmaceutical and food industries for the production of powdered materials. This method is widely used for the encapsulation of oils, flavors

and fragrances. Its large application is mostly due to its ability to evaporate moisture rapidly from a sample, maintaining a low temperature in the particles [64]. Wall materials such as polysaccharides and proteins are commonly used throughout the process [65]. In the liposome field, spray drying acts as a post-processing step to convert the liquid form of dispersions to a high stable solid form [66]. First, the liposome dispersion must be produced selecting the desired bioactive molecule by any method of interest. This solution is, then, atomized in small droplets using a spray nozzle and dried using a hot gas stream. The resulting powder is collected and can be used for further processing or packaging. Spray drying has several advantages for the production of bioactive-loaded liposomes. It is a fast and efficient method for producing large quantities of powdered material, and it can be easily scaled-up for commercial production [67]. The resulting powder is also stable and can be stored for long periods of time without degradation. However, there are some challenges associated with spray drying regarding the encapsulation of vitamins. This process can cause damage to the liposome structure, leading to a loss of the encapsulated material. Additionally, the high temperatures used during spray drying can degrade sensitive vitamins [68]. However, some recent studies on thermosensitive vitamins such as vitamin B₁, B₉ and vitamin B₁₂ showed that they may not experience significant degradation during the spray drying process depending on the wall material and process parameters [69,70]. The microencapsulation of lipophilic vitamins such as vitamin D using spray drying is rare due to the mandatory water-dispersed form that is needed for the processing. Also, the porosity of the resultant material can act as an advertisement about the risk of degradation of more sensitive materials due to oxygen exposure [68]. The use of lower temperatures or shorter drying times during the spray drying process may prevent early thermal degradation.

Spray drying was used to produce β -carotene-loaded proliposomes by Toniazzi et al. [71]. Phospholipids, sucrose and β -carotene were solubilized in anhydrous ethanol and treated by spray drying using an inlet temperature of 90 °C and outlet temperature of 85 °C, at a flow rate of 10 mL/min in a 1 mm-diameter spray nozzle with co-current airflow. Proliposomes were, then, rehydrated with deionized water and thickeners xanthan and guar gums were used as stabilizers. The MLVs produced by this method were useful to protect β -carotene over 95 days of storage with an EE% up to 96% when a 0.10% w/w mixture of xanthan and guar gums was incorporated during the hydration step.

3.2. Novel Methods

Novel methods for liposome production have been developed and improved over the years, with the aim of overcoming the drawbacks related to the traditional methods. Differently from the liposomes produced using the conventional methods, vesicles produced by these new methods are mainly unilamellar and show a more homogeneous size distribution (lower values of PDI). Therefore, post-processing techniques such as sonication or extrusion are rarely required. Moreover, conventional methods are based on the use of organic solvents and detergents, which can limit their use in food and cosmetics, and are often not environmentally friendly. Currently, research efforts around liposomes focused on the optimization of green technologies that can be scaled-up to industrial levels.

3.2.1. Heating Method

This method was first described by Mozafari et al. [73] and involves the hydration of phospholipids with a 3% v/v glycerol solution at increasing temperature up to 60 °C or 120 °C. The processing temperature depends on the absence or presence of Chol. The use of glycerol is due to its physicochemical characteristics such as water-solubility and non-toxicity, besides its ability to avoid sedimentation or coagulation of vesicles. Sterilization of the resulting liposomes is not required due to the high temperature already used during processing (120 °C), leading to cost reduction. The heating method has been improved over the years resulting in a new method called “the Mozafari method”, in which large-scale production of liposomes can be achieved without the need for the prehydration step of raw materials and without the use of toxic solvents or detergents [74]. Although vitamins are mostly thermosensitive, equally sensible molecules such as DNA are being incubated at room temperature with preformed liposomes produced by the heating method [73].

3.2.2. Membrane Contactor Method

In this technique, a porous membrane with a defined pore size is used to separate two fluid compartments [75,76]. The organic phase (generally ethanol + lipids) is flowed across one side of the membrane, while an aqueous solution is flowed across the other side. The two fluids come in contact at the pores of the membrane, and liposomes are formed thanks to the presence of water. Liposomes then diffuse tangentially through the pores of the membrane and are collected on the surface of the other side. The organic solvent is removed by evaporation under reduced pressure. This method offers some advantages over traditional liposome production methods. Firstly, it is a continuous process, able to be scaled-up, thus allowing the production of large quantities of liposomes. Secondly, it is a gentle method, with minimal shear stress, which reduces the potential damage to liposomes or to the encapsulated molecules. Thirdly, it offers a better control over the size and size distribution of the liposomes, as the pore size of the membrane can be precisely controlled [77]. Also, membranes can be regenerated by washing using a water/ethanol mixture.

This method was already used to produce vitamin E-loaded liposomes [78]. Vesicles were produced using 20-50 mg/mL of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) or Lipoid® E80, 5-12.5 mg/mL of a stabilizer (Chol, stearic acid or cocoa butter), and up to 5 mg/mL of vitamin E. Shear stress on the membrane surface ranged from 0.80 to 16 Pa. Mean particle size under optimal conditions was 84 and 59 nm for Lipoid® E80 and POPC liposomes, respectively. EE% up to 99% was obtained in MLVs produced using Lipoid® E80.

