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Review article

Skin drug delivery using lipid vesicles: A starting guideline for their development

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ABSTRACT

Lipid vesicles can provide a cost-effective enhancement of skin drug absorption when vesicle production process is optimised. It is an important challenge to design the ideal vesicle, since their properties and features are related, as changes in one affect the others. Here, we review the main components, preparation and characterization methods commonly used, and the key properties that lead to highly efficient vesicles for transdermal drug delivery purposes. We stand by size, deformability degree and drug loading, as the most important vesicle features that determine the further transdermal drug absorption. The interest in this technology is increasing, as demonstrated by the exponential growth of publications on the topic. Although long-term preservation and scalability issues have limited the commercialization of lipid vesicle products, freeze-drying and modern escalation methods overcome these difficulties, thus predicting a higher use of these technologies in the market and clinical practice.

1. Introduction

The skin offers a very attractive alternative route for the administration of drugs for several reasons [1]. Firstly, it has several advantages over the main delivery routes such as oral or parenteral. Transdermal Delivery of Drugs (TDD) has good acceptance and compliance by patients [2], it is usually painless and does not need aseptic materials, techniques, nor specialised personnel for its administration [3]. Secondly, its large surface available -an area of approximately 20000 cm^2 in adults- make it the biggest entrance to the body [4]. Finally, drug doses can be reduced as the first hepatic passage and the acidic stomachal environment are bypassed, avoiding several undesired effects [5]. In comparison, other alternative routes like sublingual, buccal and rectal mucosae have been shown as unpredictable routes with many more limitations [6].

As a result of skin structure, the application of drugs on the skin may have two aims: local therapy (dermal) or transdermal delivery of drugs [7]. Dermal delivery refers to the process of mass transport of drugs applied on the skin to various skin strata, while the transdermal delivery concept implies drug absorption through each skin layer and drug access to the microcirculation and its subsequent systemic distribution [8]. Therefore, the application of a drug onto the skin can pursue different goals: a) remain on the skin to protect the organism or fight against alive entities existent on the skin surface (*e.g.* sunscreens, repellents or antifungal/antibacterial products) [9]; b) treat different skin appendage disorders (antiperspirant or infections) [10]; c) treat different affections of the *stratum corneum* and viable epidermis (emollients, exfoliants, anti-inflammatories, antihistaminic drugs, *etc.*) [11]; d) modify the skin barrier function to improve the penetration of other drugs [12]; and e) transdermal drug delivery [1].

1.1. A brief review of skin functions and structure

The skin is the interface which separates the body and the environment. In consequence, the main function of the skin is to offer a robust barrier against the penetration of external xenobiotics, substances, allergens, and microorganisms [13]. In addition, the skin plays other important roles, such as homeostasis maintenance -preventing the dehydration of the body- [14], and protection from the harmful effects of ultraviolet radiation [15]. Finally, this tissue presents different types of receptors sensitive to pressure changes, pain and temperature, which are essential to achieve a proper interaction with the environment [16,17].

As mentioned above, the skin is the largest human organ that represents 10% of the total weight. Its thickness varies from 0.05 to 2 mm depending on the anatomical region [18]. It can be divided into three

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differentiated layers: Epidermis, Dermis and Hypodermis.

The Epidermis is the external layer of the skin, and it can be subdivided into four stratums: basale, spinosum, granulosum and corneum. The main features of each stratum are summarised in Table 1. Keratinocyte is the main cell-type present in the Epidermis layer, it is created in the *stratum basale* and migrates progressively to the next layers, giving rise to a permanent cellular renewal process [19]. Keratinocytes are surrounded by a medium with a low content of water. This amount of water decreases as keratinocyte differentiation progresses, and at the end, the most superficial layer is constituted by superposed dead cells -called corneocytes- [20]. For drug penetration and transdermal purposes, the *stratum corneum* is the most important layer as it exerts the greatest opposition to drug diffusion through the skin [21].

The Dermis is the vascularised and innerved layer located just below the Epidermis [22,23]. It is a network of proteins (mainly elastin and collagen) with notable elastic properties [24–26]. Fibroblasts [27], macrophages and leukocytes are the cells present in this layer [28]. The irrigation of this layer provides the nutrients to the dermal and epidermal cells and removes metabolites [29]. This process allows the systemic absorption of drugs after its administration, since every substance that reaches the dermis microcirculation is susceptible to absorption. The Hypodermis is the deepest skin structure and is often not considered part of the skin. It consists of a matrix made of fatty and connective tissues that connects the skin with underlying tissues and organs [30,31]. Its composition and extension vary depending on a range of factors such as gender, age, anatomical site, nutritional conditions and endocrine status [32].

The skin appendages are dermal-associated structures, such as sweet and sebaceous glands, hair and hair follicles [33]. All of them are the target of local therapies, for example, acne and alopecia affections [34,35]. The transfollicular route has often been ignored because hair follicles only occupy approximately 0.1% of total skin surface [36]. It has been demonstrated more recently that hair follicles can have an important impact on the transdermal diffusion of drugs, because their structure offers a thinner barrier between the external environment and dermal microcirculation [37]. In this sense, certain types of nanostructures can reach the bottom of the follicle and remain there as a drug deposit [38].

Current topical and transdermal applications for lipid vesicles aim to treat certain skin conditions, such as aged skin, burns or with regenerative purposes. In these cases, the cellular composition of the skin is altered. In aged skin, the dermis and epidermis become thinner, more dehydrated, and the epidermal and dermal cell number decreases. Keratinocytes suffer morphological changes and corneocytes increase in size. There is also a decrease in mast cells and fibroblasts, which leads to elastin degradation and reduction of hyaluronic acid and collagen [39]. In case of burns, the actual skin state will depend much on the severity of the burn, including cell death, destruction of all skin layers, and important changes on the transepidermal water loss [40]. Besides, the structural function of collagen is loss by heat denaturalization [41]. When skin is following a regeneration progress, it experiments a keratinocyte migration from the dermis to the wound. In addition, immune responses increase the number of phagocyte cells in the wound, such as macrophages, monocytes and dendritic cells to avoid infections [40]. These structural changes should therefore be considered when designing the different delivery systems to treat each condition.

The aim of this review is to provide a guideline for the preparation of lipid vesicles. It contains the more relevant production methods of liposomes and its related vesicle derivates, characterization methods and desirable key properties for transdermal drug delivery as size, polydispersity index, zeta-potential and stability among others. The usual *in vitro* methods used for the pre-screening, drug permeation and permeability studies are also presented. We have also included a brief section discussing the importance of these vesicles, according to the number of published articles and summarizing the ongoing and finalized clinical trials using these types of nanoparticles.

1.2. Topical administration of drugs

The access of any substance from the surface to the deeper skin layers follows a passive diffusion process. Two possible routes have been theorised as pathways: the appendageal and the transepidermal routes (Fig. 1) [46]. In the first one, the drug uses the hair follicles and glands to diffuse near the dermal vessels. The main limitation of this route is that those structures represent a minimum proportion of the total skin area and are limited to particles with an appropriate size (< 500 Da) [37,47]. The transdermal route is subdivided into two variations: the transcellular route (the molecule passes through the cell and intracellular matrix) and the intercellular route (the molecule diffuses through the lipidic matrix) [48]. Although the intercellular path is longer compared to the transcellular one, it is generally accepted as the main route [49].

This diffusion process is obviously affected by the molecule properties, which conditions its suitability as a candidate for TDD. In these terms, an intermediate partition coefficient between vehicle and membrane [50], a low degree of ionisation [51], and a molecular weight less than 500 Da have been defined as the ideal characteristics [52]. Furthermore, the integrity of the skin has a key role in cutaneous absorption, since damaged tissues facilitate the absorption process enormously.

Transdermal absorption of substances is a multistep process, where five stages are distinguished [2]: i) Partition of the drug from the vehicle into the *stratum corneum* ii) Penetration of a molecule into the *stratum corneum*; iii) Diffusion from the *stratum corneum* into the viable epidermis; iv) Partition from the viable epidermis to the dermis; v)

Table 1
Main features of the different skin layers.

	Skin Layer	Properties	Function	Ref.
Epidermis	*Stratum corneum*	-20 to 25 layers of dead and cornified cells	-Prevention of external entities entrance to thebody	[21,42]
	Stratum granulosum	-Thin layer composed of keratinocytes which contains keratin granules -Cells are rich in ceramides	-Contains important proteins and enzymes (Filaggrin, Involucrine, Loricrine) -Waterproof task	[43]
	Stratum spinosum	-Thicker layer of cells -Contains Langerhans cells	-Immunological response	[44]
	Stratum basale	-Single layer of keratinocytes which contains stem cells -Contains melanocytes and Merkel cells	-Responsible forkeratinocyte division and proliferation (peeling process) -UV protection by melanin production -Sensory perception	[45]
Deeper skin layers	Dermis	-Fibroblast is the main cell type in thislayer -Composed ofcollagen and elastin -Vascularised and innervated -Location of skin appendages	-Mechanical support of the skin -Contribution to skin elasticity -Supplies nutrients to the dermis -Thermoregulation	[26,27]
	Hypodermis	-Connective and adipose tissue -Not considered part of the skin	-Anchors skin to the different tissues and organs -Protective structure and insulator	[30,31]

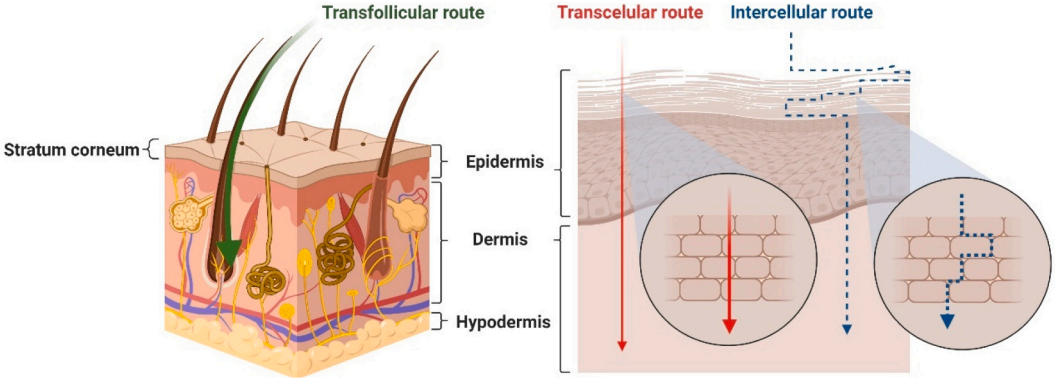

Fig. 1. Illustration of the drug diffusion routes through the skin.

Access to the systemic circulation. As a consequence of this complex process, it is difficult to suggest a biophysical model that considers all the events involved. Nevertheless, diffusion through the *stratum corneum* or full-thickness epidermis process can be modelled to estimate parameters that characterise the process [53]. The typical plot of a permeation study presents two phases: non-steady and steady states (Fig. 2).

Fick's diffusion laws describe the process in the steady state, and they establish that the change of the concentration as function of time is proportional to the gradient variation (2), according to the following equation (Eq. (1)):

$$\left(\frac{\delta c}{\delta t}\right) = D\left(\frac{\delta^2 c}{\delta x 2}\right) \tag{1}$$

The Scheuplein equation is an approximation, often used when *in vitro* models are performed under infinite dose and skin conditions. The amount of the drug that diffuses through the barrier is described by the following equation (Eq. (2)) [54,55]:

$$Q(t) = A \bullet P \bullet L \bullet C \bullet \left[D \bullet \frac{t}{L^2} - \frac{1}{6} - \frac{2}{\pi^2} \bullet \sum_{n=1}^{\infty} \frac{(-1)^n}{n^2} \bullet \left(\frac{-D \bullet n^2 \bullet \pi^2 \bullet t}{L^2} \right) \right] \tag{2}$$

Where $Q(t)$ is the amount of the drug which diffuses at a given time (t); A is the diffusion surface area (cm^2); P represents the partition coefficient of the permeant between the membrane and the donor vehicle; L is the membrane thickness; D is the diffusion coefficient of the permeant drug in the membrane; and C is the concentration of the permeant (μg/mL) in the vehicle (or the donor).

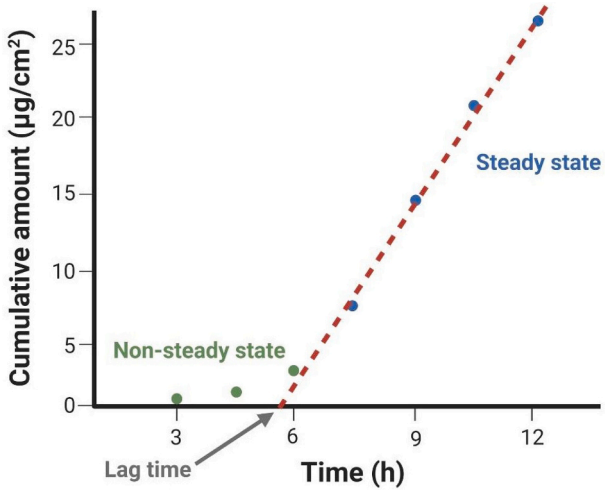

Fig. 2. Typical plot obtained from a drug permeation through the skin *in vitro* study in Franz diffusion cell set-up, with infinite dose.

Eq. (2) can be transformed to determine permeability coefficient (Kp) and lag time (tL) by substituting $P{\cdot}D/L$ and $L^2/6D$ for Kp and tL, respectively (Eq. (3)):

$$Q(t) = A \bullet Kp \bullet C \bullet \left[t - tL - \frac{12 \bullet tL}{\pi^2} \bullet \sum_{n=1}^{\infty} \frac{(-1)^n}{n^2} \bullet \left(\frac{-n^2 \bullet \pi^2 \bullet t}{6 \bullet tL} \right) \right] \tag{3}$$

The linear regression of the experimental cumulative amount data *versus* time offers the estimation of Kp and tL. Lag time is calculated as the intercept of the slope to the abscissae axis. Permeability coefficient and maximal flux per surface unit, $Jmax$ (μg/cm^2/h), can be calculated from the linear regression of the slope according to the equations (Eqs. (4) and (5)):

$$J = \frac{m}{A} \tag{4}$$

$$Kp = \frac{J}{C} \tag{5}$$

Where m is the slope of the linear part from the cumulative amount *versus* time; A is the diffusion surface area (cm^2); C is the concentration of the drug in the donor drug site.

2. Transdermal delivery systems

When talking about drug delivery *via* the skin, the most challenging aim from a technological point of view is the achievement of a systemic action. There are several well-known examples of treatments that follow this approach, specifically hormonal (estradiol) [56,57], pain (fentanyl) [58], and smoking cessation (nicotine) therapies [59,60]. However, as mentioned before, the main skin function is to prevent the entry of exogenous substances into the body. This barrier function is mainly achieved by the *stratum corneum*, due to its lipophilicity and the great cohesion between the cornified cells [61]. Transdermal Delivery Systems (TDS) are a plethora of strategies and resources that aim to increase transdermal drug absorption by modifying the skin barrier function or changing the physicochemical properties of molecules to make them optimal for this goal [62,63]. Transdermal drug permeability enhancement can be achieved by two approaches: physical and chemical methods [64]. The ideal TDS must present the following properties [48,65,66]: a) non-toxic, non-irritant or non-allergenic; b) quick action and predictable duration; c) pharmacologically inert; d) ease removal; e) allow rapid restorage of skin properties after its removal; f) compatible with excipients and drugs; g) comfortable and cosmetically acceptable; and h) inexpensive. Unfortunately, no TDS presents all these properties together to date. The main advantages and disadvantages of TDS are summarised in Table 2.

