

Development, Characterization and Use of Liposomes as Amphipathic Transporters of Bioactive Compounds for Melanoma Treatment and Reduction of Skin Inflammation: A Review

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Abstract: The skin is the largest organ in the human body, providing a barrier to the external environment. It is composed of three layers: epidermis, dermis and hypodermis. The most external epidermis is exposed to stress factors that may lead to skin conditions such as photo-aging and skin cancer. Some treatments for skin disease utilize the incorporation of drugs or bioactive compounds into nanocarriers known as liposomes. Liposomes are membranes whose sizes range from nano to micrometers and are composed mostly of phospholipids and cholesterol, forming similar structures to cell membranes. Thus, skin treatments with liposomes have lower toxicity in comparison to traditional treatment routes such as parenteral and oral. Furthermore, addition of edge activators to the liposomes decreases the rigidity of the bilayer structure making it deformable, thereby improving skin permeability. Liposomes are composed of an aqueous core and a lipidic bilayer, which confers their amphiphilic property. Thus, they can carry hydrophobic and hydrophilic compounds, even simultaneously. Current applications of these nanocarriers are mainly in the cosmetic and pharmaceutical industries. Nevertheless, new research has revealed promising results regarding the effectiveness of liposomes for transporting bioactive compounds through the skin. Liposomes have been well studied; however, additional research is needed on the efficacy of liposomes loaded with bioactive peptides for skin delivery. The objective of this review is to provide an up-to-date description of existing techniques for the development of liposomes and their use as transporters of bioactive compounds in skin conditions such as melanoma and skin inflammation. Furthermore, to gain an understanding of the behavior of liposomes during the process of skin delivery of bioactive compounds into skin cells.

Keywords: edge activators, encapsulation, inflammation, melanoma, nanotransporters, skin delivery, ultradeformable liposomes

Introduction

The skin is the largest human organ. It provides a barrier that controls the loss of water and other constituents. It also protects the body against environmental stress by its three layers; hypodermis, dermis, and epidermis (Figure 1).¹⁻³

The hypodermis is a loose connective tissue with a layer of adipose tissue that functions as insulation, nutrition storage, and protection of deeper tissues. The cells in this layer are fibroblasts, adipocytes, pre-adipocytes, and macrophages, which play important roles in adipocyte homeostasis in obesity conditions. The hypodermis seems

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to be susceptible to damage by Ultraviolet Radiation (UVR) since the subcutaneous tissue becomes thinner in photo-exposed regions of the body.^{1,4,5}

The dermis is the connector tissue between the hypodermis and the epidermis. The thickness of this layer varies from 0.3 to 3.0 mm.^{4,6} It provides most of the mechanical strength to the skin. It has two components, the superficial papillary dermis and the deeper reticular dermis, both formed by different fibroblasts.^{1,4} The papillary dermis has thin collagen fibers interspersed with elastic fibers, meanwhile, the reticular dermis has thicker collagen fibers packed and organized in bundles that form a structure around the collagen fibers.⁴

The epidermis is the most external layer of the skin. It is composed of squamous epithelium, with a thickness of about 0.05 mm to 1.55 mm. The epidermis consists of four sublayers named from the deepest to uppermost: basal layer, stratum spinosum, stratum granulosum and the most superficial stratum corneum.^{1,6} Cellular components of the epidermis are keratinocytes, melanocytes, Langerhans cells, and Merkel cells.⁶ Keratinocytes are the most predominant cell type.^{1,7} Keratinocytes are the cells that accumulate melanin produced by melanocytes, the second most abundant cells of the skin. The melanin pigment blocks the penetration of UVR into the skin.⁷

UVR causes the production of oxygen reactive species (ROS) and DNA damage. Both conditions stimulate inflammation and tumorigenesis, which unleash several reactions that promote photoaging since it alters the production of interleukin-10 (IL-10) and interleukin-2 (IL-2), as well as skin cancer by the mutation of the p53 suppressor gene leading the activation of ROS.⁸⁻¹⁰