3.2.3. Electroformation

This method was developed by Angelova & Dimitrov [79] and consists of the formation of GUVs under electric fields by the hydration of a lipid film deposited on electrodes. This method relies on the ability of lipids to self-assemble in bilayer structures in the presence of an electric field. The electric field creates a potential difference across the lipid solution, causing the lipid molecules to move towards the electrode, where they self-assemble in bilayer structures. The electroformation process typically involves the following steps: (i) preparation of the lipid solution, typically by mixing lipids in a suitable organic solvent; (ii) deposition of the lipid solution on an electrode or a conductive substrate, typically made of glass, quartz, or indium tin oxide; (iii) application of an electric field to the lipid solution by using two electrodes, typically made of platinum or gold; and (iv) collection of the vesicles from the electrode surface and further purification using size exclusion chromatography, ultracentrifugation, or dialysis [80]. The main disadvantages of this method include its low production yield and limited scalability.

Vitamin E-loaded GUVs were prepared by Di Pasquale et al. [81] in which the influence of vitamin E on the membrane organization was corroborated by SANS and fluorescence microscopy. Samples were produced using 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and Chol at a 37.5:37.5:25 mole ratio and vitamin E at a mole fraction of 0.10.

3.2.4. Nanoprecipitation

This is a scalable method that allows the production of liposomes in a single step, without the necessity of homogenizing the vesicle size. The process involves the mixing of a lipid solution and an aqueous solution, where the lipid molecules join together spontaneously to form liposomes, as they become less soluble in the aqueous phase. The size of the liposomes can be controlled by adjusting the composition and processing parameters. Due to the self-assembly process, the resulting liposomes are characterized by a narrow size range and can be produced with high reproducibility [82].

Jash & Rizvi [83] prepared vitamin C-vitamin E-co-loaded coated liposomes by the nanoprecipitation method. In this case, polyethyleneglycol and acetic acid-sodium acetate buffer 6 mM at pH 4.5 were used as solvent and non-solvent, respectively. Polyanionic block copolymer Eudragit® S100 was used to coat the liposomal surface. The resulting solution was then added

dropwise to the acetic acid-sodium acetate buffer. The coated liposomes were concentrated by centrifugation. The main results showed that Eudragit® S100 protected the encapsulated payload from deleterious gastric environment and facilitated site-specific pH-triggered release in the simulated intestinal condition.

3.2.5. Microfluidics Method

Microfluidics is a set of techniques that use channels with micrometric cross-sectional dimensions (from 5 to 500 μm), to intermediate fluid flows. Its advantages include axial mixing governed by diffusion and continuous operation at low volumes [67]. Microfluidic techniques have been demonstrated to generate uniformly dispersed liposomes and to provide a direct control over liposome size through precise adjustments to either the volumetric flow ratio or total flow rate [84]. This process requires low Reynolds numbers (laminar flow) and a diffusive mass transfer. The formation of liposomes by microfluidics is described in detail in Carugo et al. [85]. In general, a stream of lipids dissolved in alcohol is pushed through the central channel of the device. The lipid stream is sheathed and crossed by two lateral streams of water phase, generally distilled water or an aqueous buffer. By adjusting the volumetric flow rate ratio between the lipid and water phase streams and the total flow rate, the size of the focused stream can be adjusted. Liposome formation in microfluidic chips is driven by the diffusion of various molecular species (mostly alcohol and water, but also lipids) at the liquid interface between the solvent and non-solvent phases. The lipids initially solubilized in alcohol diffuse into the water (and concurrently the water diffuses into the alcohol) until the alcohol concentration decreases to a critical level below the lipid solubility limit. The decrease in alcohol concentration causes the formation of liposomes through self-assembly of lipids. Alcohol and water reciprocal diffusion across the focused alcohol/water interface is thought to cause the lipid to precipitate, resulting in intermediate structures in the form of micelles, which subsequently form liposomes [85]. Despite its high versatility and flexibility, microfluidic approach has some main drawbacks related to the use of organic solvents, the requirement for sensitive mechanical agitation, and the challenges associated with large-scale production.

Dalmoro et al. [86] prepared vitamin D₃ and vitamin K₂-loaded uncoated and chitosan-coated nanoliposomes by a novel simil-microfluidic device. PC, Chol and each vitamin were diluted in alcohol, whereas deionized water was used as the hydration solution. Higher encapsulation efficiencies were obtained for vitamin D₃-loaded liposomes (EE% = 88 - 98%) and vitamin K₂-loaded liposomes (EE% = 95 - 98%) at chitosan concentrations of 0.01% and 0.005% w/v, respectively.

3.2.6. Dual Asymmetric Centrifugation (DAC)

The DAC method involves a unique form of centrifugation in which a vial containing the liposome mixture is rotated around both the main axis and its own center. Whereas the main rotation moves the sample material outward in response to centrifugal forces, the rotation of the vial around its own center moves the sample material inward due to the adhesion between the sample material and the vial. This inward transport of the sample material is effective if the material has sufficient viscosity and adhesion to the vial material. The vesicular phospholipid gel that is formed after DAC processing can be then diluted to form liposomes. These gels can be particularly suitable for obtaining high liposome content to be applied in products such as creams, lotions or hydrogels [87]. The DAC method has also shown to produce a highly uniform population of liposomes with improved drug release properties compared to other methods [88]. Also, the equipment is of small size, easy to operate and offers good reproducibility. Liposomes with high EE% for water-soluble bioactives can be produced by this method, which does not require the use of organic solvents. Drawbacks of this method include the high shear force and the need for formulations that contain a high amount of phospholipids to increase viscosity and to form the vesicular gel [89]. To the best of our knowledge, there is no literature on the encapsulation of vitamins by the DAC method; however, it appears as a suitable method to encapsulate mainly water-soluble vitamins.