Nanotechnology aims to develop a huge variety of systems and

Table 2
Advantages and Disadvantages of Transdermal Delivery Systems (TDS) to enhance Transdermal Drug Delivery (TDD).

	TDS	Advantages	Disadvantages	Ref.
Chemical methods	Alkanes, Azone, Pyrrolidone, Urea, Fatty acids, Esters, Alcohols, Surfactants, Terpenes	High efficiency in combination with low sized molecules	-Inability to locate their effects only on the *stratum corneum* -Skin reactions (irritation, inflammation, and erythema) -Low efficiency in combination with macromolecules	[67–69]
	Prodrugs	-Higher drug stability -No degradation in the skin	Size increase can reduce its permeability through the skin	[46,70]
	Microsystems and Nanosystems	Possibility to achieve drug release in the first layers of the skin or a transdermal drug delivery	High-sized systems cannot improve drug diffusion through the skin	[71]
Physical methods	Iontophoresis, Sonophoresis, Electroporation and thermal methods	-Rapidly responsive molecular transport -Control of transport magnitude	-Expensive devices -Inability to locate the effects only on the *stratum corneum* -Intense skin reactions (irritation, inflammation, and erythema)	[72–76]
	Jet Injectors	Control of drug depth deposition	-Possible incorrect dosage and skin damage, pain, and infections	[77,78]
	Microneedles	Bypass the *stratum corneum*	-Expensive production compared to other methods -Difficult use for local skin treatments -Time that micropores remains open still unclear	[79,80]

entities ranging in the nanoscale, from 1 to 1000 nm [81]. One of the most important fields of application is nanomedicine, and more specifically, drug delivery [82]. In the 1960s, liposomes were designed as nanovehicles with specific programmed functions and precursors of several current nanoparticles [83,84]. Not long after, variations in structural components were suggested, as in the case of transfersomes and ethosomes, in order to improve their properties and achieve better results [85,86]. Nowadays, these structural variations are still emerging to fulfil the needs and demands of the pharmaceutical industry and the clinical practice [87–89].

3. Lipid vesicles: liposomes, transfersomes and ethosomes

Lipid vesicles are non-toxic and biodegradable biomembranes obtained artificially, that have gradually evolved over time and been successfully applied as drug carriers for controlled, targeted, and enhanced drug delivery [90]. Their application to TDD is highly attractive, because their lipophilic nature allows them to incorporate poorly soluble drugs in aqueous media and improve the penetration properties of drugs through the skin [91,92]. Other advantages have also been documented, such as increased stability of photosensitive molecules [93], lower drug degradation [94], and depot release [95].

3.1. Composition and structure of lipid-based vehicles

3.1.1. Components of lipid vesicles for transdermal delivery of drugs

Liposomes, transfersomes and ethosomes are mainly composed of phospholipids, and they present a lipid bilayer structure, similar to cellular membranes [96]. The amphiphilic character of phospholipids, as a result of their hydrophobic tails and hydrophilic heads, allow them to spontaneously disperse in aqueous medium and incorporate both hydrophilic and lipophilic drugs (Fig. 3) [97]. Variations in polar head and nonpolar tail functional groups lead to a broad variety of phospholipids that can be used in vesicles production. Phosphatidic acids (DMPA, DPPA, DSPA and DMPG), Phosphatidyl glycerols (DPPG, DSPG, POPG, DMPE), Phosphatidyl ethanolamines (DPPE, DSPE, DOPE, DLPC) and Phosphatidyl cholines (DMPC, DPPC, DSPC, DOPC) are the common types used in drug delivery, which condition to a certain degree the properties of the developed systems [98]. For example, phosphatidyl cholines tend to form permeable, but less stable, bilayers, whereas acyl chains-based phospholipids form more stable bilayers but more rigid

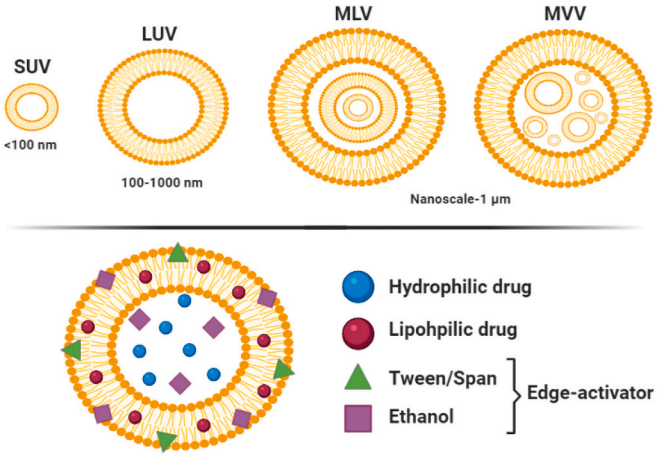

Fig. 3. Lipid vesicle classification according to size and number of lipid bilayers. Location of liposomes, transfersomes or ethosomes components in the lipid vesicle structure.

structures [99]. Lipid vesicle composition often includes stabilising agents to produce systems like cell bilayers, with cholesterol being the stabiliser *par excellence*. The phospholipid/cholesterol ratio varies from 0 to 25% *w*/w and depends on the vesicle application purposes [100]. On one hand, it is well documented that the entrapment efficiency of lipophilic drugs decreases if the percentage of cholesterol increases, since they occupy the same space in the lipid bilayers [101]. On the other hand, the increase in cholesterol content produces an increase of vesicle size and Polydispersity Index (PDI), probably as a consequence of the capacity of stabilising higher structures [100]. Finally, cholesterol can influence vesicle rigidity and, consequently, drug release and leakage. In general, the higher the cholesterol content, the higher the bilayer rigidity and lower the vesicle flexibility [102], and therefore the more flexible the vesicles, the higher the drug release and leakage [103].

The other key factor for deformability of the lipid vesicles is to include edge-activators in their composition, which leads to the obtention of transfersomes [104]. The most common edge-activators are surfactants: tweens®, spans®, bile salts (sodium cholate and sodium deoxycholate), and dipotassium glycyrrhizinate [104–107]. Usually,

those components are a single chain surfactant which causes a destabilisation of the lipid bilayers and increases its fluidity and elasticity by lowering the interfacial tension [108]. These surfactants produce micelles if they are present in concentrations greater than the micellar critic concentration [109]. However, when the molar relation phospholipid/surfactant is optimal, which happens at low surfactant concentrations, they are incorporated in the lipid vesicle structure, conferring interesting properties to the ultraflexible or ultradeformable vesicles. For instance, 15% *w*/w has been revealed to be the more efficient proportion in the case of non-ionic surfactants as tween or span for transdermal delivery purposes [110,111]. In addition, 25% *w*/w seems the most appropriate concentration in the case of sodium deoxycholate [111].

Ethosomal systems are composed of phospholipids, water, and ethanol, or other volatile alcohols at high concentrations (up to 50% *w*/w) [112]. However, the most suitable concentration of ethanol would range between 20 and 30% w/w to ensure the formation of closed multilamellar vesicles with good transdermal delivery results [86]. Characterization properties are also affected since size decreases with the increase in ethanol concentration [113].

The new designs of lipid vesicles for dermal and transdermal purposes are based on the incorporation of other components in an attempt to give additional properties to the formulations. Glycerosomes and propylene glycol-liposomes are vesicles with considerable amounts of glycerol or propylene glycol (10–30% in the water phase) [114,115]. Their objective is to confer extra flexibility to the bilayers, and hydrate the skin at the same time in the case of glycerosomes due to the humectant properties of glycerol [116]. Inspired by the features that hydrogels can offer to liposome delivery, such as an extra control of release, hyaluronosomes aim to incorporate a gelled nucleus made of hyaluronic acid, avoiding the possible inconveniences that polymeric matrices could produce, like an excessive retention of active ingredients or long-term stability issues [117]. Charged liposomes offer interesting features for skin delivery. It has been reported that anionic lipid vesicles show better penetration ability through the *stratum corneum* [118], and cationic liposomes have showed an improve stability during the storage, since the aggregation process can be hindered by the repelling effect of particle charge [119,120].

Other variants of lipid vesicles have been described, although they are not applied for dermal and transdermal purposes. These advanced-generation lipid vesicles are surface-decorated with stabilizers or specific ligands. Polyethylene glycol (PEGylated liposomes) is by far the most used steric stabiliser to avoid the clearance when lipid vesicles reach the bloodstream [119,121]. Although liposomes show a passive targeting through an enhanced permeability and retention (EPR) in cancerous cells [122], actively targeted lipid vesicles include ligands (peptides, proteins, antibodies, carbohydrates or other targeting biomarkers) conjugated covalently or non-covalently to the surface to localize the action on a specific cell type [123]. In the recent years stimuli-responsive lipid vesicles to different environments. These smart nanoparticles include in their composition a constituent that react to different stimulus -such as pH, heat, ultrasounds, light, redox potential, magnetic/electric fields, *etc.*- destabilizing the lipid bilayer's structure and triggering the release of the drug [124,125]. In addition, lipid vesicles have also been used to encapsulate medical and bioactive gases (bubble liposomes) [126,127]. Although these vesicles show very interesting features for parenteral delivery, the ligands hinder the passive transdermal diffusion of the drugs, so they have not been widely explored for these applications.

3.1.2. Structure of lipid vesicles and classification

Depending on the production method and composition, it is possible to obtain different types of lipid vesicles. According to the superficial charge given by the phospholipids, they can be classified as anionic, cationic, or neutral vesicles [128,129]. Moreover, the size and number of lipid bilayers are the usual parameters to characterise them. Multilamellar vesicles (MLV) are those that present more than one bilayer, which enclose the same number of aqueous compartments. Unilamellar vesicles have just one bilayer and a central aqueous core [130]. These well-differentiated environments make lipid vesicles a versatile TDS, as they can shelter drugs dissolved in the aqueous core or inserted within the lipid bilayer structure (Fig. 3) [131]. MLV are usually bigger in size, ranging between the nanometric scale up to 1 μm. When the number of bilayers is less than 4, they are called oligolamellar vesicles, and the drug entrapment efficiency is commonly higher than MLV [132,133]. A special case is multivesicular vesicles, where a large bilayer confines multiple vesicles inside it (MVV) [134]. Unilamellar vesicles are subclassified as small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV). SUV present a size lower than 100 nm, spherical shape and a homogeneous size distribution [135]. The entrapment efficiency of hydrophilic drugs is limited. On the contrary, LUV are larger vesicles (100–1000 nm) with a bigger aqueous core, which increases the capacity of encapsulating hydrophilic molecules [136].

3.2. Mechanisms of action of liposomes and ultraflexible vesicles

It has been reported that liposomes can effectively interact with cells by different methods [137]: a) absorption by specific interaction with cell-surface components, electrostatic and hydrophobic forces, b) endocytosis by phagocytic cells like macrophages and neutrophils; c) fusion and cytoplasm delivery by insertion of liposome bilayers into cell membranes; and d) exchange of bilayers components.

However, the mechanisms of action when applied on the skin for transdermal delivery purposes remain unclear. It is generally accepted that conventional liposomes fail to penetrate the skin layers and remain on the skin surface [138], acting as a kind of depot formulation [139]. The liposomes could directly interact with the skin and exchange the drug by the "collision complex transfer process" observed in other biological systems, or could release the free drug which is available to penetrate through the skin [140]. In the last case, liposomes perform not only a depot function, but they can induce structural changes in the *stratum corneum*, thus facilitating the drug absorption [141–143]. In the same way, a very similar mechanism based on the liposome insertion into the outer lipid layers of the *stratum corneum* can also explain the enhancement effect [144]. All these theories exclude the penetration of the intact liposome structure, which has been extensively assessed in different studies. For example, Dreier et al. combined the stimulated emission depletion microscopy (STED) and raster image correlation spectroscopy (RICS) to study the mechanism of action of conventional liposomes once applied to the skin. The images of tissue samples incubated with radio-labelled liposomes, obtained by skin cryo-section, revealed that liposomes do not remain intact beneath the skin surface and suggest that the liposomes do not act as carriers that transport their cargo directly through the skin barrier [145]. This poor performance of conventional liposomes has also been observed when crossing other biological barriers, such as the intestinal epithelia [146]. In Dreier's study, the penetration of conventional liposomes was compared to sodium cholate-based vesicles. As expected, the conclusion points out that fluorescent-labelled ultraflexible vesicles were able to deliver their content deeply. The improvement in the delivery obtained from transfersomes supports the existence of extra mechanisms involved for transfersomes. From its conception, transfersomes designers support that they can pass through the skin in a squeezing process helped by an osmotic gradient as a driving force, created by the difference in the water concentration between the skin surface (near to 15% in *stratum corneum*) and the viable epidermis layers (around 75%) [85]. Specifically, this skin environment is strong enough to push between 0.1 and 0.5 mg of lipidic material per hour and cm^2, which is substantially higher than the typical flux produced by concentration gradients [85,147]. As may occur with liposomes, the exact mechanism of transfersomes is the result of the combination of drug vectorisation, *stratum*

corneum disruption and penetration features of transfersome vesicles, bearing in mind that the destabilisation of *stratum corneum* produced by transfersomes is more intense due to the action of surfactants incorporated in the vesicles. One confirmation of these squeeze-mediated mechanisms is the study done by Cevc et al. [148]. In their study they assessed the fracture of liposomes during the transport through a semipermeable barrier, while transfersomes maintained their initial sizes. They also present the liposome and transfersome behaviour after its application on a murine skin model using Confocal laser scanning microscopy (CLSM), and confirmed the presence of intact vesicles in the blood stream of transfersome-treated mice by size exclusion chromatography. Similar findings have been obtained in several studies performed by other researchers in different types of ultraflexible vesicles, as Niu et al. and Manconi et al. [149,150].

Ethosomes are considered a variation of transfersomes because they have the same ability to penetrate intact through the epidermal layers. The underlying mechanisms of action are essentially similar, with little differences. Besides being a bilayer fluidiser, ethanol is also a chemical enhancer that promotes *stratum corneum* disruption by its interaction with the polar groups of skin phospholipids [151,152]. Nevertheless, it has been reported that the enhancement produced by an ethosomal formulation is greater than those produced by ethanolic drug solutions [153,154]. Moreover, the ethanol evaporation once applied on the skin surface could increase the effect of concentration gradient in the transdermal absorption process.

3.3. Production methods of lipid vesicles

A wide variety of lipid vesicle preparation methods are available, and each of them has certain advantages and disadvantages, and confer specific characteristics to the vesicles [155]. Its selection depends on different parameters, such as: a) physicochemical properties of the encapsulated drug and lipid vesicle components; b) effective therapeutic drug concentration and its toxicity; c) desired vesicle size, polydispersity index and surface charge according to its further applications; and d) batch reproducibility and scale up production.

3.3.1. Liposomes and transfersomes production methods

The specific molecular mechanism of liposome formation depends to a large extent on the production method used. The basic underlying principle is the hydrophobic/hydrophilic interactions between lipid and water molecules, regardless of the chosen methodology [156]. The application and transference of energy in form of heating, stirring, shaking or sonication contributes to the arrangement of the lipid molecules into bilayer vesicles.