Melanoma is a type of skin cancer that occurs in areas barely exposed to the sun. Melanoma tumors are found mainly on the back of men and the legs of women. This type of tumor develops from an existing nevus and is often irregular-shaped and has ragged edges.¹¹ This type of skin cancer is a disease that could lead to fatal outcomes according to the stage when it is diagnosed (Figure 2). Patients with metastatic melanoma have a five-year survival rate less than 15%. Almost all organs are targeted for metastasis; however, the most frequently affected are the liver, bones, and brain.¹² Topical chemotherapy is becoming a promising strategy to avoid the sequels of conventional treatments. Therefore, newly developed alternative treatments, such as the use of liposomes as drug carriers to protect against several skin pathologies need to be implemented.¹³

Liposomes have been used in the cosmetic and drug industries because they can carry and protect encapsulated substances, improve the solubility of certain substances, and provide controlled drug release.⁹ Some liposomes have been prepared for treatment of certain pathologies, such as melanoma, in which their use has a positive effect in reducing tumor size/weight and melanoma cell proliferation.¹⁵⁻¹⁷

The objective of this review is to provide an up-to-date description of existing techniques for development and use of liposomes as transporters of bioactive compounds for treatment of melanoma and reduction of skin inflammation. Furthermore, to gain an understanding of liposome behavior during the application and delivery of bioactive compounds into skin cells.

The keywords used for this review were skin cancer, inflammation, liposomes, skin delivery, antioxidants, ultra-deformable liposomes, nanoparticles, squalene, bioactive compounds among others. Databases used were scholar.google.com, sciencedirect.com, library.illinois.edu, ncbi.nlm.nih.gov/pubmed/, patents.google.com, and <https://ghr.nlm.nih.gov>. Mainly scientific papers published within the last 5 years were considered for the review.

Definition of Liposome

The word liposome has its origin in the Greek words “Lipos” meaning fat; and “Soma” meaning body. Liposomes are small vesicles composed of natural or synthetic phospholipids that can be used to carry polar, non-polar, and amphiphilic substances (Figure 3A).¹⁸⁻²⁰ They have an aqueous core surrounded by a lipidic bilayer, their particle size can range from micro to nanometers.²¹

Classification of Liposomes

The size of liposomes varies from 0.025 μm (very small liposomes) to several μm (large liposomes). Their classifications, based on the number of double layers, as described in Figure 3B, are the following:^{22,23}

- Multilamellar vesicle (MVV) range from 0.5 to 5 μm
- Multi-vesicular vesicle (MLV) range from 2 to 4 μm
- Unilamellar vesicles
 - Giant unilamellar vesicles (GUV) range from 10 to 100 μm
 - Large unilamellar vesicles (LUV) larger than 50 nm
 - Small unilamellar vesicles (SUV) less than 50 nm