3.2.7. Crossflow Filtration Detergent Depletion

This method was developed by Peschka et al. [90] by combining the conventional detergent depletion technique with a cross-flow filtration system, providing a solution for a rapid detergent removal. The cross-flow filtration unit consists of a starting reservoir, a pump, a filtration device (membrane system), tubing with an integrated rotary slide valve, and a manometer to monitor the retentate pressure. By increasing the pressure on the membrane, the retentate pressure also increases, leading to the rapid removal of the detergent. The starting reservoir contains a mixed micelle solution that undergoes tangential filtration. The filtration unit includes a single membrane or membrane cassettes with a selected molecular weight cutoff. This cross-flow filtration process enables the production of liposomes with uniform size, homogeneity, and high stability [89]. Compared with other detergent removal methods, large quantities of liposomes can be produced in a significantly shorter time [91]. This method also allows for the production of sterile products by using sterile filtered mixed micelles and autoclaved devices. Additionally, the wasted filtrate can be recycled to minimize production costs [91].

3.2.8. Inkjet Method

The ethanol injection method has been modernized by the development of the inkjet method, which allows a precise control over liposome size and has the potential for scalable production [92]. Amphiphilic compounds are dissolved in ethanol and printed into an aqueous solution using an inkjet device, resulting in uniform liposome droplets in the range of 20 - 100 nm. Additionally, the inkjet method has been utilized to produce unilamellar lipid vesicles by transforming a bioactive solution into a jet of uniform droplets, which then collide with a solution containing a lipid bilayer membrane at the liquid/air interface. This sequence of events leads to the formation of each lipid vesicle, as the membrane undergoes deformation, collapse, and separation [93]. Although it is not currently used for the encapsulation of vitamins in liposomes, inkjets have been used to produce edible films for oral delivery of B-complex vitamins [94].

3.2.9. Supercritical Technologies

Supercritical fluid technologies have emerged as attractive methods for producing liposomes [56]. CO₂ is the most commonly used gas in supercritical fluid technology for several reasons, including low cost, non-toxicity, safety, and large availability. It is therefore considered a safe gas for use in food, pharmaceutical, and cosmetic applications [56]. Most processes of liposome production that uses supercritical CO₂ (sc-CO₂) involves dissolving a lipid mixture in a solution of CO₂ at high pressure and temperature. The CO₂ solution then acts as a solvent for the lipids, leading them to self-assemble and to form vesicles. The pressure and temperature of the CO₂ solution can be easily controlled to modify the size and shape of the samples. One of the key advantages of sc-CO₂-based processes is the absence of organic solvents, which can be toxic and difficult to remove from the final product. Besides being non-toxic, CO₂ readily evaporates, leaving no residue in the final product. Over the years, many supercritical technologies for liposomes production have been explored, and some of them are discussed below.

- Depressurization of expanded liquid organic solution into aqueous solution (DELOS-susp): this is a compressed fluid-based method that enables the reproducible and scalable production of nanovesicular systems with exceptional physicochemical properties, including uniformity, morphology, and particle size [95]. To prepare the samples, the reagents are initially dissolved in an organic solvent and subsequently treated using pressurized CO₂ until saturation. Then, the sample is rapidly depressurized from the bottom, experiencing a significant pressure drop from 10 MPa to ambient pressure. CO₂ molecules are released from phospholipid bilayers to temporarily disrupt them into highly dispersed phospholipids that undergo a rapid reorganization due to hydrophobic and van der Waals interactions, then packing themselves into liposomes [96,97]. This process produces small and uniform liposomes due to the high rate of depressurization and can be used to encapsulate thermo-sensitive materials since works under slight conditions [98]. Adaptations of this method have been proposed to remove the need for organic solvents or surfactants during production, some of them still resulting in SUVs at high storage stability [99].

- Depressurization of expanded solution into aqueous media (DESAM): this is an alternative dense gas technology, in which pressure requirements for liposome production are reduced to 4 - 5.5 MPa [100]. This method involves dissolving lipids in a solvent and pressurizing the solution with a dense gas to create an expanded lipid solution. The expanded solution is, then, released in a controlled manner into heated aqueous media, with pressure maintained through the addition of more dense gas. Care is taken to keep the pressurization and expansion below a certain threshold to avoid solute precipitation. The dense gas and solvent can be separated and reused. The resulting fine droplets disperse the lipid in the aqueous phase and improve component interaction, resulting in uniform liposome formation. The high temperature in the vesicle formation chamber can also aid in removing organic solvent from the product [100]. Liposomes produced by this method are mainly unilamellar, with sizes ranging from 50 to 200 nm, PDI not exceeding 0.29, and highly stable for periods of 8 months [142]. Recently, a continuous process named nano-carrier by a continuous dense gas (NADEG) technique appeared as an evolution of the DESAM method [101,102].

- Rapid expansion of supercritical solution (RESS): this is a technology currently used for micronization, co-precipitation and encapsulation. Lipids are dissolved in a mixture of sc-CO₂ plus 5 - 10% v/v of ethanol within an extractor. This primary dissolution in a co-solvent (ethanol) is strictly necessary because the natural phospholipids are poorly soluble in sc-CO₂ [103]. This solution is then released through a heated small nozzle in a low-pressure chamber and mixed with an aqueous solution. A rapid depressurization follows, and the pressure drop results in the lipids desolvation, which favors the formation of layers around the droplets due to solute supersaturation. Small particles are obtained from the gas stream [104,105]. This process produces small particles, with a uniform size. However, this method shows problems such as the difficult separation between vesicles and co-solvents during depressurization, which increases production costs [106]. Nevertheless, the RESS method is one of the most studied supercritical technologies for vitamin-loaded liposome production [107-112]. Han et al. [154] produced vitamin E acetate-loaded liposomes using an optimized RESS process without any organic solvent. Operating conditions were controlled using the single-factor analysis and the response surface methodology combined with Box-Behnken design. Samples were produced using polyvinyl acetate grafted phospholipids and vitamin E acetate in a 6.35:1 mass ratio and resulted in vesicles characterized by EE% = 93%, size of 247 nm, PDI of 0.295, and zeta potential of -42.55 mV. Jiao et al. [108] also used the Box-Behnken design to optimize the process parameters for the production of vitamin C-loaded liposomes using PC as wall material. Vesicles presented size of 270 nm, PDI of 0.254, zeta potential of -41.7 mV, and EE% of 75%. Sharifi et al. [111] produced ironized multivitamin-loaded liposomes containing lecithin, Chol, iron sulfate, and hydrophilic and hydrophobic vitamins (C and E, respectively) by a new venturi-based method called Vent-RESS, in which RESS was combined with Bernoulli principles. Liposomes with unimodal size distribution were obtained and EE% of bioactive molecules were improved when operating pressure increased from 12 to 18 MPa. The Vent-RESS process was also used in Jash, Ubeyitogullari, & Rizvi [110] to produce vitamin C-vitamin E-co-loaded liposomes in milk fat globule membrane phospholipids (MFGM) or sunflower phosphatidylcholine (SFPC). These authors verified that MFGM-based ULVs were smaller in size than SFPC-based ones (533 nm vs. 761 nm, respectively), with higher zeta potential (-57 mV vs. -37 mV, respectively).