The Bangham's method, also known as film method, is the first described procedure to obtain lipid vesicles (Figs. 4 and 5) [83]. It presents three clear steps, consisting of the preparation a phospholipid/cholesterol/edge activator organic mixture; removal of organic solvent using a rotary evaporator to produce a thin-film on the round-bottom flask wall; re-hydration with an aqueous solution above the phospholipid transition temperature to produce the lipid vesicles. Despite the method simplicity, the low entrapment efficiency in many cases, and the production of MLV heterogeneous populations makes further procedures to obtain useful lipid vesicles for transdermal purposes necessary [157]. Furthermore, different methods have emerged over the years to overcome the limitations of the original method (Table 3).

3.3.2. Ethosome production methods

In 1996 Touitou et al. introduced for the first time a lipid vesicle with ethanol as an edge-activator (Fig. 6) [86]. The proposed method consists of preparing a 20–50% *w*/w ethanolic phase at 30 °C and stirred at 700 rpm and its dropwise addition into an aqueous phase, using a constant rate of 12 ± 0.5 mL/h. The incorporation of the active ingredients can be done in the organic blend or in the aqueous solution, depending on its physicochemical properties. Then, when the proper volume of water is incorporated, the ethosomal dispersion is kept under stirring for 5 min and homogenised in terms of size and PDI by different methods. This procedure is defined as a classical cold method, and one of its main advantages is that it is done almost at room temperature, so it allows the preparation of thermo-sensitive drug-loaded lipid vesicles [151]. Alternative methods can be used to produce ethosomes in a more efficient way (Table 3), as it is for conventional lipid vesicles.

3.4. Purification methods of lipid vesicles

A mandatory step in the production process to obtain homogeneous final products is to remove the excess of vesicle components [137]. In addition, the elimination of non-entrapped drug fraction, these purification methods can remove the rest of non-incorporated components guaranteeing a high-quality formulation [167]. As the molecular weight of lipid vesicles is considerably higher than drugs and edge-activators, most of the methods are based on this size difference.

Even though these methods allow the vesicles purification, they have

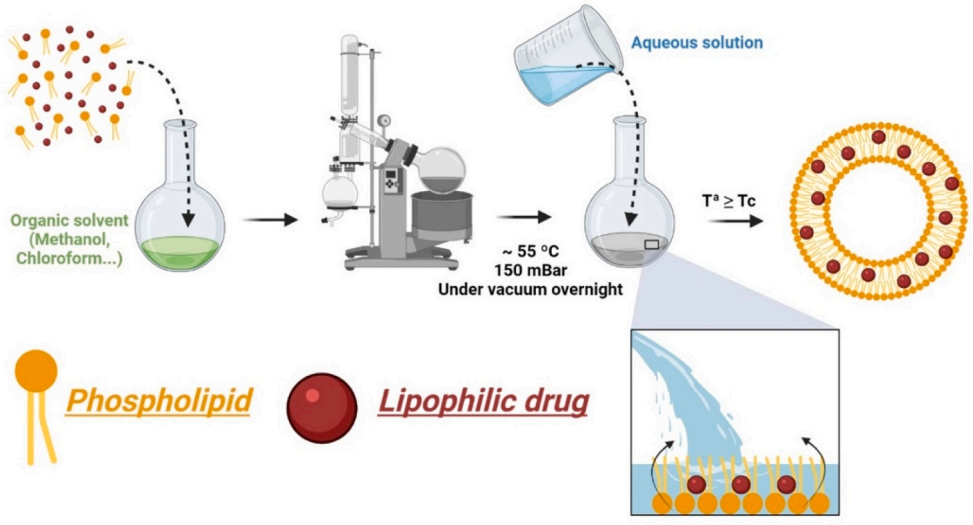

Fig. 4. Lipophilic drug-loaded lipid vesicle batch production. Lipophilic drugs are included in the phospholipid organic blend and re-hydrated with an aqueous solution.

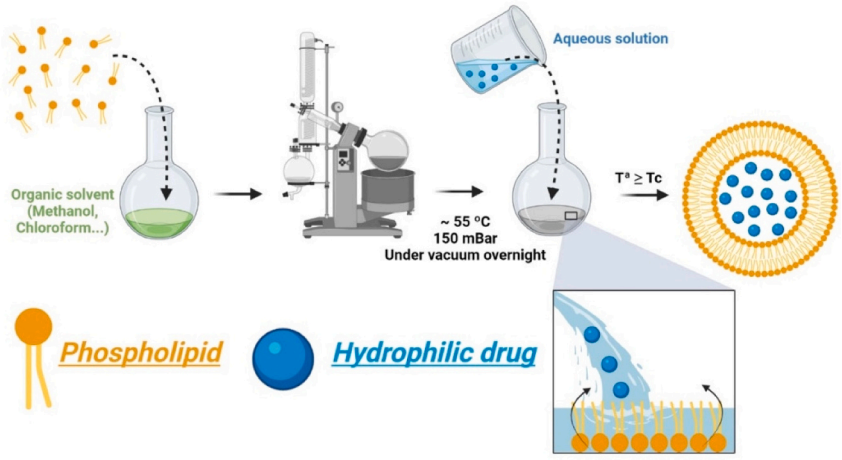

Fig. 5. Hydrophilic drug-loaded lipid vesicle batch production. Hydrophilic drugs are included aqueous solution for re-hydrating the phospholipid thin-film.

Table 3
Basic lipid vesicle production methods.

Method	Lipid Vesicles	Procedure	Outcome	Ref.
Thin-Film (Bangham's method)	Liposomes Transferosomes Ethosomes	It can be used for preparing ethosomes if the thin-film is rehydrated with an ethanolic solution	MLVs	[158]
Ultrasonication	Liposomes Transferosomes	Strong ultrasound pulses applied to an aqueous phospholipid dispersion produce lipid vesicles	SUVs LUVs	[159]
Reverse-phase evaporation	Liposomes Transferosomes Ethosomes	Phospholipids are dissolved in an organic solvent and then hydrated by mixing with the aqueous phase. When organic solvent is removed a gel is produced, which turns into a vesicle dispersion after stirring	MLVs LUVs	[132,137,160]
Eter/Ethanol injection	Liposomes Transferosomes Ethosomes	Phospholipids are dissolved in an organic phase which is injected through a syringe system into the aqueous phase	SUVs LUVs	[161,162]
Hot method	Ethosomes	Drug is dissolved in ethanol and propylene glycol mixture. Then it is added to a 40 °C phospholipid aqueous dispersion and mixed for 5 min.	SUVs LUVs	[163]
Freeze-Thaw	Liposomes Transferosomes Ethosomes	Requires the use of blank lipid vesicles that are suspended with the drug solution and several cycles of freezing and posterior thawing are carried out	LUVs	[164,165]
Dehydration-Rehydration	Liposomes Transferosomes	Requires the use of blank SUVs formulations which are mixed with drug solutions and dried (usually by freeze-drying) and then rehydrated	LUVs	[166]

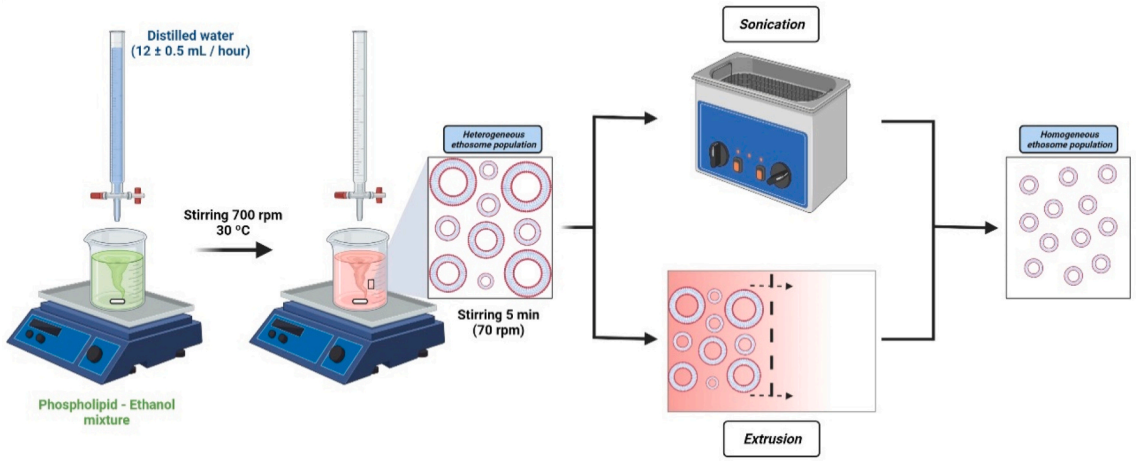

Fig. 6. Touitou's method for ethosomes production. Further size reduction by sonication or extrusion is needed to produce a homogeneous ethosomes batch.

drawbacks in most cases, such as vesicle dilution or decreased product yield [168]. These difficulties explain why large-scale purification is limited and the purification process remains nowadays a challenge. According to the drug physicochemical properties and lipid vesicle final application, researchers must choose the appropriate method in each case. The most used purification methods are dialysis, centrifugation, ultrafiltration, and size exclusion chromatography (Fig. 7).

3.4.1. Dialysis method

Dialysis removes non-entrapped molecules from liposomal dispersions with the use of a semi-permeable membrane, whose pores allow molecules to pass through, while the diffusion of large structures is restricted [169]. The simplicity of the process, the possibility to purify large volumes of vesicle formulations and its low cost are the main advantages of this technique. The success of this method resides in the use of an appropriate dialysis bag and dialysis media. Both free drug and other molecules should be highly soluble in the dialysis media for an adequate extraction [170]. Moreover, osmotic pressure must be considered to preserve the vesicles' integrity and avoid the forced leakage of entrapped drugs [100]. This operation is generally successful for hydrophilic molecules, since an aqueous media generally fulfils these requirements, and it is usually performed at low temperatures, around 4 °C [103,171,172]. The time required to complete the process is one of the main disadvantages of this method, as it takes at least 24 h to ensure complete free-drug removal [103,171]. However, an excessive dialysis time can lead to an encapsulated drug depletion as a consequence of the drug affinity for the media [173]. Dialysis of lipophilic molecules is often more complex as drugs do not pass to aqueous media, and double-way flow of organic media through the pores in the diffusion process could make the vesicle structure unstable. Nevertheless, if the volume ratio between vesicle dispersion and aqueous dialysis media is considerably balanced in favour of the media, the concentration gradient for drug diffusion remains active and the dialysis of lipophilic components is possible [172].

3.4.2. Centrifugation method

The application of a centrifugal force to heterogeneous dispersion produces the separation of its components according to their size and density. It can be used in lipid vesicle purification, leveraging the obvious differences regarding size, weight and density between non-encapsulated components and lipid vesicles. Under the centrifugal force, free drugs remain in the supernatant, while lipid vesicles tend to accumulate in the bottom of the centrifuge tube or Eppendorf. The optimal rotor speed depends on different factors, such as the density of the medium or vesicles size and stability, but as a general rule, the rotating speed to achieve an effective separation should be up to 10,000 rpm and applied between 30 and 90 min at 4 °C [103,174,175]. Differential high-speed centrifugation precipitates vesicles under forces up to 100,000 xg. However, excessive forces may cause particle aggregation and the bilayer fusion, with the subsequent drug entrapment loss.

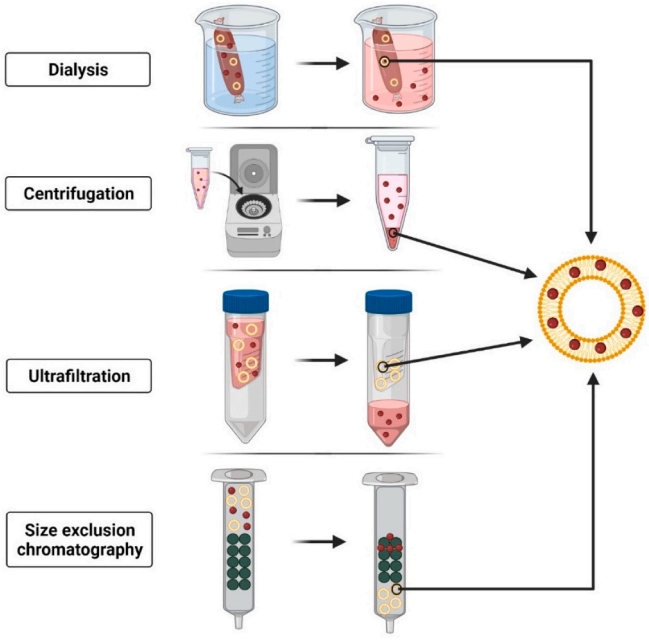

Fig. 7. Purification methods of lipid vesicles: dialysis, centrifugation, filtration and size exclusion chromatography.

3.4.3. Ultrafiltration method

Ultrafiltration is a method that combines the centrifugation and the pass of free molecules through a semipermeable membrane [176]. As with dialysis, the pore size plays a key role in the purification process. Usually, to achieve an effective purification, the pore size used is ranged at least one tenth of the vesicle size (between 50 and 300 kDa) [176–179], and centrifugation force around 4000 rpm or 2000 xg [177,178,180]. High weight structures are retained in the membrane while water and other smaller components freely pass through the membrane pores. The method divides the formulation in two fractions: the ultrafiltrate which contains the extra-vesicle components in the vesicle dispersion medium, and the sediment which contains the lipid vesicles [177]. Thus, ultrafiltration is especially useful for hydrophilic drugs and its main advantages are that drug leakage is partially avoided as the liposomes do not immerse in a purification media, and it reduces the time to complete the purification (around 1–2 h) in comparison with the dialysis method [179].

3.4.4. Size exclusion chromatography method

Size exclusion chromatography is based on the separation of the components of a blend as a consequence of their interaction with the porous beads contained in chromatographic columns, which leads to a different retention time [181]. The non-entrapped drug and other free components with low molecular weight and size are capable of interacting with the pores, whereas lipid vesicles or other large components do not enter the pores [182,183]. Consequently, the elution of vesicles is relatively quick, while the free drug is retained in the column for a longer period. To achieve an efficient elution, the type and diameter of gel particles and pores is essential. Sephadex, Sepharose and Sephacryl columns are the most used. For example, Sephadex G-25, G-50 and G-100; Sepharose 2B, 4B and 6B; and Sephacryl S200 and S1000 usually offer good results in liposomes purification [137,167,182,184,185]. The time needed to complete the purification process is intermediate, no more than 6 h [167]. The inconveniences of this method are possible reactions between vesicles and reactive groups of gel particles, drug leakage [177], and issues in removing lipophilic compounds as the eluent solutions are usually of a hydrophilic character, like HEPES buffer [186].

3.5. Size reduction procedures of lipid vesicles

MLV formation is often spontaneous after using most of the lipid vesicle production methods [187,188]. Nevertheless, the topical and transdermal application mostly requires the use of vesicles with a smaller and specific size (SUV and LUV). The reduction of the size and lamellarity is possible once the vesicles are obtained by different methods. Usually, the techniques used for those purposes are sonication, extrusion, and homogenisation.

Lipid bilayers present different structural states depending on the temperature [189]. There is an ordered phase, also called gel state, which exhibits high rigidity at lower temperatures. On the other hand, beyond the transition phase temperature, which is specific for each lipid type, they become a disordered phase, known as liquid-crystalline state. At this phase, lipids have soft mechanical properties making the bilayers

more fluid [189,190]. In that way, it is important to work above the phospholipid transition temperature [189,191], while reducing size vesicles, to prevent a sharp drop in the efficiency process [192].