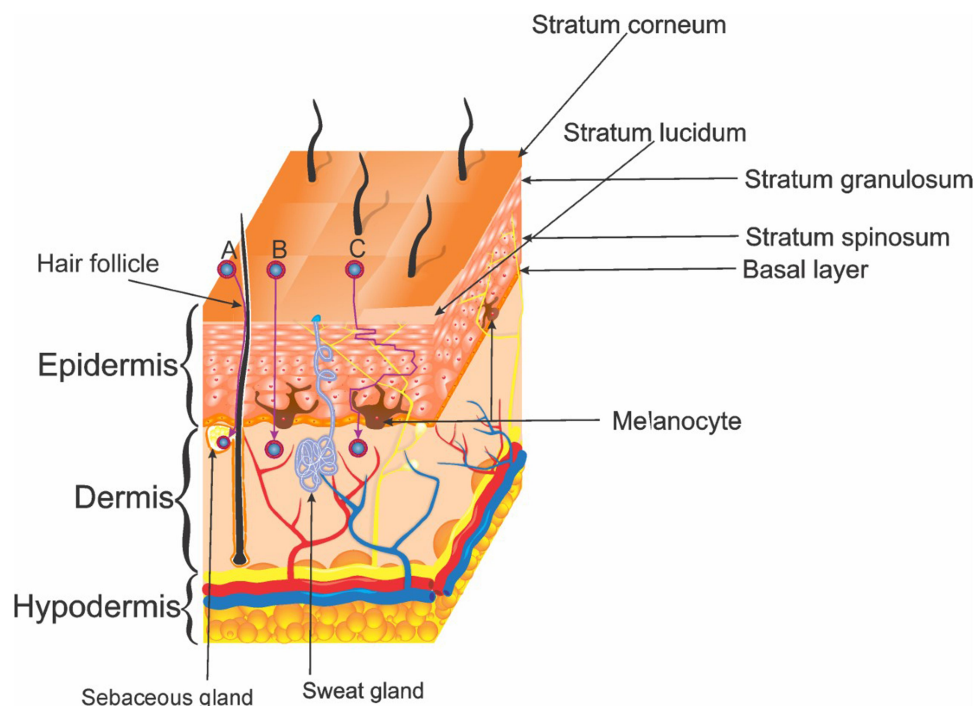


Figure 1 Skin microstructure and skin penetration mechanisms of nanoparticles. (A) Penetration through sebaceous glands and hair follicles. (B) Transcellular penetration. (C) Intracellular penetration.

For skin delivery of compounds, the major type of liposomes reported by most authors is LUV. In addition to liposome size, researchers are also looking for improving the permeability of the vesicles by adding edge activators (EA).

Ultradeformable Liposomes

Ultradeformable liposomes (UDL) or transferosomes are highly flexible and elastic unilamellar liposomes composed typically of 85:15 w/w ratio phospholipids: EA.²⁴ The use of Tweens and bile salts, such as sodium cholate (SC) are preferable over Span or other EAs since they increase membrane fluidity, therefore deformability is improved.²⁵

The physical properties of EA in UDL allow the modification of the structure of the liposome at lower energy facilitating a faster rearrangement of the lipid bilayer. Under stress conditions, EA relocates in the areas with higher curvature.^{25,26} Other types of liposomes can be prepared and characterized following the same methodology as for conventional liposomes, as will be discussed later.

Ethosomes

Ethosomes are other type of liposomes developed to improve skin permeation ($15.1 \mu\text{g}/\text{cm}^2$); in comparison to conventional liposomes which have lower permeation ($4.4 \mu\text{g}/\text{cm}^2$). These kinds of vesicles are composed of phospholipids, water, and ethanol.²⁷ The properties of ethosomes, such as

stability, encapsulation efficiency, and penetrability in the stratum corneum have been improved.²⁸ Ethanol provides flexibility to the nanoparticles, which allows ethosomes to reach deeper skin layers. Furthermore, the phospholipids of the stratum corneum fuse easily with the ethosomes leading to enhanced drug penetration.²⁸

Mentosomes

Mentosomes are liposomes prepared with phospholipids, EA, and menthol.²⁹ Menthol is a permeation enhancer which affects the organization of the vesicle.³⁰ This type of liposome can be prepared by thin-film hydration.³¹

Components of Liposomes

The structure of the molecular arrangement of a solution of amphiphilic compounds can be determined by the geometry of the molecules. It can be estimated by the Critical Packing Parameter (CPP) using the following equation:^{32–36}

$$CPP = \frac{v}{a_0 \ell c} \quad (1)$$

Where:

CPP=Critical Packing Parameter

v = hydrophobic tail volume

a_0 = area of the polar/nonpolar interface

ℓc = hydrophobic tail length

STAGES OF MELANOMA

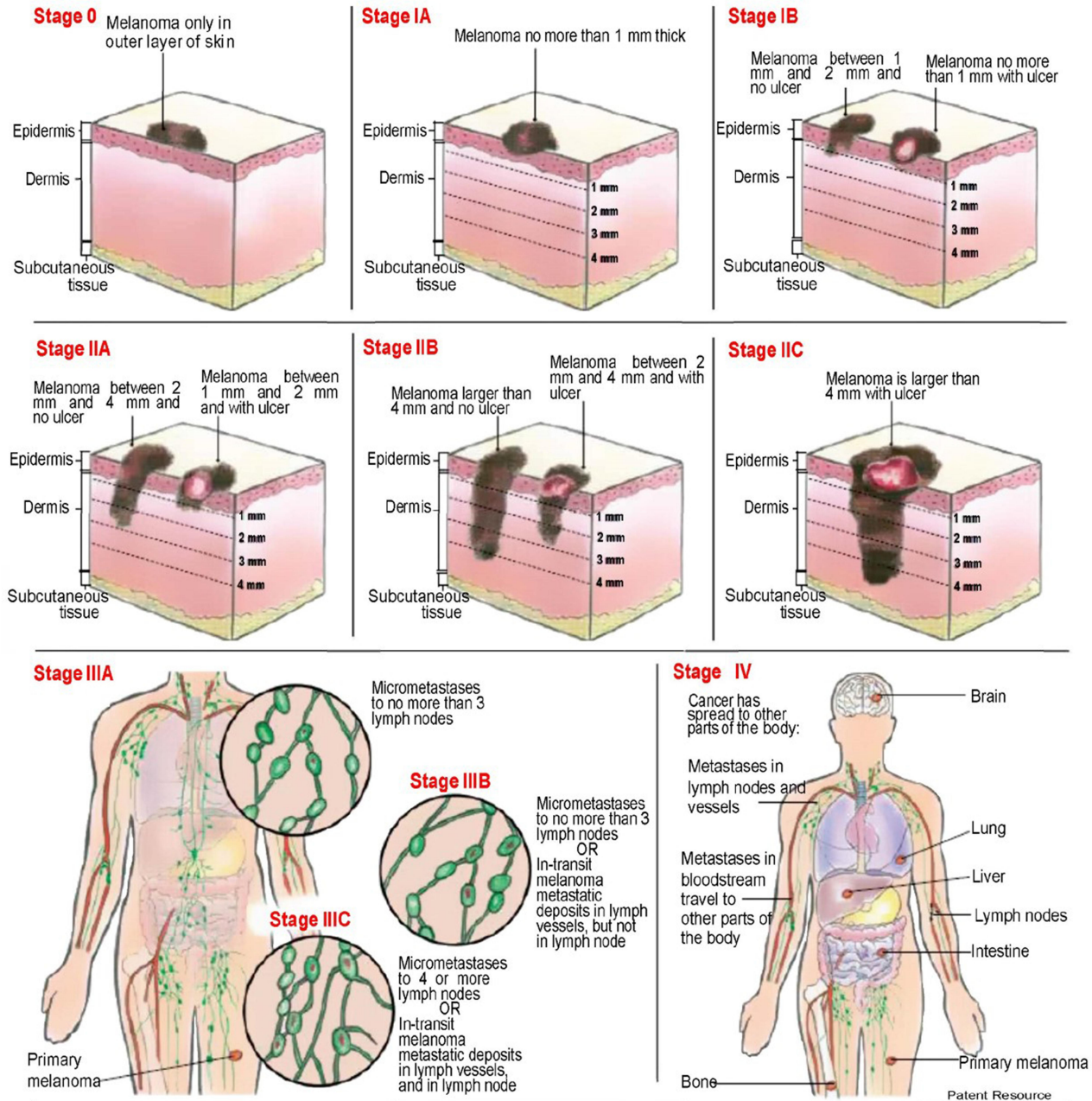


Figure 2 Melanoma stages.
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The bilayer micelles are formed when the value of CPP ranges from 1/2 to 1. If CPP value is <1/2, formation of spherical micelles will take place, in contrast when >1, formation of inverted micelles will occur.³²⁻³⁶

Since liposomes are vesicles composed by a lipidic bilayer, CPP of the amphiphilic molecules used for their elaboration should lie between 1/2 and 1. Phospholipids with two hydrocarbon chains, such as phosphatidic acid,

phosphatidylcholine, phosphatidylethanolamine, and phosphatidylglycerol would generate CPP in this range.³²

The CPP of dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, and cholesterol is ~1/2, >1/2 and >1, respectively.³³ Egg yolk contains phosphatidylcholine, and soybean contains lecithin, phosphatidylethanolamine, phosphatidylinositol, and phosphatidic acid.^{35,37} The CPP of these phospholipids lies between 1/2 and 1.³²

Phospholipids

The main components of liposomes are phospholipids (Figure 4). They are molecules with a polar head group that contacts the aqueous core, the external medium, and a non-polar hydrocarbon chain placed in the center of the lipidic bilayer (Figure 3).

Phospholipids are the main components of the biological encasement of the cell membrane. The most common phospholipid is phosphatidylcholine, which has a positive charge given by the quaternary ammonium group and a negative charge given by the phosphate group at physiological pH.^{18,22} This phospholipid is also widely used in the cosmetic industry for skincare and in shampoo products because of its softening and conditioning properties.²¹

The type of phospholipids used to prepare liposomes is established by their stability and rigidity, determined by their phase transition temperature (T_c). T_c is

determined mainly by the properties of the fatty acid, such as chain length, unsaturation degree, and the polar head groups.¹⁹

The behavior and elasticity of liposomes are properties given by the nature of the fatty acids, for instance the number of double bonds in the hydrocarbon chain.²²

Phospholipids that can be used in the preparation of liposomes vesicle are shown in Figure 4. Examples are specified in Table 1.