- Supercritical reverse phase evaporation process (scRPE): this is a batch method developed by Otake et al. [113] that enables the efficient formation of liposomes using a one-step process. It acts similarly to the RPE method; but, in this case, sc-CO₂ substitutes the organic solvent. It involves the mixing of sc-CO₂, lipids, and ethanol and, then, the introduction of small amounts of water to generate a liposome dispersion through an emulsion formation. The procedure is carried out in a stirred volume cell at a temperature above the lipid phase transition temperature. As the aqueous solution is gradually added to the reactor, sc-CO₂ is released, resulting in the formation of liposomes upon depressurization [97,114]. Some years later, Otake et al. [115] optimized the scRPE method in a way that organic solvents were no longer needed. Zhao & Temelli [116] developed a similar process in which liposomes are formed by simple pressurization and depressurization of a sc-CO₂-lipid-

based aqueous solution. Liposomes size can vary from 100 nm to 1.2 μm by using this method, being SUVs or MLVs [117].

- Supercritical antisolvent (SAS): it involves a continuous spraying of an organic solution of phospholipids into sc-CO_2 , which serves as an antisolvent for phospholipid precipitation. As soon as sc-CO_2 contacts the liquid phospholipid phase, it quickly diffuses and divides the liquid phase in tiny droplets. At the same time, the organic solvent evaporates from the droplets as a consequence of the dissolution in sc-CO_2 . This mass transfer creates a supersaturation of phospholipids within the droplets, leading to the formation of small phospholipid particles through nucleation and aggregation. A pure CO_2 washing step can be performed to remove any trace of the organic solvent. Finally, spherical micro- or nano-liposomes are formed upon hydration with an aqueous buffer. Xia et al. [118] produced vitamin D_3 -loaded proliposomes using a SAS based technology. Hydrogenated PC was used as a lipid source. Conditions as $T = 45^\circ\text{C}$, $P = 8\text{ MPa}$ and 15% w/w lipid to vitamin D_3 ratio resulted in hydrated liposomes with an $\text{EE}\% = 100\%$ and an effective loading of 12.9%.

- Supercritical assisted liposome formation (SuperSomes): this is a continuous sc-CO_2 based process proposed by Reverchon and co-workers [119] in which, differently from the other methods, water particles are first formed by atomization and, then, covered by lipids dissolved in an expanded liquid mixture. The expanded liquid mixture is composed of phospholipids, ethanol and sc-CO_2 . The main idea is that lipids reorganize themselves around the water droplets forming inverted micelles, which tend to form liposomes as soon as they come in contact with a water pool located at the bottom of the vessel [120,121]. Process parameters, as water flow rate, injector diameter, phospholipid concentration, pressure, and gas to liquid ratio, have been constantly optimized during SuperSomes studies [122]. This apparatus has been efficiently used to encapsulate both hydrophobic and hydrophilic bioactive molecules [123-126]. The process is reproducible, therefore, allowing a good control of vesicle size distribution, and nanometric vesicles at high $\text{EE}\%$. Recent studies showed the feasibility of using SuperSomes apparatus to encapsulate vitamin D_3 into nanoliposomes [127,128]. In both studies, liposomes were produced using different ratios of soy hydrogenated and egg-yolk nonhydrogenated phospholipids. In Chaves et al. [127], samples produced using only egg-yolk phosphatidylcholine presented sizes of 132 nm. Also, a 10 mL/min water flow rate also led to highly homogeneous vesicles produced using a maximum of 20% of soy hydrogenated phospholipids with size of 218 nm, PDI of 0.253, and an $\text{EE}\%$ of 89%. In Chaves et al. [128], the effect of the incorporation of vitamin D_3 in curcumin-loaded liposomes was investigated. The addition of vitamin D_3 reduced the overall size of liposomes from approximately 220 nm to 130 nm; but also promoted a decrease in $\text{EE}\%$ of curcumin. The authors stated that this behavior was probably due to the competition between the two hydrophobic bioactives for the inner region of lipid bilayers.

- Aerosol solvent extraction system (ASES): this method was originally developed to produce a sterile product containing a biologically degradable carrier and a molecule embedded within it [129]. Using this technique, organic liquids are sprayed through a nozzle into a bulk of sc-CO_2 , which quickly extracts them and facilitates the rapid precipitation of solutes from the solution. After a short period of drying with circulating sc-CO_2 , the residual solvent can be easily removed from the precipitates. This is because the pressure reduction during particle formation and drying only affects the circulating supercritical gas phase containing the organic liquids. The ASES process has been successfully applied to liposome preparation, enabling the production of dry and reconstitutable pharmaceutical liposomes suitable for large-scale manufacturing [130,131].