3.5.1. Sonication

Sonication provides energy to the vesicle systems by high frequency ultrasound. Usually, it allows the obtention of a homogeneous population of SUV from the sonicated MLV [193]. The obtained SUV show a size not larger than 200–100 nm (303,304). In particular, ultrasounds can be applied in short intervals (around 30 s) or pulses of a few seconds to the vesicles [194–197]. The number of pulses (from 3 to 8 cycles) depends on their energy and frequency (ranging up to 600 W and 450 kHz) [194,197,198]. The main limitations of the sonication technique are the oxidative reactions that phospholipids could suffer and the loss of encapsulated drugs, especially if they are hydrophilic, as a result of the reduced aqueous volume entrapped within the bilayers [132].

3.5.2. Extrusion

Extrusion converts MLV into homogeneous LUV and SUV vesicle populations [199,200]. The technique consists in the passage of the vesicle dispersion through polycarbonate or polyethersulfone extrusion membranes with uniform pore networks [201–204]. The pore size ranges usually between 0.1 and 5 μm [200,202,204]. Variables that define the lower size attainable are pore size, number of extrusion cycles, applied pressure, extrusion temperature and vesicle flexibility [204]. Different protocols have been proposed, but around 20 passages, combined with the appropriate extrusion membranes, have commonly been performed to achieve sizes lower than 300 nm [172,205]. However, Ribeiro et al. demonstrated that after 6 passages, the particle size of liposomes was reduced significantly from 569 ± 61.2 nm to 162.5 ± 1.4 nm, accompanied with a reduced PDI lower than 0.150 [199].

3.5.3. Industrial methods

Homogenisation, French press, and micro-fluidification methods can be considered an evolution of the extrusion technique. They are the preferred methods in the industrial context [192]. Operating at high pressure levels, the first two consist of pumping the vesicle formulation through a stainless holed wall, while a microfluidiser aims to provoke vesicle collisions between them [206–209]. The main drawbacks of all of them is their own working high pressure and the equipment costs, but on the other hand, the reproducibility in downsizing and the possibility of processing higher vesicle formulation volumes makes them ideal methods for scale-up process [192].

3.6. Drug-loading strategies

Drug encapsulation in lipid vesicles is probably one of the critical steps in vesicle production, as their pharmacological activity is subjected to achieve drug concentrations within the therapeutic range after the drug release process. Two loading strategies can be distinguished: active and passive loading.

Passive strategies just rely on the spontaneous capacity of vesicles to capture a certain aqueous volume (where a water-soluble molecule is dissolved) during their formation [210,211]. As hydrophilic drugs are located in aqueous core of vesicles, their entrapment efficiency is affected by the vesicle size and lamellarity, and these, in turn, by phospholipid concentration and production methods [212]. Regarding lipophilic molecules that are embedded within the lipid bilayer, the entrapment efficiency mainly depends on phospholipid concentration and vesicle number, regardless of the size or morphology [213].

Active loading consists of the incubation of empty vesicles with a drug solution to push the cargo through bilayers if they are permeable enough. In the simplest case, a concentration gradient is the pushing force to complete the loading until the equilibrium between the interior of vesicles and the surrounding medium is achieved [214]. In consequence, this type of loading is restricted to hydrophilic and small molecules, with the packaging structure of bilayer components being the main opposition to the loading [215]. Efficient and quick loadings can be achieved using other pushing impulses, such as pH gradient [216]. It involves the preparation of liposomes using a pH buffer around 4.0 and then adjusting the external pH to more than 7.0, adding a basic character compound or directly exchanging the surrounding medium by the optimal buffer solution [217].

Freeze-thaw in cycles is also a processing technique to encapsulate drugs into vesicles or to improve the entrapment efficiency [164,165]. It works as an extra force to achieve the equilibrium in drug concentrations inside and outside the vesicles [218]. The optimal number of freeze-thaw cycles reported in the literature is highly variable (up to 10 in some cases) [219], because of the different types of drugs involved. Zhao et al. showed that 4 cycles are optimal in the case of protein-loaded vesicles [220]. However, Costa et al. reported that 2 freeze-thaw cycles resulted in the maximum predicted entrapment efficiency of Tenofovir, a polar anti-HIV nucleoside with low permeability [218].

4. Key properties and parameters in the design of lipid vesicles for transdermal delivery purposes

In order to achieve a highly efficient transdermal permeability of drugs, lipid vesicles should present some key properties, namely: size, morphology, PDI, zeta-potential, entrapment efficiency, deformability degree, drug release properties, stability, and biocompatibility. These properties are connected with each other, and therefore, changes in one of them lead to variations in the other. Consequently, it seems almost impossible to get the perfect or ideal vesicle and the real challenge focuses on the development of a prototype with a suitable balance between all the key parameters.

4.1. Entrapment efficiency

The drug-to-lipid ratio influences the drug therapeutic index for any lipid vesicles application [221,222]. Hence, high entrapment efficiencies are desirable since they contribute directly to achieving significant effects after vesicles administration. In turn, entrapment efficiency is dependent on various factors such as physicochemical properties of drugs, vesicle size and lamellarity, and vesicle production method.

Firstly, hydrophilicity or lipophilicity of drugs have a huge influence in the encapsulation process when vesicles are loaded through passive strategies. Lipophilic drugs attain a high encapsulation ratio, around 90% or above, as a consequence of their intercalated location between lipids of vesicle bilayers, while hydrophilic drugs present low entrapment rates, between 10 and 50% [223–225]. Encapsulation of water-soluble drugs depends on the fact that an important proportion of drug dissolved into the water phase remains outside the vesicles, decreasing the encapsulation ratio. This is the reason why size conditions, especially the entrapment efficiency of water-soluble drugs: LUV, in comparison to SUV, have larger amounts of water in their core [160]. In the same way, the increase in the number of lipid bilayers in MLV plays in favour of lipophilic drug entrapment, as the concentrical membranes increase the available space for hydrophobic drugs. Moreover, MLV also improve encapsulation of hydrophilic drugs since the several bilayers tend to retain inside this type of drugs [212].

Secondly, as each production method provides different types of vesicles regarding size, it can be considered that the entrapment efficiency is also subjected to the selection of the manufacturing technique [226].

Finally, the drug loading method is the last factor which has a notable influence on entrapment efficiency. Active loading strategies have an excellent uptake response from vesicles which can improve the entrapment efficiency, since it is independent of the lipid composition and presents high drug retention properties [227].

Entrapment efficiency can be directly or indirectly calculated. The

indirect method consists of determining the amount of drug present in the outer medium of vesicles to estimate the entrapped fraction of drug by difference to the initial amount of drug formulated [228]. For this, usually vesicle dispersions are centrifuged to isolate them and analyse the supernatant, which contains the non-entrapped drug fraction [228]. The direct estimation of the entrapped amount of drug requires the destabilisation of vesicles to force the release of their content [229]. It is achieved by adding high amounts of methanol or surfactants like sodium dodecyl sulphate or Triton X-100 [103,223,229,230].

4.2. Determination of phospholipid concentration

The efficiency of lipid vesicle fabrication and their percentage in phospholipids in establishing the drug-to-lipid ratio can be estimated through different methods [231]. Chemical methods like Steward, Rouser and Barlett assays are frequently used, as they are relatively simple and cost-effective. In Steward's assay, the vesicle solutions are mixed with chloroform, $FeCl_3$ 0.1 M, NH_4SCN 0.4 M in a ratio 0.5:3:2:2 and vortexed for 20 s. After centrifugation (1000 rpm) for 5 min, 2 mL of the lower chloroform fraction is carefully transferred into a quartz cuvette, and absorbance is measured at 485 nm [232]. Rouser's method consists of heating 100 μL of vesicle solutions at 270 °C until complete liquid evaporation, followed by the addition of 450 μL of $HClO_4$ (70% *v/v*). After, the blend is heated to 250 °C for 30 min. Then, 3.5 mL of water, 500 μL of ammonium molybdate (2.5% *w/v*) and 500 μL of ascorbic acid (10% w/v) are added. Finally, the mixture is vortexed and incubated at 100 °C for 7 min and cooled down again. The absorbance is measured at 820 nm [233]. In Bartlett's method, up to 2 mL of samples and 0.5 mL of H_2SO_4 10 N are mixed and heated at 150–160 °C for at least 3 h. Subsequently, 2 drops of H_2O_2 (30%) are added and maintained at 150–160 °C again for 1.5 h to ensure a complete combustion and decomposition of all the peroxide. Then, 4.6 mL of ammonium molybdate (0.22% w/v) or 4.4 mL of water, plus 0.2 mL of ammonium molybdate (5% w/v) and 0.2 mL of Fiske-SubbaRow reagent are added, mixed, and heated for 7 min in a boiling water bath. The optical density is measured at 830 nm [234].

Alternatively, enzymatic assays have been suggested to determine phosphatidylcholines in liposomes [235]. For example, a colouring reagent solution is prepared by adding 45 mL of buffer solution (Tris buffer 50 mM, calcium chloride 5 mg/dL, and phenol 0.05%) to the dry colouring reagent (phospholipase D 20 U, choline oxidase 90 U, peroxidase 240 U, 4-aminoantipyrine). Microwell plates are filled with 50 μL of vesicle dispersion and 250 μL of the colouring agent and warmed to 37 °C for 5 min. The quantification is performed measuring the absorption at 492 nm. Other techniques have seldom been applied based on phospholipid and its degradation product determination purposes, such as gravimetric assays [236], magnetic resonance spectroscopy (H-RMN) [232], and ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS) [237].

4.3. Size and morphology

The particle size of lipid vesicles is probably one of the parameters with a stronger influence on the transdermal delivery. Generally, the smaller the vesicles, the higher the transdermal flux is achieved, even though it is dependent on other parameters [238]. Vesicles with a diameter of 300 nm or below enable the delivery of their 'cargo' to the deeper skin layers, especially when they are flexible. Verma et al. used CLSM to visualise the effect of the size penetration ability of liposomes, loaded with the fluorescent molecule. The maximum levels of fluorescence in the cryo-sectioned skin samples were observed with <70 nm diameter, while liposomes with a size of 120 nm diameter also showed statistically enhanced skin penetration as compared to larger ones [239]. On the contrary, vesicles sized 600 nm or above remain on the *stratum corneum* layers where they release their cargo [240,241]. Absorption through the skin of vesicles ranging between 200 and 600 nm is possible, but their performance is more influenced by other aspects, such as edge-activators composition or the deformable behaviour of vesicles [242].

The exact mechanism that nanovesicles follow in the skin permeability process is still under discussion, however, it also seems to be linked to vesicles size. Small nanovesicles may be absorbed through transepidermal routes including aqueous pores [243], whereas larger entities up to 210 nm also follow the transfollicular route as an extra option [47,244]. As mentioned before, size and morphology can be controlled choosing the appropriate vesicle production method, and are commonly assessed by Dynamic Light Scattering (DLS) and microscopy techniques respectively [245,246].

DLS is a reliable method for determining the size of nano and micro particles [199,247]. It is based on the property of vesicle dispersions to produce a variable light scattering over time because of the constant changing position of vesicles as result of a Brownian motion. DLS measures this intensity and its fluctuations to establish a correlation function. An exponentially decaying function is correlated with decay times, which are related to diffusion coefficients and particle radius by the Stokes-Einstein equation [248].

Microscopy techniques offer the possibility to visualise the lipid vesicles and estimate their preliminary size. These techniques are not a surrogate of DLS since vesicles can show certain variations in size due to the sample preparation protocol. Optical microscopy is a basic tool unable to provide complete information about lipid vesicles and their bilayer in comparison with other microscopy techniques [249]. Nevertheless, it can be used to obtain a quick image of lipid vesicles and general information, although not precise, about the size, shape, and homogeneity of big vesicle types such as Giant Unilamellar Vesicles (GUV) and even MLV (361–363) [250–252].

Electron microscopy is the most extended method for the visualisation of lipid vesicles. It offers a high magnification and super-resolution images that allow us to obtain a clear view of vesicles [253]. This technique focuses a beam of electrons onto the vesicles' surface, which are scattered by the sample. These electrons are refocused and magnified to produce a projected image [246]. Scanning electron microscopy (SEM) utilises an electron beam that is scattered across the surface of the sample to produce a magnified image of an object [254]. SEM is now not usually used for imaging lipid vesicles because it requires the sample to be fixed or air-dried prior to imaging, which can cause damage to the particle's integrity due to the surface tension of the evaporating water [253,255]. However, special drying techniques, such as the critical point drying, or paper absorption, followed by ethanol concentration gradients, have been successfully used to prepare the sample before SEM analysis [253,256]. Also, freeze-dried powders of lipid vesicles can be analysed using this technique to study the morphology of the vesicles [254,257].

Environmental scanning electron microscopy (ESEM) is an improved imaging system that does not require the use of fixing, staining or freezing of vesicles to visualise wet systems [258]. ESEM allows the presence of vapour in the sample chamber, since a multiple-aperture vacuum system lets the imaging chamber be maintained in a partial vacuum environment, unlike other parts of the equipment [259].

Transmission electron microscopy (TEM) is probably the most frequently used imaging technique for lipid vesicle imaging [172,260]. It provides a better contrast and contour of vesicle structures than other microscopy techniques, thus it easily denotes information about structure and surface modifications of vesicles [246]. In contrast to scanning-based microscopy, the electron beams cross the samples and are refocused by different lenses to form an image. It requires sample-preparation procedures prior to visualisation, specifically negative staining, freeze-fracture and cryo-TEM [261–263]. The negative staining is a fast preparation technique and allows the use of hydrated samples. It consists of embedding the vesicles in electron-dense materials, typically heavy metal salts like phosphotungstic acid or uranyl acetate, which enhances the contrast between them and the background

[264]. The most relevant inconvenience is the difficulty in evaluating the vesicle morphology. The possibility to introduce artefacts that may be mistaken for vesicle structure and entities [265], and the changes in the structure of vesicles as a consequence of vacuum atmosphere and dehydration of the samples are other important limitations [259]. The freeze–fracture technique involves vitrification by quick freezing using cold liquids such as propane or nitrogen [266]. Then, samples are fractured and surfaces etched to get a negative replica of the fracture sample [267]. The main advantages of freeze-fracture TEM are that it can provide information about the internal structure and does not require any drying pre-treatment. However, it requires the use of organic solvents to clean the replicas of the samples that are removed prior to visualisation [246]. Cryo-TEM is the most evolved technique of microscopy currently available [268,269]. The native state of lipid vesicles can be evaluated through it, receiving complete information about size, shape, internal structure, and lamellarity [270,271]. The aqueous films are vitrified in liquids such as ethane, and the quick freezing of samples prevents the crystallisation of drugs and vesicle components and minimises the appearance of artefacts [246].

As a variation of the scanning microscopy technique, atomic force microscopy (AFM) is an interesting alternative to visualise nanoparticles and analyse their surface modifications and ligands detection, thanks to its high resolution in the order of fractions of a nanometer [272–275]. AFM consists of a sharp tip attached to a cantilever and connected to an optical sensor with a laser beam that records and quantifies the cantilever deflection [276]. The system explores the sample surface and it moves up and down the cantilever as a result of the surface relief. The main advantage of AFM is that it can operate in a liquid or air environment, and it does not need a vacuum condition [246]. On the other hand, the main limitations are the need to adsorb the nanoparticles onto mica or silicon surfaces, which can potentially modify the size and shape of the lipid vesicles [277], and their displacement and dragging as result of their contact with the tip and cantilever [246].