Cholesterol

The aim of incorporating sterol in liposomal formulations is to regulate membrane stability and rigidity. The most common sterol included in mammalian membranes is cholesterol (Figure 4). Cholesterol in the membrane is located in the first few carbons of the fatty acid chain. The tricyclic rings are inside the hydrocarbon core and the hydroxyl groups are facing the hydrophilic phase.²²

Cholesterol is not a molecule that could form a bilayer on its own, but its inclusion between the polar heads of phospholipids increases the packing of phospholipids. This improves the stability of the bilayer by reducing its permeability and enhancing the resistance to aggregation. Thus, liposomes become more rigid and improve intravesical interactions. Cholesterol could also lead to a decrease in encapsulation efficiency of hydrophobic molecules because this molecule takes more space in the bilayer leading to a reduction in the potential encapsulation surface.^{18,19}

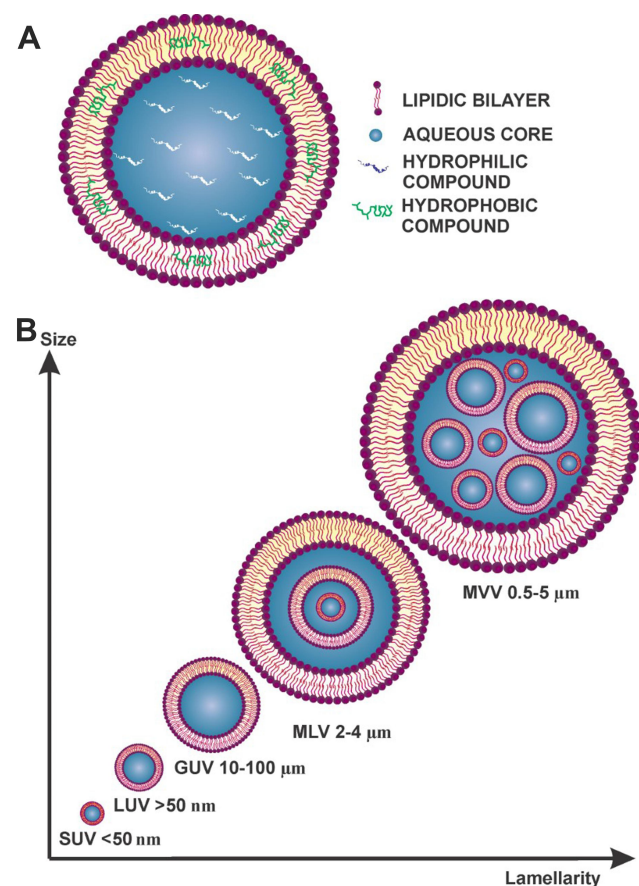


Figure 3 (A) Schematic representation of a liposome. (B) Classification by vesicle number.

Abbreviations: MLV, multilamellar vesicle; MVV, multi-vesicular vesicle; GUV, giant unilamellar vesicle; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles.

Techniques of Liposome Elaboration and Particle Size Reduction

The methods of liposome preparation have not changed significantly since they were discovered. The most used method is thin-film hydration followed by sonication or extrusion for particle reduction.

Liposome Preparation

There are several methods to prepare liposomes. Most of them consist of one lipidic phase (LP) composed of a mixture of lipids. The hydrophobic compound to be encapsulated should be first dissolved in the desired volume of an organic solvent, such as methanol. The solvent is then dried or poured directly into an aqueous phase (AP), prepared with phosphate-buffer saline (PBS) and the hydrophilic compound that needs to be encapsulated.