- Particles from gas saturated solution (PGSS): this is a cutting-edge method that uses supercritical fluids to produce particles of a precise size. This process operates at mild temperatures, generally between $40 - 60^\circ\text{C}$, in an inert environment, and uses CO_2 and water as solvents [132]. It offers an alternative to conventional techniques such as spray drying and freeze drying. One of the main advantages of the PGSS-drying method is its efficient atomization, due to the rapid release and expansion of gas from the solution during depressurization from supercritical to ambient conditions. This technique also allows a drying at lower temperatures in the spray tower, reducing the exposure of the bioactive material to harmful high temperatures [133]. Moreover, it enables the use of carrier

materials that cannot be processed by conventional spray drying due to their low melting temperature.

4. Techniques for the Quantification of Vitamins into Liposomes

In 2018, the Center for Drug Evaluation and Research of Food and Drug Administration (CDER-FDA) developed a guidance summarizing the main characterization techniques that producers should consider when developing liposomes at industrial scale. In this context, the liposome formulation should be able to contain and retain as much as possible the molecule in the appropriate structure. Vesicles should be characterized in terms of morphology, surface characteristics, encapsulation efficiency, bioactive loading, particle size, phase transition temperature, in vitro release of the molecule, leakage rate from liposomes throughout shelf life, and integrity changes in response to changes in factors such as salt concentration, pH, temperature, or addition of other excipients.

Liposome composition, production method, and bilayer membrane rigidity can impact EE%. EE% is calculated as the percentage of bioactive inside the liposomes compared to the total amount of bioactive used. Separation of the free bioactive is necessary to quantify the amount within liposomes. Several techniques are used for separation, including chromatography, gravitation or centrifugation, dialysis membrane, and ultracentrifugation. Indirect and direct methods are used to determine EE%. Conventional techniques for measuring drug concentration include UV-Vis and fluorescence spectroscopy, enzyme or protein-based assays, HPLC, UPLC, LC-MS, and GC-MS. ESR and ^1H NMR can also be used for bioactive quantification. Table 3 summarizes some studies in which EE% tests were performed on vitamin-loaded liposomes. Solvents and other chemicals commonly used to disrupt the liposome membranes, besides the techniques carried out to quantify the amount of hydrophobic and/or hydrophilic vitamins released from vesicles, are also presented.

Table 3. Chemicals and methods currently used for the extraction and quantification of vitamins in liposomes.

Vitamin	Liposome type/size	Chemical used to demulsify/precipitate the liposomes	Method for vitamin quantification	EE%	Reference
Vitamin A	SUV	Methanol	HPLC	50.6-56.2%	[134]
Vitamin A	MLV	Chloroform /Methanol 2:1 v/v	UV-Vis spectrophotometry	99%	[135]
Vitamin A	MLV/SUV	Chloroform	HPLC	15.8%	[136]
Vitamins A, D, E, and K	n.s.	Tween 80	HPLC	20-100%	[137]
Vitamin B ₁	MLV	n-octyl β -D-glucopyranoside	Optical density	31.2%	[138]
Vitamin B ₁	n.s.	PBS	HPLC	97%	[139]
Vitamin B ₂	n.s.	Triton X-100	Indirect method (photolysis)	42.3%	[140]
Vitamin B ₅	n.s.	Chloroform/Methanol	Direct method (mass weight)	75%	[141]
Vitamin B ₉	n.s.	Ethanol	Ultrafiltration in centrifuge tube followed by HPLC	87.4%	[142]
Vitamin B ₁₂	MLV	Methanol/Water 1:10 v/v	HPLC	70%	[143]
Vitamin B ₁₂	LUV	-	Centrifugation followed by UV-Vis spectroscopy	27%	[33]
Vitamins B ₁₂ , D ₂ and E	SUV	Ethanol	UV-Vis spectrophotometry	56-76%	[144]
Vitamin C	n.s.	Ethanol	Dialysis followed by UV-vis spectrophotometry	77.9%	[145]
Vitamin C	n.s.	Methanol, chloroform, and ammonium buffer	HPLC	99.2%	[146]

Vitamin C	n.s.	Ethanol	HPLC	75.4%	[108]
Vitamins C and E	n.s.	Triton X-100 / DMSO	UV-Vis spectrophotometry	93-95%	[83]
Vitamins C and E	n.s.	Assay kit BC1235	Indirect method	98.5%	[147]
Vitamin C	SUV/LUV	Meta-phosphoric acid	Titration with indophenol solution	94.2%	[148]
Vitamin C	SUV/MLV	-	Gel permeation chromatography	5-16%	[149]
Vitamins C and E	MLV/MVV	Protamine sulfate solution followed by Triton X-100	Protamine aggregation method followed by UV-Vis spectrophotometry	12-88%	[111]
Vitamins C and E	MLV	Acetic acid and protamine solution	UV-Vis spectrophotometry	38%	[150]
Vitamin C	n.s.	-	Dialysis followed by UV-Vis spectrophotometry	100%	[151]
Vitamin D	MLV	Triton X-100	Ultracentrifugation followed by HPLC	61.5%	[152]
Vitamin D ₃	n.s.	Methanol	Ultrafiltration in centrifuge tube followed by HPLC	74%	[127]
Vitamin D ₃	SUV/LUV	Methanol	HPLC	90.2%	[72]
Vitamin D ₃	SUV/MLV	Water	Ultrafiltration in centrifuge tube followed by RP-HPLC	100%	[153]
Vitamins D ₃ and K ₂	SUV/LUV	Ethanol	UV-Vis spectrophotometry	98%	[86]
Vitamin E	n.s.	Methanol	Dialysis followed by HPLC	92.5%	[154]
Vitamin E	n.s.	Ethanol	UV-Vis spectrophotometry	98.6%	[155]
Vitamin E	n.s.	Chloroform/Methanol 2:1 v/v	UV-Vis spectrophotometry	78%	[156]
Vitamin E	n.s.	Water	Centrifugation followed by HPLC	94%	[157]
Vitamin E	n.s.	-	Centrifugation followed by UV-Vis spectrophotometry	83.8%	[158]
Vitamin E	n.s.	Chloride acid, Tween 80:ethanol and n-pentane	UV-Vis spectrophotometry	97%	[159]
Vitamin K ₁	n.s.	-	Ultracentrifugation followed by HPLC	79.2%	[160]
Vitamin K ₁	n.s.	Methanol	HPLC	3.4-154 µg/mg	[34]