4.4. Polydispersity index

As mentioned before, vesicle size has an important impact on transdermal permeability as well as on therapeutic efficacy. Successful formulations should be efficient, stable and safe, which demands homogeneous populations in the case of pharmaceutical nanoparticles [278–280]. PDI is the parameter that evaluates the size distribution of a population of particles [281,282]. As for size, PDI is accurately determined using DLS. It is scaled to the unit, so that its values are ranged from 0 to 1. In general, values lower than 0.5 refer to monodisperse systems, whereas results above 0.7 indicate high polydisperse populations. In drug delivery, PDI values of 0.3 and below are considered acceptable for lipid-based carriers, and indicate homogeneous populations of phospholipid vesicles [238]. Size reduction methods like sonication, extrusion, and high-pressure homogenisation have been reported to reduce excessive PDI values [283–286].

4.5. Vesicle flexibility

The degree of deformability is the differential feature of flexible lipid vesicles in comparison to common liposomes. It seems to be the key aspect that allows the improvement in drug permeation and permeability by transfersomes and ethosomes [287]. The flexibility of a lipid vesicle depends on the composition and the incorporation of the edge-activators. Different simple methods have been proposed for the determination of this degree of deformability to compare the flexibility of different vesicle batches. Essentially, two techniques based on the extrusion procedure are used. The first one consists of the measurement of the vesicle suspension volume recovered after extrusion, which is directly proportional to the flexibility. Thus, the more deformable the vesicles are, the more freely they will pass through extrusion membranes without saturating the extrusion membrane and leading to a minimum volume loss [103,172,288]. The alternative method compares the size before and after the extrusion process. The reduction in size is inversely proportional to the flexibility, as the more flexible vesicles can squeeze through the membrane pores maintaining their size [289,290]. A deformability index has been proposed using the following equation (Eq. (6)) [291]:

$$D = J \bullet \left(\frac{rv}{rp}\right)^2 \tag{6}$$

Where J is the volume of formulation extruded; rv is the particle size after the experiment; rp is the membrane pore size.

4.6. Drug release

The design and optimisation processes of lipid vesicles imply the analysis of drug release mechanisms that constitutes the first and indispensable step after *in vivo* administration. The *in vitro* methods to study drug release are similar to the purification ones, such as the centrifugation/filtration and dialysis method, but applied under completely different experimental conditions [292]. Dialysis methods are commonly used since they are simple and cost-effective [100]. The solubility requirements of drugs in the acceptor medium and the maintenance of sink conditions are the main limitations [293]. Even though drugs released from vesicles must cross an extra barrier (the dialysis membrane), it does not interfere if first and zero order kinetics are representative of the process, and the release rate from vesicles is higher than or equal to the diffusion rate through the dialysis membrane. Thus, the limiting step here is the release process. The experimentally obtained data reflects the release behaviour in all cases [100,103]. The dialysis methods are usually carried out using FDC setups or dialysis tubes systems in a physiologically tempered environment at 32–37 °C [294,295].

Rigidity and structure of vesicles can modify the release patterns of drugs. MLV present a higher number of barriers that drugs should overcome, which increases drug retention inside the structures and delays the diffusion process through the membranes [212]. Vesicle composition modulates the rigidity because cholesterol acts as a membrane stabiliser, diminishing the fluidity of phospholipids and increasing the rigidity. In fact, it has been reported that rigidity increases the resistance to drug transport and therefore diminishes drug release [102,296]. On the other hand, the hydrophilic or lipophilic character of molecules is the other main factor that influences drug release, as hydrophilic drugs present higher and faster release processes in aqueous media in comparison to lipophilic substances (227,408) [100,297].

Release data is often fitted to different kinetic models that propose the underlying release mechanisms and allow quantitative release rate comparisons. Theoretical models such as zero and first order kinetics have been used, but empirical models generally offer more complete information [298]. Higuchi, Korsmeyer-Peppas, and Peppas-Sahlin are the semi-empirical mathematical models usually applied to the drug release from vesicular systems. Among them, the Higuchi model seems to be the most limited as it assumes that the process is carried out exclusively by passive diffusion [299]. The Korsmeyer-Peppas and Peppas-Sahlin models consider both a relaxation mechanism and passive diffusion with different impact in the whole release process [300–302]. A modification of the Korsmeyer-Peppas model, also called the Power-law model, was introduced by Kim and it considers a possible burst effect during the initial moments of drug release [303]. However, burst effects can be attributed to free drugs present in the media when vesicles are not adequately purified or if the drug has been released from the TDS during the storage.

The suitability and accuracy of the different fitting models to the experimental data is commonly evaluated by the correlation coefficient (R^2) and the Akaike information criterion (AIC) (Eq. (7)) [304–306]. The AIC is very useful to compare models with a different number of

parameters, since it compensates for the statistical versatility of equations with lower degrees of freedom using a penalising factor. The correlation coefficient is usually reported as an indicator of the proportion of the results variability that is explained by the model.

$$AIC = n \bullet Ln\left(\frac{SSr}{n}\right) + 2 \bullet p \quad (7)$$

Where n is the number of experimental data; SSr is the residual sum of squared; and p is the number of parameters in the mathematical model considered.

4.7. Stability

The stability of lipid-vesicles formulations is a key issue in drug delivery, as it determines the feasibility of any pharmaceutical product to be commercially available [307]. Stability-related phenomena can be classified into two groups: immediate or long-term changes. In general, zeta-potential works as a good predictive stability parameter of colloidal particles such as lipid vesicles [308]. High values above ±30 mV assure a great repulsion between the surface charged particles, which produces an electrical stabilisation of the particles and leads to a low aggregation or flocculation [309]. Temperature clearly affects the stability of vesicles, for which they are stored at 4 °C until their use [310]. However, they still present some stability issues, which are in fact one of the main limitations of lipid-vesicles [94].

4.7.1. Immediate or short-term stability

Physical stability refers to phenomena occurring in the days or weeks following lipid-vesicles production. Creaming-clarification, aggregation, coalescence, sedimentation, and flocculation are typical events of instability that vesicles can show relatively often (Fig. 8). These phenomena are easily detected by size and PDI measurements carried out with DLS (Table 4) [311]. Additionally, Turbiscan® is a complementary tool to check the phenomena that vesicles experience during the early storage period. It records the variation of transmitted and backscattered light by the sample over the time (Table 4). In addition, it offers the Turbiscan Stability Index (TSI), which expresses a global idea about the stability of the formulation.

Coalescence and flocculation lead to an increase in particle size. Coalescence is irreversible as it entails the fusion of vesicles, while flocculation is an aggregation of particles without the creation of single drops [312,313]. Flocculates are often easily dispersible, unless they coagulate. Moreover, flocculation can lead to coalescence at some point. Creaming is rarely observed in lipid-vesicle storage. It takes place when the dispersed phase has lower density than the continuous phase and goes up to the top of the vial sample. Creaming can be coupled with coalescence, flocculation and even phase separation. On the other hand, sedimentation is a similar process. The density of the dispersed phase is higher than the density of the continuous phase, and the fraction of particles migrate to the bottom of the vial sample [314]. Clarification is an associated phenomenon that implies particle depletion of a part of the sample.

Table 4
Possible changes of lipid vesicles observed in a short-term period by DLS and Turbiscan®. ΔBS: variation of backscattering; ΔT: variation of fransmitance; ↑: increase; ↓: decrease.

	Vial Bottom		Vial Middle		Vial Top			
	ΔBS	ΔT	ΔBS	ΔT	ΔBS	ΔT	Size	PDI
Sedimentation	↑	↓	–	–	↓	↑	↑	↑
Creaming	↓	↑	–	–	↑	↓	↑	↑
Flocculation	–	–	↑/↓	↑/↓	–	–	↑	↑
Coalescence	–	–	↑/↓	↑/↓	–	–	↑	↑

4.7.2. Long-term stability

It has been reported that lipid-vesicles may be stable for 2–3 months [315,316]. During this period, the phenomena described above can continue. Additionally, if vesicles suffer the destabilisation of lipid bilayers or changes in storage conditions, they can disintegrate. Nevertheless, the main issue during the long-term storage is drug leakage, which takes place especially in vesicles loaded with hydrophilic drugs, as a consequence of their affinity for the media where the vesicles are dispersed [317]. Rigid vesicles, with high amounts of cholesterol, can control drug leakage, but will slow down in drug release.

4.7.3. Freeze-drying

Freeze-drying or lyophilisation is the easiest method to preserve lipid vesicles during long periods of storage. It removes the aqueous content of the formulation and therefore avoids chemical reactions, such as oxidation, which can affect the vesicle structure [318,319]. Moreover, drug leakage is prevented since there is not a media to which to diffuse.

Freeze-drying is a complex process that has three main steps: freezing, sublimation, and secondary drying. There is not a general recipe that works with all formulations, and each one requires the development of a specific protocol of temperature, pressure, and time according to its own characteristics. It is particularly important to attain the whole sample freezing prior to the vacuum phase to get an optimal result. Therefore, it is essential to determine the critical collapse temperature for the preparation, which is the maximum temperature that can be used during drying [320]. As amorphous structures, lipid vesicles substances have a glass transition temperature instead of a eutectic point [321], and the collapsing temperature is usually a few degrees higher [322,323]. The use of organic solvents is also a problem, as lower temperatures are required to freeze, and they can easily bypass the condenser during the following phases and damage the equipment. On this matter it seems impossible to proceed to ethosomes lyophilisation, as ethanol is also removed from the formulation as a consequence of its high volatility and vesicles would be reconstituted in water [324]. The next step dries the sample by removing the bulk of the water *via* sublimation, and it is divided into two steps (Fig. 9).

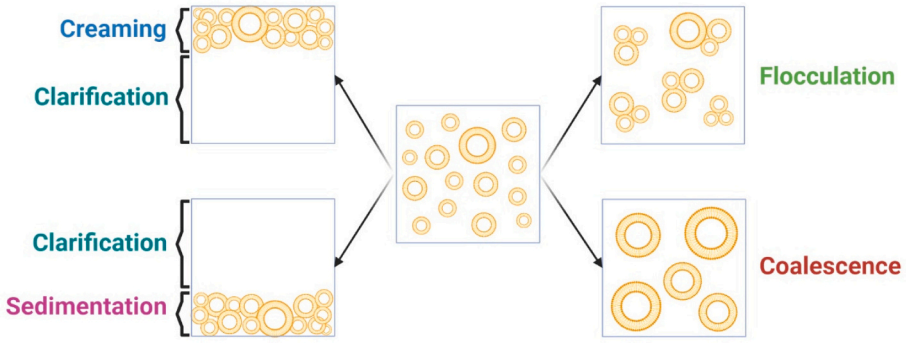

Fig. 8. Short-term instability phenomena that lipid-vesicles can show during the storage period: flocculation, coalescence, clarification, creaming and sedimentation.

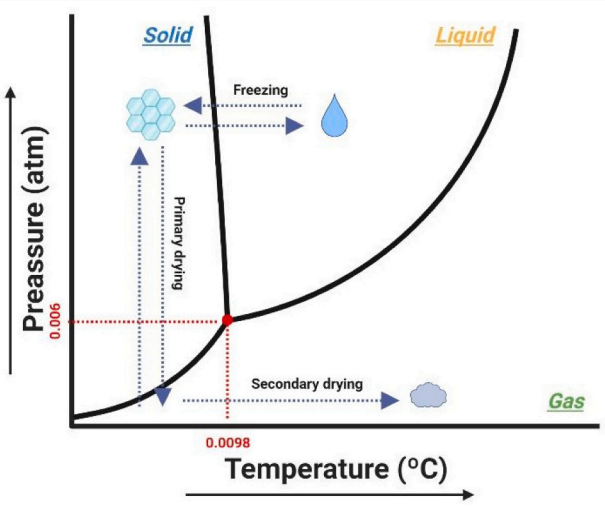

Fig. 9. Standard phase diagram of water. The process starts with the freezing of the formulation, and the drying steps take place by dropping down the pressure level and increasing the temperature.

Primary drying is a slow phase, conducted at cooler temperatures than the critical collapse temperature of the freeze-dried formulation. The product temperature depends on its vapour pressure, which in turn, is determined by the heat transfer to the product (controlled by the shelf temperature) and the vacuum level. Provided the optimal temperature of the formulation is identified, the parameters to control during the primary drying are the shelf temperature and the vacuum level [325]. The amounts of residual water still bound to the formulation after this process is desorbed in the secondary drying by increasing the temperature [326,327]. This increase must be controlled and slowed to avoid the collapse of the formulation.

Depending on the further applications of the formulations, the final water content varies from 0.5 to 3%, but in most cases, the driest products will have a longer shelf life. Thermogravimetric analysis (TGA) can be used to set up the freeze-drying conditions and as a control of the final product, since it measures the mass loss and its rate as a function of temperature. In thermograms, different steps of mass loss can be detected and the first one is used to correspond with the moisture content of the sample (Fig. 10) [328,329].

Lyophilisation of lipid vesicles can be achieved with just the primary drying step [330,331]. Vesicles must be previously frozen around

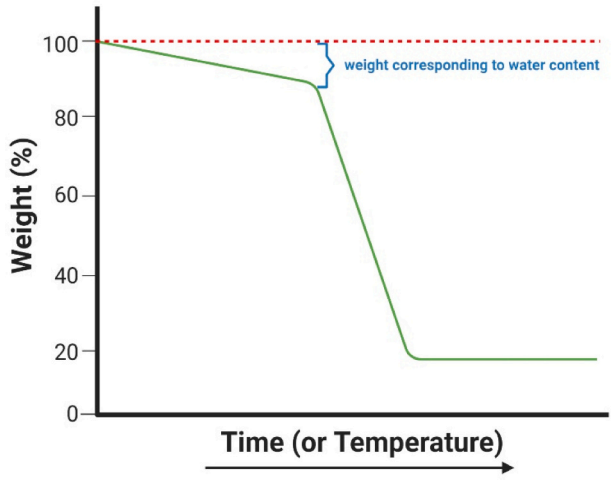

Fig. 10. Standard profile of a thermogravimetric analysis. The first phase of weight loss corresponds to the water content which must range between 1 and 3% for freeze dried products.

−80 °C for at least 6–12 h to assure a complete freezing of the sample. For the primary drying (24–48 h), shelf temperature must be set around −40 °C, condenser temperature − 55/−60 °C, and pressure 1–6 Pa. In the case of a multiple step drying, a constant rate of 0.25 °C/min or additional cycles of 5 h with a decreasing shelf temperature are used, for instance −30 °C, −16 °C and 20 °C [332]. The final products can be stored during long periods at 4 °C prior to use. An optimal lyophilisate should be a cake composed of a uniform, fine and smooth powder, easily hydratable and dispersible in water.