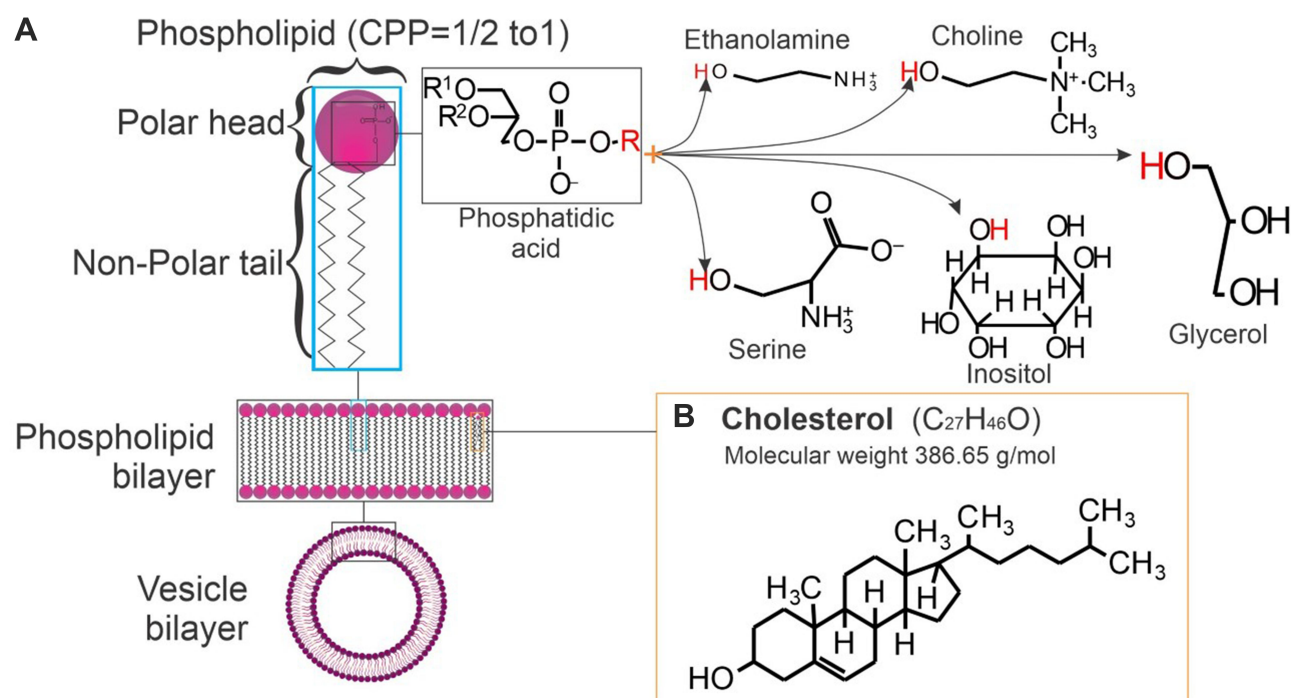


Figure 4 Main components of liposomes. (A) Phospholipids (nomenclature=phosphatidyl + choline, + ethanolamine, + glycerol, + inositol, + serine) the molecular weight of the lipids will change depending on the R groups; (B) Chemical structure of cholesterol.

Abbreviation: CPP, critical packing parameter.

Thin-Film Hydration (Film Dispersion-Homogenization) Method

The LP is evaporated, by rotary evaporation, then rehydrated with AP on a vortex system.⁹

Ethanol Injection Method

The LP must be injected fast into an AP and kept under vortex mixing. The ethanol is removed by rotary evaporation.⁹ Examples of this preparation method are given in Table 1.

UDL has been used for vitiligo therapy.³⁸ In this investigation, liposomes were prepared by ethanol injection and thin-film hydration. The best method in this study was ethanol injection with particle size below 150 nm to enhance the therapeutic efficacy in vitiligo. The high hydrophobicity and crystalline nature of 5-methoxypsoralen and 8-methoxypsoralen, used in the film hydration method, produced precipitation of the LP when adding AP, resulting in non-optimal particle size and polydispersity index.³⁸

When liposomes were formulated to contain vitamin D3 against photoaging, ethanol injection produced smaller liposomes (92.5 ± 1.8 nm) when compared with those prepared by film dispersion-homogenization (169.4 ± 2.0 nm). Moreover, encapsulation efficiency (EE) was higher, 80.3 ± 0.4 vs 62.2 ± 0.9 , respectively.⁹