5. Applications of Vitamin-Loaded Liposomes

5.1. In Food

Liposomes have become increasingly popular in the food industry for functional purposes due to their ability to increase bioactive dissolution rate and bioavailability, protect sensitive ingredients, improve stability during processing, storage and digestion, and to confine undesirable flavors. They have also been used to achieve controlled release to specific targets. The encapsulation of food bioactives using liposomes has been investigated, and there is a wide range of opportunities for research in real applications of liposomes in different food formulations. A proper application of bioactive-loaded liposomes in the food industry should involve excipient materials and bioactives that are generally recognized as safe (GRAS), fully incorporated within the liposomal structure, and

not reactive with the core ingredients. Liposomes can be used to encapsulate an aqueous phase in order to decrease the vapor pressure of a matrix, allowing the lowering of the water activity without decreasing the moisture content, thus preventing the growth of microorganisms in foods that contain nutrients such as proteins or sugars [161]. Studies on the evaluation of shelf-life and sensorial acceptance, as well as oral processing, digestibility, and bioaccessibility of encapsulated compounds in liposomes designed for food applications, should receive more attention in the literature.

Vitamins are not easily incorporated into foods. Liposoluble vitamins are hardly introduced into aqueous-based food formulations and are easily oxidized in the air. Most vitamins are also thermolabile and can be readily degraded after thermal treatments as pasteurization. On the other hand, the addition of vitamins into liposomes can protect their activity, besides increasing their absorption and bioavailability [86]. Some studies have been carried out in order to test the effects of the incorporation of vitamin-loaded liposomes in food matrices. Marsanasco et al. [150] produced SPC-based liposomes encapsulating vitamins E and C for orange juice fortification. The hydration of a thin film lipid method was used to prepare these vesicles. To substitute cholesterol, which is related to health issues as atherosclerosis and high blood pressure, the authors tested other molecules to increase the rigidity of membranes as stearic acid (SA) and calcium stearate (CaS). The lipid films were constituted of mixtures of SPC and SA or CaS in a 1:0.25 molar ratio and vitamin E from a stock solution, and were further hydrated with acetic acid 3% v/v containing vitamin C to form liposome dispersions. These dispersions were, then, added to orange juice samples, which were pasteurized at 65 °C for 30 min and stored at 4 °C. In another study, Marsanasco et al. [162] incorporated SPC:SA and SPC:CaS based vitamin E-folic acid-co-loaded liposomes to enrich chocolate milk. Vesicles were prepared using the thin lipid film hydration at a 1:0.25 molar ratio of SPC and SA or CaS. Vitamin E was incorporated in the lipids before hydration step; whereas folic acid was incorporated in the hydration buffer. Samples were pasteurized and, then, added in a 1/100 ratio in chocolate milk kept at 4 °C. These authors observed a protective action of vitamin E over folic acid as oxidative stability of folic acid remained unchanged in the presence of vitamin E after pasteurization.

Banville, Vuilleumard, & Lacroix [152] produced vitamin D-loaded liposomes aiming to fortify Cheddar cheese. Vesicles were prepared by hydrating a proliposome mixture (Prolipo-Duo™) using a vitamin D solution. The resulting MLVs were then incorporated into raw milk, which was employed for the cheese manufacturing. The results showed that vitamin D-loaded liposomes promoted a higher final concentration of the vitamin in the processed cheeses than a commercial water-soluble vitamin D emulsion (Vitex-D). Wechtersbach, Ulrih, & Cigić [163] incorporated vitamin C-loaded liposomes based in DPPC and Chol in both apple juice and fermented milk that underwent through pasteurization at 72 °C; they observed higher retentions of vitamins after encapsulation than when added in free form. These authors also verified a higher retention of the molecule in vesicles containing DPPC and Chol than those containing DPPC alone. Mohammadi, Ghanbarzadeh, & Hamishehkar [164] produced vitamin D₃-loaded liposomes aiming at beverage fortification. Samples were produced using SPC and Chol at various concentrations by the thin film hydration method. The resulting MLVs were subjected to homogenization at 60 °C for 15 min and to probe sonication at 20 kHz and 70% of strength in order to decrease their size and turn them undetectable by the human eye. Size of vesicles were up to approximately 87 nm after 30 days of refrigerated storage for samples produced at a 50:10 PC:Chol ratio with an EE% up to 95%. Vitamin D₃-loaded liposomes were also incorporated into dark and white chocolate by Didar [165,166]. The ethanol inject method was used to prepare these liposomes based on phospholipids and Chol. A concentration of 5 µg/10 g of vitamin D₃ in chocolate was fixed for free form-enriched and encapsulated enriched samples. The author verified a reduction of vitamin D₃ after 60 days of storage in samples fortified with the free form of the vitamin instead of encapsulation in liposomes. Also, no impacts on rheological, colorimetric or sensory characteristics of chocolate were observed in the samples enriched with liposomes. Another study regarding the enrichment of foods using vitamin D₃ was conducted by Chaves et al. [167], in which pineapple yogurt was fortified with nanoliposomes coencapsulating vitamin D₃ and curcumin. The nanovesicles were produced using different ratios of purified and unpurified phospholipids by the hydration of proliposomes, which in turn were produced by coating of micronize sucrose.