Different phenomena can lead to changes in the lyophilised cake appearance with critical, major, or minor consequences in the product efficacy and safety. The most common are collapse, melt-back, and puffing. Collapse is usually a consequence of a drying (primary or secondary) above the collapse temperature of liposomal formulation and results in a viscous flow and loss of the microstructure previously established by the freeze process [333]. Melt-back takes place due to traces of ice remaining at the end of primary drying or in the early moments of secondary drying [334,335]. Consequently, it happens if primary drying occurs above the eutectic point or if primary drying is incomplete. Both are undesirable in all cases since they have an important impact on the final product quality and attributes. Puffing consists of the presence of bubbles on the top surface of the dried product. Different reasons can cause its appearance, such as minimum collapse or small melting during drying. During the freezing process small bubbles of air are included, which can migrate to the surface and, if they are stable enough to withstand the drying, remain in the final product [333,336]. Contrary to collapse and melt-back, puffing does not imply in all cases critical consequences in the quality of the final product and is acceptable when it is observed as a characteristic of the formulation in all vials within a batch without any impact on the product attributes. In case of observing it in a few vials of a batch, the product showing puffing evidence should be discarded [333].

The use of cryoprotective agents is generally recommended to preserve the starting characteristics and vesicle integrity. Amino acids and complex polysaccharides have been used as cryoprotecting agents [337,338]; however, simple saccharides are generally considered the first-choice excipients. Their mechanism of action is not completely understood, and different theories have been proposed which are not exclusive and may work together. On one hand, the water replacement theory suggests that the H-bonds interactions between water and polar head groups of phospholipids are replaced by those with the carbohydrates, stabilising the vesicle structure [339]. On the other hand, the vitrification model proposes that sugars produce a phase with high viscosity and low mobility, which allows to maintain the distance among vesicles acting as a barrier between adjacent bilayers. This matrix protects the bilayers from damages caused by ice formation and prevents the fusion of vesicles. In consequence, sugar avoids the increase of Tm and the possible leakage of hydrophilic drugs induced by the effect of extra-vesicular ice [340]. For example, glucose, lactose, sucrose, mannitol and maltose have been used in a 5–10:1 M ratio (cryoprotectant: lipid) (Table 5) [341]. Sorbitol has been prepared at 0.7–2.5% (*v*/v) solutions and then mixed in a 1:4 ratio (vesicle sample: cryoprotective solution) [172]. After the reconstitution of freeze-dried vesicles, parameters such as size and PDI are maintained in most cases. Nevertheless, some reports show that the use of sugars can produce a tiny but

Table 5
Glass transition temperature (Tg) of some common cryoprotectant sugars. Taken and modified from Levine and Slade work [344].

Cryoprotectant	Tg (°C)
Glucose	−43
Lactose	−28
Sucrose	−32
Mannitol	−40
Maltose	−29.5

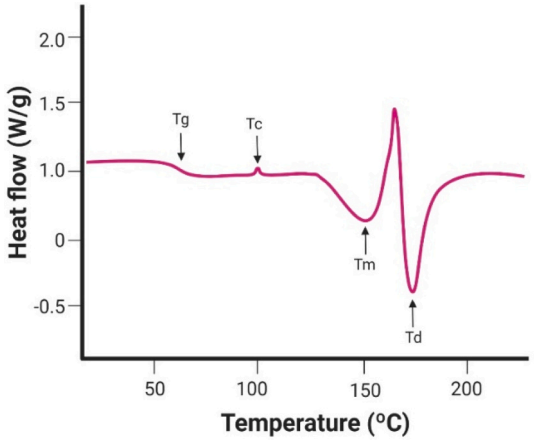

Fig. 11. Standard DSC analysis profile. Glass transition temperature (Tg) is observed as a slight change in the slope that can take place in a wide range of temperatures. Crystallisation (Tc), melting (Tm), and degradation (Tg) temperatures are observed as well-defined peaks.

significant size decrease after lyophilisation, which is higher when sugar concentrations are increased [330,342]. As an explanation of this, several reasons have been accounted for, but the loss of water content in the membrane for preventing vesicle damage as a consequence of kosmotropic effects seems to be the most reliable cause (454) [343].

4.7.4. *Differential scanning calorimetry*

DSC has been widely used to characterise solid components and stability [345]. The technique supplies heat at a constant rate to a sample and a reference material -usually between 1 and 20 °C/min from 25 to 300 °C range- [346–349], with the aim of comparing and determining the difference in the heat flow needed to keep both at the same temperature [350]. Thermal analysis represents the heat flow difference between the samples and the reference material, which is plotted *versus* temperature (Fig. 11). It allows the identification and determination of different material state transitions like glass transition temperature (*Tg*), degradation temperature (*Td*), melting temperature (*Tm*) or crystallisation temperature (*Tc*). First-order transitions, such as crystallisation, melting and degradation, imply an abrupt absorption or release of latent heat and are depicted as well-defined peaks in thermograms [351]. Second-order transitions, like glass transitions, do not involve a change in volume or latent heat, since they are just associated with variations in molecular mobility [352]. Consequently, it is observed as a slight change in the slope that may take place in a large range of temperatures. These parameters and phase transitions are essential for a complete understanding of the relationship between the properties and the structure of materials. As a result, DSC provides a characterization of the solid materials or powders (including lyophilised lipid vesicles), providing key information about the interaction of their constituent components and its evolution over the time.

The main limitations of DSC are, on one hand, the analysis of multicomponent samples that generate thermograms with complex signals, which can be different from those provided by pure constituents that make the correct identification of transitions and thermodynamic changes difficult. On the other hand, the overlapping thermogram profiles are also dependent first, on the heat rates used and second, on the fact that melting and degradation can occur in a narrow range of temperatures [350].

4.8. *Biocompatibility*

Lipid nanocarriers are composed predominantly by phospholipids, which are considered by regulatory agencies as safe components [353]. However, the chemical modifications in its structure and the inclusion of other additional components may potentially lead to an increase of the toxicity of the resulting liposomes. Even though lipid vesicles increase the therapeutic index of many drugs by decreasing drug accumulation in other organs and healthy tissues, they can produce toxic effects in the target tissues or elicit immune responses [354]. In particular, the interaction with proteins and vesicular systems can trigger the innate immune responses such as the activation of complement cascade, cytokine production, and hypersensitivity reactions. In the same way as the other aspects related to lipid vesicles, the final biocompatibility or toxicity is the result of the combination of different properties and features such as size, surface charge, and composition [354,355]. Thereby, *in vitro* and *in vivo* evaluation of lipid vesicle biocompatibility has become a compulsory step in their design and development.

In vitro toxicity is assessed as viability tests on cell lines such as diploid fibroblast strain MLD and 3 T3 mouse fibroblast [356,357], NEB-1, SCC, HaCaT and HEK as representative of keratinocyte lines [358–360], B16F10 murine melanoma cells [361], and RAW 264.7 murine macrophages [362,363]. These assays include 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or lactate dehydrogenase (LDH) tests [364–366]. Cell viability results are generally expressed as percentage of viability, and those above 80% are considered non-toxic, between 80 and 40% as weak-moderate and below 40% as cytotoxic [367]. The main limitation of these protocols is the possible *per se* interference of lipid vesicles as a consequence of their lipidic nature, since certain vesicle components can induce and stimulate cell growth [368].

For the *in vivo* evaluation of side effects, the Buehler test has been used extensively. It is considered a conservative test with relevance to the clinical condition [369]. In the Buehler sensitisation test, a single occlusive patch containing a minimally irritating concentration of the tested formulation is applied to the shaved flanks of guinea pigs for 6 h. This procedure is repeated 2 times more on the same site once a week. After a 2-week rest period, the animals are challenged with an occlusive patch with the highest non-irritating concentration of the test material at a naive skin site. The patch challenge test sites are evaluated 24 h and 48 h after removal for the presence of erythema using a scale of 0–3, in which 3 represents intense erythema. Additional control groups of animals are needed and similarly challenged. The incidence of severity of responses in the test group is calculated relative to that in the control group.

If the point of application is the eye, the Draize test was the most used assay to assess the irritating properties of the formulation [370,371]. However, there are many ethical concerns related to animal testing and the scientific community is searching for alternative methods. This test is based on the blinking produced by 0.1 mL of the studied formulation in the rabbit eye compared to a saline-treated control eye. Irritation levels are registered at 24, 48, 72 h and 4, 7, 14 and 21 days following application using a score from 0 (non-irritative) to 100 (maximally irritative) [372]. As an *in vitro* alternative for testing ocular irritation, the Bovine corneal opacity and permeability (BCOP) test has been proposed. After *in vitro* experiments where cornea or sclera are used, ocular tissues are visually inspected looking for any damage or appearance modification that indicates irritation [373–375]. However, in many cases this test is not considered strong enough to assess the complete safety of formulations and other tests are needed. Hen's Egg Test Chorioallantoic Membrane (HET-CAM test) is probably one of the most accepted alternatives to the Draize test [376,377]. In order to conduct it, fertilised eggs are placed and maintained for 8 days in the incubator at 37 ± 0.5 °C with 40 ± 5% environmental humidity. They are turned 3–5 times per day to prevent the attachment of the embryo to one side of the egg. At the end of the 8th day, eggs are placed with the large end facing up for 24 h to ensure the moving of the embryo to the bottom of the egg. On day 9, eggshells are cut in the air chamber area without damaging the membrane, which is moistened for 30 min with 2 mL of 0.9% NaCl solution before removing it to expose the chorioallantoic membrane (CAM). Around 200 μL are deposited in the CAM and eggs are observed for bleeding, vascular lysis, and coagulation of the CAM vessels for 300 s

[378]. The irritation score (IS) is calculated according to the following equation (Eq. (8)) [379]:

$$IS = \frac{(301 - tH) \bullet 5}{300} + \frac{(301 - tL) \bullet 7}{300} + \frac{(301 - tC) \bullet 9}{300} \quad (8)$$

Where *tH* is the haemorrhage time (s), *tL* is the lysis time (s) and *tC* is the coagulation time (s). Substances are classified as no irritation (IS <1), weak irritation (1 ≤ IS <5), moderate irritation (5 ≤ IS <9), and severe irritation (9 ≤ IS <21) (483).

Luckily, topical application of lipid vesicles does not entail frequent side effects in *in vitro* studies, since cells are often insensitive to the toxic effects of vesicles showing higher compatibility than the corresponding drug solutions, and well tolerated in *in vivo* models when they are given topically [380]. On this matter, the administration of liposomes containing a high proportion of negatively charged phospholipids has been related to the drying effect on the skin, and stearylamine to irritation if applied topically in the eye [369].

5. Common *in vitro*, *ex vivo* and *in vivo* methods to evaluate drug transdermal permeability and penetration

Even though *in vivo* methods are the ideal procedures to evaluate the effects of any drug delivery system, many studies show that systemic and local drug concentrations can be estimated and predicted using skin permeation parameters, such as the permeability coefficient (*Kp*), the permeation coefficient (P), the diffusion coefficient (D), the lag time (tL) and the transdermal flux (*Jmax*), all of them calculated by *in vitro* and *ex vivo* methods.

5.1. *In vitro methods: diffusion cells*

Since their conception, diffusion cells have become the reference method for assessing transdermal drug permeation parameters. Specifically, Franz diffusion cells (FDC) of static and vertical type are widely used in a huge number of studies [381]. FDC are composed of two compartments, also called chambers. They are clamped together incorporating a membrane or tissue in between. The donor chamber harbours the drug formulation, and the receptor chamber is filled with an acceptor medium to receive the drug after its passage through the barrier under study, in this case, the skin (Fig. 12). To mimic the *in vivo* conditions, as a surrogate method, this receptor compartment is stirred using a magnetic bar and warmed to the physiological temperature of the skin (32–37 °C) introducing it in a tempered water bath or using and additional jacket system surrounding the cell [382]. The receptor chamber includes a sampling port that allows the sampling procedure at preset intervals of time. Afterwards, the samples are analysed by a suitable analytical method according to the drug nature and its permeated amounts.

As an essential premise for optimal FDC studies, they should be conducted under conditions that ensure the real performance of the formulations. Finite dose condition is an approach based on the use of an insufficient drug dose that causes a final equilibrium between donor and receptor drug concentrations [383]. This method is closer to the *in vivo* and clinic practice conditions. However, it requires complex calculations and complicates the interpretation of the results [384]. On the other hand, infinite dose conditions entail the existence of a sufficiently high drug concentration in the donor chamber that produces a concentration gradient between chambers and the maintenance of the skin conditions [385]. These conditions ensure a constant thermodynamic dragging force over the drug and results in a practical simplification that makes the perception of the absorption process easier. In these terms, it is accepted that in the acceptor medium, the drug concentration should not increase above 10% of the drug solubility in the medium or decrease beyond 10% of the initial concentration in the donor medium [386,387]. However, not only a high drug solubility in the acceptor medium is important towards the maintenance of the sink conditions. Temperature, pH, stirring and degasification have an undeniable influence on the experimental variability. In fact, FDC studies often show a lack of reproducibility, which can be minimised when validated conditions and procedures are followed. Acid or basic drugs solubility is directly correlated with the pH and denotes the importance of selecting the proper acceptor medium to avoid the breakage of sink conditions [386]. It has also been reported that an increase in temperature of around 10 °C leads to a 2-fold transdermal flux as a consequence of its marked impact in drug diffusivity and solubility [387]. Although it seems that stirring and stirrer-type could have a trivial role in the outcome from permeability studies, they determine the correct mixing and homogenisation of drug in the receptor chamber and, hence, the correct sampling procedure and maintenance of the sink conditions. In general, the use of a common magnetic stirrer with a low stirring speed (200 rpm) does not offer proper mixing conditions and higher speed rates are needed (800 rpm) [386]. Nevertheless, speeds as high as this can produce air bubbles on top of the receptor chamber that notably reduce the effective diffusional area at the membrane. Therefore, the evaluation of the suitability of the stirrer-speed combination must be assessed in each case, since there are examples where a simple PTFE cylindrical magnetic bar (12 × 4.5 mm) operating at 200 rpm demonstrated to be optimal [387]. As mentioned, degassing must be carried out in any study by tilting the FDC and letting the bubbles come out through the sampling arm, but it must be done as quick as possible in order to avoid temperature fluctuations.

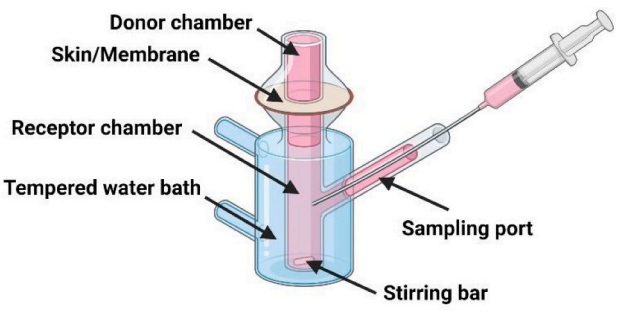

Fig. 12. Schematic illustration of a jacketed-FDC set-up.

Excised human skin is considered the self-standard as a diffusional barrier for *in vitro* experiments [388]. However, ethical and supply issues restrict its use. Moreover, in the case of obtaining human skin samples, they frequently present considerable inter-individual variations, as a consequence of the difficulties to obtain donors with a similar profile (age, gender, ethnic group, general state, *etc.*) [389–391]. Thus, animal skin is used as an alternative, since it is easier to obtain and there is higher homogeneity in the population [391]. Porcine skin is, from a histological point of view, the most similar to human [392–394]. Rat skin is structurally the most similar among all rodents, but several studies point that it is more permeable in comparison to human skin [395–397]. The thickness of the skin, and particularly the *stratum corneum*, has a notable influence on drug penetration and determination of transdermal absorption parameters [398]. This thickness varies depending on the species and the anatomical region chosen, as shown in Table 6. Standard experimental protocols and guidelines for *in vitro* skin

Table 6
Skin thickness among species and anatomical region. Taken and modified from Todo, 2017 [388].