Reverse-Phase Evaporation Method

LP is prepared in AP emulsion under stirring conditions. The solvent is evaporated by rotary evaporation and the dried lipid layer is then resuspended in PBS.¹³ UDL has been prepared by reverse-phase evaporation, optimized by response surface methodology and loaded with papain to treat hypertrophic scar in New Zealand white rabbits. As result, the scar became thickened, collagen fibers had regular arrangement, and micro-vessels decreased, demonstrating that UDL is an efficient topical treatment against hypertrophic scars.³⁹

Ethosomes Preparation Method

The preparation of ethosomes is similar to ethanol injection. The lipidic compounds are dissolved in ethanol in a glass-sealed container under stirring conditions. The addition of the AP should be dropwise. The solution is then placed into an ice bath and sonicated.²⁸

Particle Size Reduction

Particle size reduction is important to consider after liposome preparation, to determine if needed. The particle size is going to play an important role in terms of skin delivery because it affects the penetration of encapsulated compounds through the skin to the deeper layers.^{26,40}

Table 1 Physical Properties of Liposomes with Different Compounds and Lipid Ratio After Being Prepared with Thin-Film Hydration

Bilayer Compounds (Best Conditions)	Ratio	Size Reduction Method	Particle Size (nm)	EE%	Zeta Potential	Encapsulated Compound	Generalities	Characterization Methods	Ref
DSPC:Chol	55:45	Extrusion	117.8±2.7 to 143.4±11.4	0 to 49.9±2.1	-13.6±1.4 to -1.0±0.4	5-Fluorouracil, Cetuximab	Drug loaded: Drug-to-lipid ratio at 0.1 and 0.2	DSL (Particle size, Zeta potential, and PDI) EE	[13]
DPPC	100	Extrusion	108.0±2.0 to 133.0±28.0	31.1±16.9 to 43.0±5.6	-16.7±5.7 to 4.3±1.7	Curcumin	Curcumin loaded: 0.1–15% w/w	DLS (Particle size, Zeta potential, and PDI) Liposome deformability trough extrusion	[87]
SL:P80	1:48	Extrusion	86.0±1.0 to 90.2±1.6	88.7±0.2 to 92.72±0.1	-33.9±1.4 to -27.7±1.1	Naringin	Naringin loaded: 3, 6 y 9 mg/mL	DSL (Particle size, Zeta potential, and PDI) EE TEM (negative staining)	[97]
DPPC:SC:T80 or S80	max 84.5:15.5	Sonication/extrusion	127.0±7.8136.0 ±10.0	Max 99		Oestradiol	Oestradiol loaded: 1 mg/mL	DSL (Particle size, Zeta potential, and PDI) EE	[3]
PC:T80 or SC	70:30 and 80:20	Sonication/extrusion	81.6±6.5 to 102.6±3.5	30.6±1.2 to 37.4±1.1	-22.1±2.3 to -11.7±1.2	Methotrexate	Drug loaded: Drug-to-lipid ratio at 1.5 w/w	DSL (Particle size, Zeta potential, and PDI) EE TEM (negative staining) Liposome deformability trough extrusion	[25]
SFC:SC	86:14	Sonication/extrusion	167.0±6.0 to 191.0±4.0	0.8±0.8 to 22.7±0.63	-58.0±6.0 to -32.0±6.0	Pirfenidone	Drug loaded: 5 mg/mL	DSL (Particle size, Zeta potential, and PDI) EE Stability for a period of 4 weeks	[86]
DPPC:MPPC: SC	80:10:10 and 75:15:15	Sonication/extrusion	141.0±20.0 to 190.0±70.0	91.9±1.9 to 93.2±1.2	-6.1±2.1.5 to 14.3±3.8	Tamoxifen	Drug loaded: 2 mg/mL	DSL (Particle size, Zeta potential, and PDI) EE	[108]
DPPC:MPPC: SM	70:15:15	Sonication/extrusion	156.0±4.0 to 188.0±6.0	54.6±2.7 to 73.2±1.5	12.3±1.1 to 14.7±0.8	Imatinib mesylate	Drug loaded: 2–3 mg/mL	FTIR (Interaction of the drugs with the liposomal system)	
DPPC:MPPC: SM	70:15:15	Sonication/extrusion	168.0±7.0 to 180.0±4.0	Tamoxifen: 92.8±1.5 Imatinib: 73.8±4.4	14.5±1.2 to 16.7±3.6	Tamoxifen: Imatinib mesylate	Drug loaded: 2–3 mg/mL each		