Dispersions were produced upon hydration of proliposomes, which were stabilized by xanthan and guar gums at a 0.02% w/v concentration. Liposomes were incorporated into formulated pineapple yogurts at 5% v/v and maintained under refrigeration at 4 °C. These authors verified that increased concentration of purified phospholipids were beneficial to increase the EE% of both bioactives in dispersions. Liu et al. [168] produced vitamin C-loaded nanoliposomes by self-assembly of alginate and chitosan to enrich mandarin juice. Samples were prepared by the thin lipid film hydration method combined with microfluidization at 120 MPa for 2 cycles. The main ingredients included SPC, Chol, Tween 80 and vitamin E at a 6:1:1.8:0.12 ratio. Samples were rehydrated using PBS 0.05 M at pH 7.4 containing 5 mg/mL of vitamin C. Vesicles were, then, coated with alginate and chitosan using electrostatic deposition. Finally, liposomes were incorporated into mandarin juice samples and pasteurized at 90 °C for 10 s and stored at 4 °C. Liposome coverage by alginate and chitosan was responsible for an increase in size and a reduction in zeta potential of the samples, besides promoting aggregation of vesicles at day 90. However, lipid peroxidation and vitamin C release were lower than non-coated samples, suggesting a protective effect. It is worth mentioning that several other bioactive molecules that are not vitamins have been incorporated into liposomes and tested in food matrices, such as nisin in Minas frescal cheese [170], rutin for chocolate coating [171], quercetin in cornstarch [172], betanin in gummy candy [173], baicalin in mushrooms [174], basil essential oil in pork [175], clove oil in tofu [169], ginger extract in wheat bread [176], and fish oil in yogurts [177].

5.2. In Cosmetics

Nanotechnology-based approaches in cosmetics are growing exponentially with the aim of developing novel formulations that can confer aesthetic and therapeutic benefits to people [178,179]. In particular, such cosmetic formulations are referred to as “cosmeceuticals” when they have both cosmetic and medicinal functions [180]. Liposome-based nanoformulations are gaining particular interest since can represent a promising strategy to prepare antiperspirants, creams, lipsticks, deodorants, moisturizers, hair care products, etc., and can be also successfully used to deliver vitamins, like vitamin A, B₁₂, E, K, antioxidants (like coenzyme Q10, lycopene, carotenoids, etc.), and other bioactive molecules [46,143,181].

In the literature, *in vitro* and *in vivo* studies demonstrated the improved cosmeceutical effect of bioactives when incorporated into vesicles; e.g., in reducing pigmentation disorders, skin aging and solar exposure problems. These positive effects are due to the incorporation of the bioactives into vesicles that favors the penetration through the stratum corneum of the skin and promotes their activity at the damaged site [182,183]. For instance, the loading of vitamin C into liposomes, by facilitating its percutaneous transport, can increase its concentration in the dermis more than five times compared with the application of the pure vitamin [184]. The encapsulation of vitamin B₁₂ in vesicles and its slow release enhances the bioactive absorption and bioavailability, in addition to the protection of the vitamin from the degradation induced by heat, light, air, and a no correct storage [143]. Jiao et al. [142] to improve vitamin C (VC) and folic acid (FA) stability, co-loaded these antioxidant molecules in liposomes (VCFA-Lip) and chitosan-coated liposomes (CS-VCFA-Lip). The mean vesicles size of VCFA-Lip and CS-VCFA-Lip was 138 nm and 249 nm, respectively; whereas the encapsulation efficiency of both drugs in CS-VCFA-Lip was much higher than that measured for VCFA-Lip. Moreover, the stability study revealed that the chitosan coating can efficiently improve the physical stability of VCFA-Lip. Dreier et al. [185] suggested that the main mechanism of liposomes action is their rupture and merging with the lipids in the stratum corneum. However, the effectiveness of liposomes in the skin delivery of bioactive is influenced by numerous parameters, such as their size, surface charge, number of layers (uni- or multi-lamellar vesicles), and the flexibility of the bilayer. Touti et al. [186] showed that systems at higher lipid bilayer flexibility can effectively pass through narrow constriction of the skin; whereas multilayer liposomes at high flexibility and larger dimensions can penetrate much deeper into the skin than smaller vesicles. This behaviour is due to the “abrasion” of liposomes and the loss of their outer layers during the diffusion in the skin. On the other side, the ultradeformable lipid carriers with sizes below 150 nm would be able to penetrate the skin and to release an active molecule in the deeper layers without allowing adsorption

on keratinocytes. Transdermal transport through the skin can also be improved by the positive charge of phospholipid carriers: e.g., the stronger electrostatic interactions with the skin surface favor the accumulation of liposomes on it or within hair follicles, through which they can diffuse more easily. Choi et al. [187] showed that flexible cationic liposomes can promote a larger penetration than their less charged counterparts.

6. Discussion and Concluding Remarks

From the data and methods presented in this review, emerged that the encapsulation of vitamins in liposomes is a relevant field to be explored, both for food and cosmetics. Several methods of liposome production, from the most conventional to the highly innovative ones, have been described and the application of liposomes in vitamin incorporation was found and contextualized. However, the scale-up and the use of liposomes in food and cosmetic products are in distinct technological stages, as in cosmetics there are already products on the market.