Animal specie	Anatomical region	*Stratum corneum* (μm)	Epidermis (μm)	Whole skin (mm)
Human	Forearm	17	36	1.5
Pig	Back	26	66	3.4
Pig	Ear	10	50	1.3
Mouse	Back	5	13	0.8
Rat	–	18	32	2.09

permeation studies allow the use of full-thickness skin, dermatomed-skin, heat separated epidermis, and *stratum corneum* sheets [388]. Heat separated epidermis can be easily obtained by the Kligman method, which consists of plunging the skin for 90–120 s in a 60 °C water bath and then removing the epidermal layer using forceps [399]. For *stratum corneum* layer isolation, the epidermis is immersed for 24 h in a 0.0001% *w/v* trypsin solution (pH 8.0–8.6) [399].

Even though *in vitro* permeation through human skin presents high variability in general, animal skin samples are not exempt from it. Artificial cellulose and sulfone based-membranes have been used as diffusional barriers [400–402]. Nonetheless, they do not replicate the structure and characteristics of the skin and its use has been relegated to drug delivery mechanistic studies. As a real alternative, reconstructed skin models and 3D-bioprinted tissues have been set-up in recent decades [403]. These artificially generated tissues replicate, either partially or completely (epidermal and dermal layers), the native histological skin structure, but they are rather expensive due to the equipment involved [404]. For example, histological architecture is mimicked sandwiching a fibroblast layer between two collagen-based layers and, eventually, including immune cells as well [405]. EpiSkin®, EpiDerm® or Labskin® are other artificial commercially available skin models.

5.2. *Ex vivo methods: tape-stripping, differential-striping, and confocal laser scanning microscopy*

Drug depth-penetration in the skin and dermatopharmacokinetics are usually estimated using *ex vivo* methods [406]. Tape-stripping stands out for being a minimally invasive technique that removes the *stratum corneum* [407]. Briefly, a skin biopsy is placed onto a glass slide and covered with an aluminium mask, leaving the application area uncovered. A feasible amount of drug formulation is applied, and the set-up is incubated at 32 °C for between 2 h and 6 h [103]. Longer incubation times can cause the disintegration of the epidermis as a consequence of excessive exposure to occlusive conditions [54]. After incubation, 20–25 strips of adhesive tape are applied sequentially to the skin under standardised conditions of pressure to progressively remove the corneocyte layers (Fig. 13) [408]. For this purpose, the percentage of *stratum corneum* removed can be monitored through infrared densitometry devices, differential weighing methods, microscopic measures or transepidermal water loss (TEWL) measurements [408,409]. The drug is extracted from the strips, immersing it overnight in an extractive medium where the drug is highly soluble, and then quantified using an appropriate analytical method. To avoid drug concentrations under the quantification limit, strips can be grouped in different pools, for example: 1, 2, 3–5, 6–10, 11–15, 16–20, 21–25. Eventually, tape-stripping can be performed under *in vivo* conditions [410,411]. High performance liquid chromatography (HPLC, and UHPLC) [412], Attenuated total Reflection-Fourier transform infrared (ATR-FTIR) [413], Differential scanning calorimetry (DSC) [414,415], and Raman microscopy have been successfully coupled with tape stripping as analytical methods [416,417]. Although the tape-stripping method is widely used to study the barrier activity of skin, it is a technique associated with considerable variability and errors if it is not done under standardised conditions. Therefore, a standardised protocol which specifies the material, pressure, and operating conditions reduces considerably the associated variability [418]. Additionally, to reduce the variability, the first strip can be discarded since immediate drug availability in the first layer, or deficient cleaning of the skin surface before the stripping step, can introduce an overestimation [419].

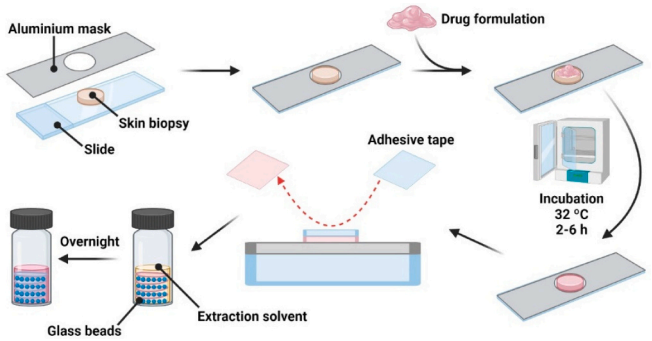

Fig. 13. Schematic illustration of the tape-stripping technique steps.

Differential-stripping is a technique that consists of a combination of the tape-stripping technique with a cyanoacrylate skin surface biopsy [420]. It is considered the most straightforward technique to quantitatively determine the follicular uptake [421]. For this technique, human skin is not recommended because hair follicles are contracted after the skin excision. Therefore, pig ear skin is considered here as the gold standard, as the ear cartilage prevents the closure of hair follicles and because they are anatomically quite similar (density and follicular diameter) to human skin [422]. Briefly, after carrying out a tape-stripping procedure, as described above, a suitable amount of instant quick-drying cyanoacrylate adhesive is applied onto the pre-stripped skin [421]. Upon the glue polymerisation, the cyanoacrylate layer is peeled off to recover the follicular cast and is then processed similarly to common strips. A mass balance study is highly recommended in both techniques to assure complete drug extraction by the method, where 100 ± 20% is considered a satisfactory final balance [423].

Confocal laser scanning microscopy (CLSM) has been applied to skin research for multiple purposes, for example, the determination of vesicle pathway, vesicle-skin interactions or the effect of different penetration enhancers [148,424]. The CLSM approach is based on linking covalent fluorescent dyes (*e.g.* rhodamine) to the xenobiotic in order to track the system diffusion [144,425]. It allows the virtual depth-sectioning of the skin, slicing horizontal planes, and then reconstructing them to obtain an image of the skin structure [426]. The main advantage of CLSM is its low invasiveness [427], and its drawbacks are the interferences as a consequence of skin autofluorescence and short periods of analysis [149].

5.3. *In vivo methods: microdialysis, skin biopsies, in vivo plasma level-time profiles and pharmacodynamic response-correlation studies*

Microdialysis (MCD) is the reference method to obtain a complete study of drug pharmacokinetic processes in the skin [428]. Unlike the tape-stripping technique, MCD is not only restricted to the *stratum corneum* structure, and *in vivo* conditions provide more realistic outcomes. MCD set-up consists basically of a semipermeable dialysis probe lodged in the skin structure and connected to a perfusion pump, which infuses a physiological receiver fluid (Fig. 14) [429]. The main difficulties of MCD are those related with the surgical procedure to install the dialysis probe (anaesthetic pre-treatment and need of trained personnel) [429], the perfusion conditions like flow or drug concentration in retro-microdialysis (R-MCD) variation [430], the requirement of a highly sensitive analytical method for sample analysis [431], and a compulsory calibration before and after the experiment [432]. When an optimal equilibrium of the system is achieved, an exchange of molecules takes place by diffusion, as an effect of the concentration gradient. The drug molecules, previously released by the drug formulation and permeated until the extracellular fluid in the dermis, diffuse to the receiver fluid and are collected for analysis [433]. R-MCD variation uses the same principles, however, the drug under study is dissolved in the perfusion fluid and diffuses from the dialysis probe to the dermis (Fig. 14) [430]. Beyond the information about drug diffusive properties provided by R-MCD, its application seems more limited, as it overlooks the *stratum corneum* effect on drug absorption.

When a drug is applied topically with systemic purposes, *in vivo* plasma concentrations-time profiles offer a direct measurement of the

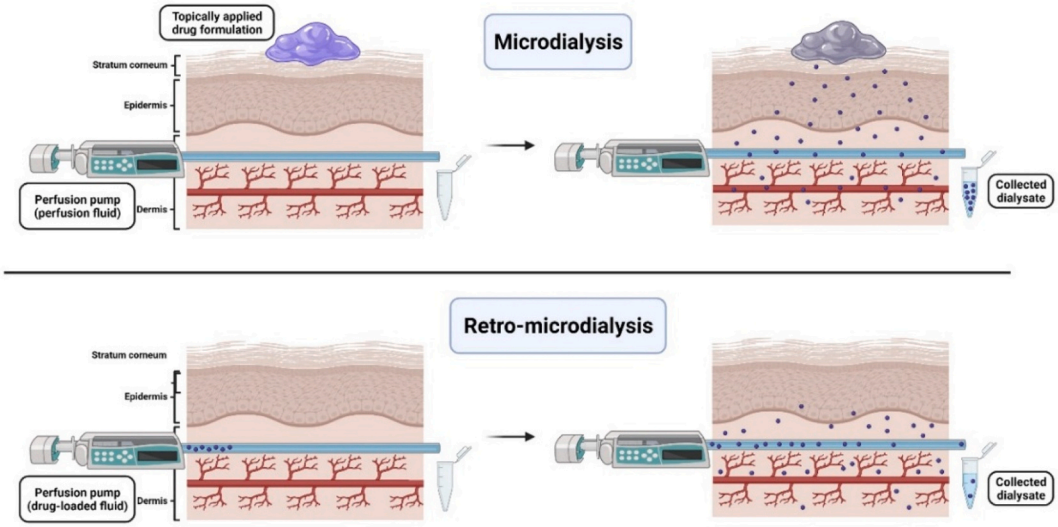

Fig. 14. Schematic illustration of skin microdialysis and retro-microdialysis basic set-up.

absorption, distribution and elimination processes from blood or plasma samples [434], obtained at pre-set times *via* tail vein, retro-orbital puncture, jugular cannulation, or other ethically approved bleeding techniques [435]. In these experiments, topical formulations are compared to oral, parenteral, or subcutaneous as reference administration routes, to determine the feasibility of topical application as an effective alternative by comparison of certain parameters, such as the area under curve (AUC), maximal plasmatic concentration (Cmax), time of the peak plasma concentration (Tmax) and elimination half-life ($t_{1/2}$) [434]. Local or systemic pharmacological effects and safety of topical treatments usually require the use of animal models which trigger the pathologic condition under study. Atopic dermatitis, psoriasis and melanoma models are often employed since there is currently a great interest in these skin diseases [436–438]. Diabetes, hypertension and pain are other focus points of pharmaceutical research nowadays [439–441].

The obtention of skin biopsies is an extremely-invasive approach, where skin samples are removed (under anaesthetic procedure) using a punching device or a controlled-deep blade [442]. It is used for the study of certain skin affections, such as skin tumours or immuno-inflammatory skin diseases, like atopic dermatitis or psoriasis [443,444]. The tissues are further processed to extract the drug and determine drug concentration or the metabolite of interest in the skin. Afterwards, the tissue is homogenised and the drug is extracted from the homogenate to release the interstitial and extracellular analytes [442]. Homogenisation is performed usually using either a fast-rotating device that shreds or grinds the skin into smaller pieces or a mortar to pulverise a frozen skin sample [445]. Additionally, to ensure a complete release of the intracellular and interstitial content, skin samples can be solubilised using chemical agents (for example hydrogen peroxide 30% and ammonium hydroxide [446], or enzymatic digestion (collagenase) [447]. The extraction procedure is performed similarly to the *ex vivo* methods, with a drug-affine medium, or using a precipitation protocol in the case of proteins [448].

6. Summary and future prospects

From their development in 1960's decade, the study and use of lipid vesicles has shown a constant increase, even though only a few applications have been approved by regulatory authorities. Cancer therapy, antifungal treatments and viruses vaccines represent the main applications of those authorizations and they are all developed for parenteral delivery [449]. A Boolean search in Scopus database combining the terms "liposome", "transfersome", "transferosome", "ethosome", "drug delivery", "skin" and "transdermal" with the proper operators (TITLE-KEY-ABS, AND, AND NOT and OR) offered 92,134 manuscripts (in English language) published between 1968 and 2022. Numerous researchers consider lipid vesicles as a promising tool to enhance transdermal delivery of drugs since around 8% of published papers related to liposomes, transferosomes or ethosomes are devoted to this subject currently (Fig. 15).

The increasing interest might contrast with their long life, making lipid vesicles a field of knowledge that it is still evolving and attract the attention of pharmaceutical research and industry. In fact, 9051 patents have been registered to the date in the European Patent Office under the concept "liposome", "transfersome", "transferosome", "transethosome" or "ethosome" (according to Espacenet service) and 1150 clinical trials have been carried out (according to http://ClinicalTrials.gov) (Figs. 16 and 17). The percentages of de stages both for completed and ongoing trials are almost similar, being Phase I, Phase II and Phase III the most common (around 23%, 45% and 24% respectively. As mentioned above, cancer and antifungal therapies account for the vast majority of them, and only a few of them are revolve around topical or skin applications. Psoriasis, dermatitis, vitiligo and skin cancer are the main skin conditions studied in these trials (Table 7).

Despite the large number of studies and clinical trials using lipid vesicles conducted in the past, only 22 liposomal-based formulations are approved by regulatory agencies for clinical use, and no one for dermal or transdermal purposes (Table 8). Nevertheless, lipid vesicles-based formulations are commercially available marketed as cosmetics, thus

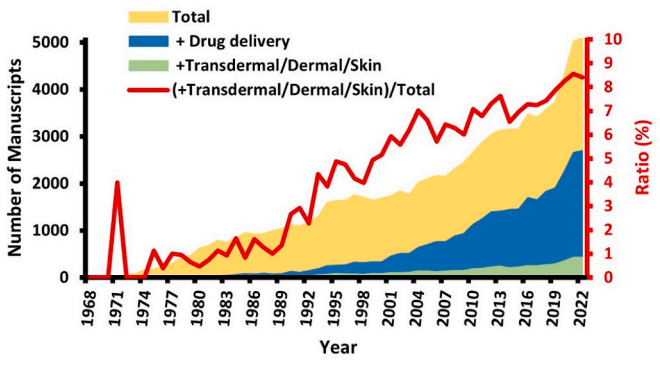

Fig. 15. Bibliometric study of lipid vesicles field (liposomes, transfersomes and ethosomes) based in the number of manuscripts published.

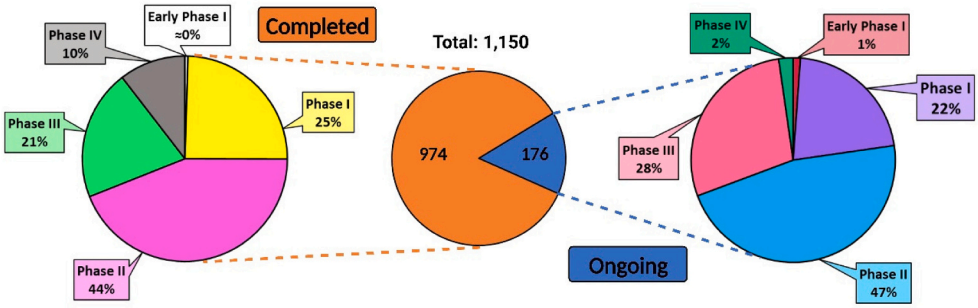

Fig. 16. Number of active and completed clinical studies carried out under the concept "liposome" to the date and percentage of clinical trial phases: Early Phase I, Phase II, Phase III, and Phase IV.