It is quite important to highlight that the number of publications in this subject has been constantly growing over the last 20 years; but particularly in the last ten years, according to Figure 1.

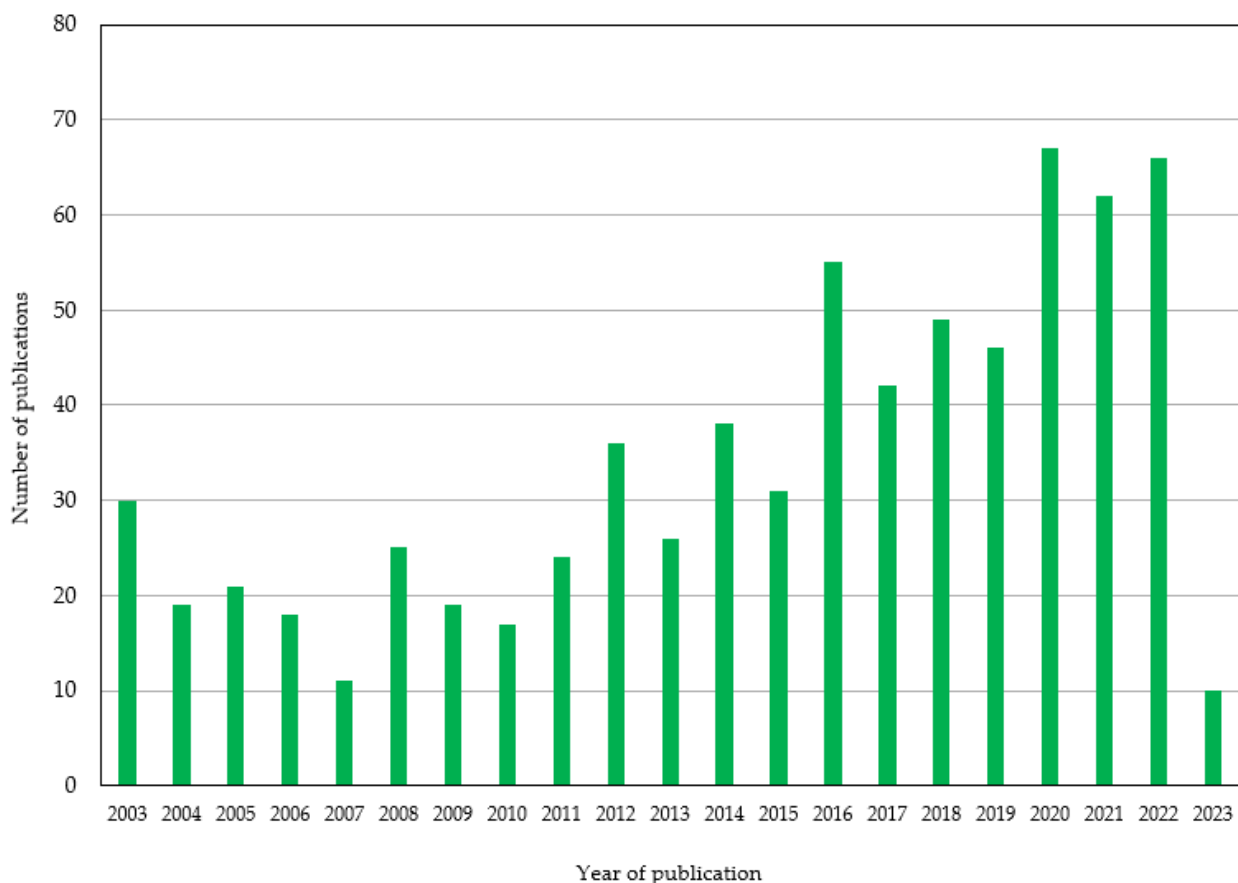


Figure 1. Evolution of the publications about the encapsulation of vitamins in liposomes over the last 20 years.

In the case of food, the oral route is the main challenge to be faced, mainly due to the known sensitivity of liposomes to the gastric environment. Most vitamins should be released at the end of the gastric phase or only in the intestine to be absorbed, which often requires the liposomes to be gastroresistant. This is one of the main challenges in vitamin-loaded liposomes that need to be overcome, and some approaches have been tried, such as the coating using polysaccharides (chitosan and pectin, for example) [188,189]. However, studies about the digestion (both *in vitro* and *in vivo*) to determine the bioaccessibility and bioavailability of vitamin-loaded liposomes in food products are still scarce. Other challenges that can be cited for the liposomes to be used in foods are: (i) the high

price of phospholipids when considering a large scale; (ii) restrictions about the use of organic solvents, which is a limitation for the use of some of the most common methods to produce liposomes already scaled-up.

On the other hand, as the main route for the application of vitamin-loaded liposomes in cosmetics is through the skin, the elasticity and flexibility of the vesicles are pointed out among the main challenges, together with their average size. Some important rewards come from the topical administration in comparison to the oral route, such as the possibility of lower fluctuations in plasma bioactive levels, site-specific delivery, avoidance of first-pass metabolism, and better patient compliance [190]. Also, as the skin is the first defensive barrier against external factors and prevents several bioactives from penetrating the underlying layers or going into systemic circulation, there are some limitations to deliver the active ingredient to the target site [191]. Ultradeformable liposomes, as transfersomes, ethosomes, and transethosomes are the new generation of elastic liposomes suitable for a more effective transdermal delivery of therapeutics, including vitamins [190]. Other challenges related to the use of liposomes in cosmetic products, including their use as cosmetics, are low drug loading, physical and chemical instability, and scarce reproducibility of the results [192].

Therefore, although both types of utilization (food and cosmetics) of vitamin-loaded liposomes are in different stages of technology maturity, the development of new technologies to face the challenges presented in this work can be mutually beneficial.

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