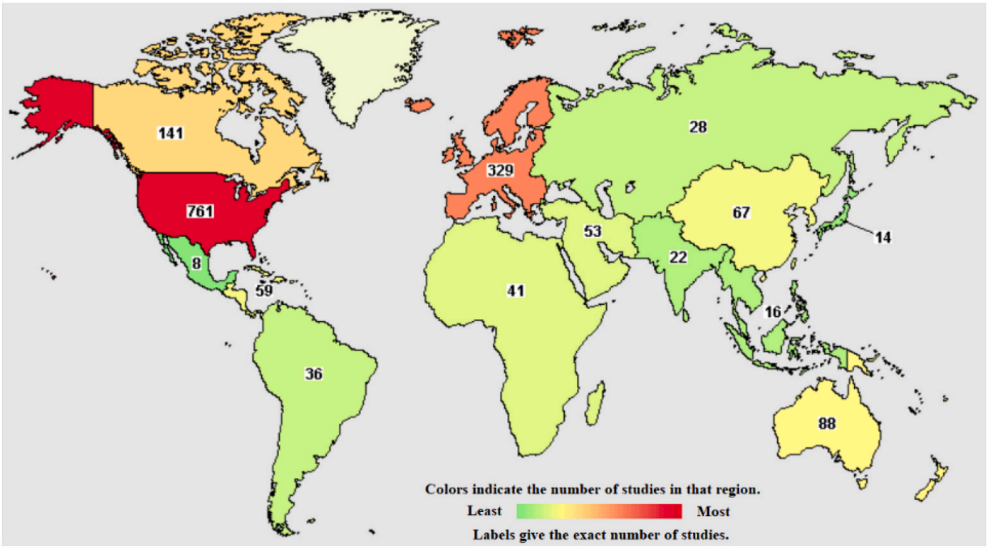

Fig. 17. Number of active and completed clinical studies carried out worldwide under the concept "liposome" to the date (source: http://ClinicalTrials.gov).

avoiding the standard guidelines, quality controls and needs of permission demanded for drugs by regulatory agencies in many countries, falling in a loophole that clearly needs a more specific regulation.

The main advantages of ultraflexible vesicles in skin delivery is the enhancement in drug absorption they produce. They also allow the skin delivery of drugs of a higher molecular weight than possible without using these vesicles (> 500 Da).

However, to obtain an efficient manufacturing procedure is not an easy task, as the properties balance of these vesicles should be maintained because they are all related. Slight changes in vesicle composition can lead to differences in other strongly related properties, which will influence their final performance in drug delivery, as manufacture is a global process.

For transdermal delivery purposes, lipid vesicles should present, on one hand, the lowest size and PDI possible if the aim is to carry the drug into the deeper skin layers. This is valid when vesicles of different size are compared if they contain the same dose. According to our experience, bigger vesicles allow the incorporation of higher doses and, probably due to higher thermodynamic activity, drug access to deeper layers is improved. On the other hand, apart from the higher drug loading, flexibility, stability and biocompatibility also play determining roles.

Composition certainly determines the size, flexibility, and drug release, but at the same time, the manufacturing method has a considerable impact in size and PDI.

Biocompatibility issues are not expected since lipid vesicles are formulated generally with safe components previously tested, although the incorporation of the drug can lead to unexpected toxicity effects.

Probably, one of the main limitations in the lipid vesicles translation from basic research to the clinical practice is the stability, especially for hydrophilic drugs which have a leakage trend from vesicles over the storage time. In this way, long-term stability can be predicted by z-potential values, but vesicles rarely show a > 2–3-month storage period. Freeze-drying can be successfully applied to lipid vesicle formulation under optimised protocols, which makes it an easy solution to stability inconveniences.

Although several commercially available lipid vesicles-based formulations are already in the market, they are generally only used parenterally, except for some cosmetic applications as mentioned above. The recent COVID-pandemic has entailed the use of similar lipid particles massively in the vaccines, thus demonstrating their safety and familiarity of the population with such systems, which will facilitate their acceptance. Topically applied vesicles are still a challenge, as their performance when crossing such a barrier still needs to be fully addressed. However, the positive outcomes in basic research pave the way to further studies to translate their use to the clinic.

In addition, in any lipid vesicle development project *in vitro* and *in vivo* testing methods have an essential role that shows the real performance of new formulations and determines if they present good perspectives to continue with further studies, development, and scalability processes to bring them from bench to bedside.

Finally, it is worth to mention that exosomes have appeared as a new emerging tool in dermatology and cosmetics fields. Exosomes and extracellular vesicles (EVs) are lipid enveloped nanoparticles that are

Table 7
Ongoing and completed clinical trials using liposomal formulations for treating the main skin conditions (source: http://ClinicalTrials.gov). *Melanoma treatments are intended for IV administration. **Unknown: study has passed its completion date and status has not been verified in more than two years.

Condition	Liposomal drug	Stage	Year/Status	Patients	Information
Dermatitis	Cobamamide (HL-009) liposomal gel	Phase II	2012–2013, Completed	120	-Randomized -Double-blind -To evaluate the Safety and efficacy of HL-009 liposomal gel in adult patients with mild to moderate atopic dermatitis
	Liposomal Human Cu/Zn-Superoxide Dismutase (APN201)	Phase I, Phase II	2012, Completed	20	-Randomized -Double-blind -To prevent radiation-induced dermatitis in women with breast cancer
Psoriasis	Ethosomal and liposomal preparations of Anthralin	Phase V	2017–2020, Completed	20	-Randomized -Parallel assignment -To develop an ethosomal delivery system anthralin and evaluate its effectiveness and safety in treatment of psoriasis and comparing it with liposomal delivery system anthralin
	MSC Exosome ointment	Phase I	2022, Completed	10	-Interventional -Open-label (no masking) -To determine safety and tolerability of the topical application of mesenchymal stem/stromal cell (MSC) exosome ointment to treat Psoriasis in healthy volunteers
Melanoma*	Liposomal Vincristine	Phase I	2005–2007, Completed	7	-Interventional -Single group assignment -To evaluate the pharmacokinetic profile of Vincristine sulphate liposomal injection in patients with malignant melanoma and hepatic dysfunction secondary to metastases
	Marqibo®	Phase II	2007–2014, Completed	54	-Interventional -Non-randomized (patients were enrolled into the study in two cohorts) -To determine if Marqibo® (liposomal vincristine) can help to control metastatic uveal melanoma
	RNA-nanoparticle vaccine	Phase I	Estimated dates: 2023–2027), Active	18	-Interventional -Open-label (no masking) -Sequential assignment -To evaluate the toxicity and feasibility of a tumour-specific RNA-NP vaccine in patients with stage IIB-IV melanoma who have progressed on anti-PD1 (a-PD1) adjuvant therapy
	Lipovaxin-MM (dendritic cell-targeted liposomal vaccine)	Phase I	2009–2012, Completed	12	-Interventional -Open-label (no masking) -Sequential assignment -To evaluate the safety and immunogenicity of escalating doses of Lipovaxin-MM in patients with metastatic melanoma
	Liposomal interleukin 2	Phase II	1998–2000, Completed	32	-Interventional -Randomized trial -To study the effects of Interferon Alfa-2b on the immunogenicity of a polyvalent melanoma antigen vaccine in patients with stage III malignant melanoma and comparison with liposomal interleukin 2
	PEGylated liposomal doxorubicin	Phase I	Estimated dates: 2020–2025), Active	240	-Interventional -Non-randomized trial -Sequential assignment -To investigate the safety, tolerability, pharmacokinetics, and biological activity of ATRC-101 as Monotherapy and in combination with other anticancer agents in adults with advanced solid malignancies and comparison with results derived from PEGylated liposomal doxorubicin.
	PNT2258-loaded in liposomes	Phase I	2010–2012, Completed	22	-Interventional -Open-label (no masking) study -To determine the safety and pharmacokinetic profile of PNT2258 in patients with advanced solid tumours
	MicroRNA miR-RX34 liposomal Injection	Phase I	2013–2017, Completed	155	-Interventional -Open-label (no masking) -Single group assignment -To determine the pharmacokinetics and maximum tolerated dose (MTD) for MRX34 and the recommended phase 2 dose (RPh2D) and observing the number of patients with evidence of clinical activity of MRX34
	Glutathione (GSH) PEGylated liposomal doxorubicin	Phase I, Phase II	2011–2014, Completed	84	-Interventional -Non-randomized

(*continued on next page*)

Table 7 (*continued*)

Condition	Liposomal drug	Stage	Year/Status	Patients	Information
	hydrochloride formulation (2B3–101)				-Single group assignment -To determine the safety, tolerability, pharmacokinetics and efficacy of 2B3–101 both as single agent and in combination with trastuzumab
	PEGylated liposomal doxorubicin	Phase I	Estimated dates: 2019–2024), Active	166	-Interventional -Non-randomized -Parallel assignment -To determine the dose, safety, efficacy and pharmacokinetics of TRK-950 when used in combinations with selected anti-cancer treatment regimens (PEGylated liposomal doxorubicin) in patients with selected advanced solid tumours
	Pbi-shRNA STMN1 liposomes	Phase I	2012–2017, Completed	20	-Interventional -Open-label (no masking) -Single group assignment - To determine the safety of intertumoral administration of pbi-shRNA™ STMN1 LP in patients with superficial advanced and/or metastatic cancer who have no acceptable form of standard therapy
Xeroderma Pigmentosum (precancerous condition)	Liposomal T4N5 lotion	Phase III	1996, Unknown**	30	-Interventional -Randomized -Double masking -To compare treatment using T4N5 liposome lotion with treatment using placebo in reducing actinic keratoses and other sun-induced skin damage in patients with xeroderma pigmentosum
	Liposomal DNA repair enzymes	Phase I	2004–2006, Completed	13	-Observational -To determine the effect of an intensified daily photoprotection over 24 months with an SPF30 sunscreen and an after sun-lotion both containing liposomal DNA repair enzymes in a population of patients at high-risk for skin cancer, including xeroderma pigmentosum and basal cell nevus syndrome
Wound healing	Liposomal gene constructs	Early Phase I	1999–2014, Completed	1164	-Interventional -Randomized -Open-label (no masking) -Single group assignment -To determine if the use of foetal membrane (human amnion) improves the wound repair and study if the incorporation of liposomal gene constructs enhance the functionality and efficacy of human amnion
Vitiligo (pigmentation disorder)	Lithium liposomes	Phase I	2019–2020, Unknown**	12	-Interventional -Randomized -Parallel assignment (3 groups) -Double masking -To evaluate the efficacy and the cutaneous acceptability of the dermocosmetic formulation (lithium liposomes) in the re-pigmentation of vitiligo

produced by cells and take part in the intercellular transfer of biological material such as proteins or RNAs [452]. This paracrine and autocrine activity aims to alter the functions of other local and distant cells [453,454], thus playing important modulatory roles in physiological and regenerative process such as tissue repair, stem cell maintenance and immune processes [455,456]. In dermatology field, it has been proved that EVs are involved in immunomodulatory roles in a range of inflammatory skin conditions as psoriasis, atopic dermatitis and chronic wound healing [457,458], besides their participation in the modulation of cell senescence, angiogenesis induction and *de novo* collagen and elastin synthesis [459–461]. These therapeutic effects have been observed from plasma, mesenchymal, dendritic, fibroblasts and keratinocytes derived-EVs, however, plant-based sources also provide EVs with bioactivity on keratinocytes and anti-melanogenic activity [462]. Moreover, using active-loading techniques they could be used as carriers for a wide range of drugs enhancing the cellular targeting and uptake. The main advantage that exosomes offer is their inherent biocompatibility, size (< 200 nm) for dermal applications and the possibility for scaled-up EVs production when used immortalized cell lines [463]. EVs-containing injectable products have not received permission for commercialization yet, which is mainly related to the high heterogenicity of EVs preparations since it depends on the parental cell type, culture procedure (culture environment, seeding density, passage protocol, EVs collection frequency, *etc.*) and health state of the donor [464]. Therefore, standardised potency assays and obtention, purifying and dosage protocols are highly needed. However, EVs are only allowed for topical administration applications and a few studies have assessed the penetration of EVs across the *stratum corneum.* Although no-access to deeper skin layers had been observed after 24 h, positive results were obtained, as the vesicles were able to induce immunoregulatory effects [465,466]. These data encourage current and future clinical trials.

7. Conclusions

Ultraflexible lipid vesicles for topical applications are delivery systems of high interest as they are very compatible with the delivery route anatomy and include chemical enhancers in their composition. Parameters such as size, flexibility and drug loading mainly determine their efficacy. Vesicle access to deeper layers is still uncertain, as some studies suggest vesicles interact with the lipid bilayers of the stratum corneum destabilizing the systems and allowing a more efficient drug passage. Stability and scalability are their main limitations, which can be

Table 8
Approved liposomal based products commercially available for clinical use. IV. Intravenous; IT: Intrathecal; EP: Epidural; LI: Local infiltration; OI: oral inhalation; IM: intramuscular. Taken and modified from Bulbake et al., 2017 [449]; Liu et al., 2022 [450] and Nsairat et al., 2022 [451].

Drug	Product	Year approval	Agency	Admin. route	Indication
Doxorubicin	Doxil®	1995	FDA	IV	-Ovarian cancer
		1996	EMA		-Kaposi's sarcoma
					-Myeloid melanoma
	Lipodox®	2012	FDA	IV	-Ovarian cancer
					-Kaposi's sarcoma
					-Myeloid melanoma
	Myocet®	2000	EMA	IV	Breast cancer
Daunorubicin	DaunoXome®	1996	FDA	IV	Kaposi's sarcome
Cytrabine	DepoCyt®	1999	FDA	IT	Lymphomatous meningitis
		2001	EMA		
Cytrabine: Daunorubicin (5:1)	Vyxeos®	2017	FDA	IV	-Therapy-related acute myeloid leukaemia -Acute myeloid leukaemia with myelodysplasia-related changes
Mefamurtide	Mepact®	2004	FDA	IV	Osteosarcoma
		2009	EMA		
Vincristine	Marqibo®	2012	FDA	IV	Leukaemia
Irinotecan	Onivyde™	2015	FDA	IV	Pancreatic adenocarcinoma
		2016	EMA		
Paclitaxel	Taxol®	1998	FDA	IV	-Breast cancer
	Lipusu®	2006	FDA	IV	-Breast cancer
					-Ovarian cancer
					-Non-small cell lung cancer
Amphotericin B	Abelcet®	1995	FDA	IV	Severe fungal infections
	Amphotec®	1996	FDA	IV	Severe fungal infections
	Ambisome®	1997	FDA	IV	Presumed fungal infections
	Fungisome®	2003	FDA	IV	Systemic fungal infections
Verteporphin	Visudyne®	2000	FDA	IV	Wet aged-macular degeneration
		2000	EMA		
Morphine sulphate	DepoDur™	2004	FDA	EP	Postoperative analgesia
Exparel	Bupivacaine®	2011	FDA	LI	Post-surgical analgesia
		2020	EMA		
Amikacin sulfate	Arikayce®	2018	FDA	OI	Lung disease
		2020	EMA		
Recombinant varicella-zoster virus glycoprotein E	Shingrix®	2018	EMA	IM	Shingles and post-herpetic neuralgia
Inactivated hepatitis A virus	Epaxal®	1993	EMA	IM	Hepatitis A
Inactivated hemagglutinin of Influenza A/B virus	Inflexal® V	1997	EMA	IM	Influenza

overcome through different approaches. More research should be performed to successfully translate them to the clinic.

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Data availability

No data was used for the research described in the article.

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