Abbreviations: EE, encapsulation efficiency; Chol, cholesterol; DLS, dynamic light scattering; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; FTIR, Fourier transform infrared; MPPC, monopalmitoyl-2-hydroxy-sn-glycero-3-phosphocholine; P80, Polysorbate 80; PDI, Polydispersity Index; PC, phosphatidylcholine; SL, soybean lecithin; SFC, soy phosphatidylcholine; S80, Span 80; SC, sodium cholate; SM, sorbitan monooleate; T80, Tween 80; TEM, transmission electron microscopy; YL, yolk lecithin; w/w, weight/weight.

Extrusion

In this technique, the liposomal solution is extruded through a filter with different pore sizes. There are a variety of parameters that can affect particle size in this method such as, applied pressure, number of extrusions, and filter pore size. Ong et al⁴¹ concluded that the smallest particle size was achieved by extrusion (103.3 ± 13.5 nm) and the higher particle size (279.1 ± 15.8 nm) by the homogenization, among several particle reduction methods tested. The extrusion technique also produced the most homogenous liposomes.

Microfluidization

This technique has been used in the pharmaceutical industry during the preparation of pharmaceutical emulsions of liposomal products using high-pressure homogenization of the liposome solution.⁴² The pressure forces the solution through the microchannel and the particles collide with each other leading to particle shear. The shear forces are the reason for the resulting particle reduction.^{43,44} Gulzar and Benjakul⁴⁵ proposed to use 7000 psi, and ten passes for the microfluidizer. However, nanoencapsulation showed better efficiency than microfluidization and it was suggested for the fortification of astaxanthin in foods.

Sonication

It is one of the simplest methods to achieve particle reduction and liposome homogenization. This method consists of the exposure of the liposomal solution to acoustic energy which induces a breakdown of MLV to SUV. The time of contact with the sound waves determines the size of the liposome.⁴⁶ This technique can be performed by either probe sonicator or bath sonicator. The probe sonicator can produce overheating of the suspension causing degradation of the liposomes. In contrast, in bath sonication, the operator can set up the temperature of the bath. The recommended temperature is just above Tc.⁴⁷ He et al⁴⁸ found that probe sonication can lead to an increment of encapsulation efficiency; the best conditions for sonication were 120 W, for 20 min using an ice-water bath. Gulzar et al⁴⁵ compared particle size of liposomes prepared with micro fluidization vs sonication. The particle size was larger with micro fluidization (214–928 nm) than with sonication (40–284 nm), indicating that for skin delivery, sonication was better than micro fluidization.

Characterization of Liposomes

Characterization of the vesicles plays an important role in the understanding of the functionality of the liposomes. The size

of the particles is important in terms of skin delivery since large particles are not able to diffuse through the skin layers and reach the desired location within the target tissue. For instance, the penetration of liposomes with 80 nm particle size after 24 hours of administration into the skin was $588 \mu\text{m}$ reaching viable epidermis, while liposomes with particle size of 500 nm barely reached $504 \mu\text{m}$.⁴⁹ In terms of encapsulation, drug bioavailability of liposomes with particle size 311.4 ± 2.9 nm and with encapsulation efficiency of $97.9 \pm 0.3\%$ had a plasma concentration of around 175 ng/mL, while liposomes of 329 ± 1.8 nm and with encapsulation efficiency $31.8 \pm 0.9\%$ had a plasma concentration of around 100 ng/mL.⁵⁰ It is also necessary to know the behavior of the particles in the medium to be transported. This characteristic will be given by the charge of the particles as assessed by zeta potential.⁴²

Particle Size Distribution and Zeta Potential

Dynamic Light Scattering (DLS), also known as Photon Correlation Spectroscopy, is used to perform liposome size distribution analysis. It measures the fluctuation of light scattered from particles undergoing Brownian motion. With this technique, the particle size, polydispersity index (PDI), and zeta potential can be calculated.⁵¹

The particle size is one of the factors that plays an important role in the diffusion of particles through the stratum corneum.^{40,52} Particles with a size of 50 nm have better diffusion than those of 200 nm.⁵² Liposomes with a particle size 120 nm showed higher accumulation of loaded compound in stratum corneum ($72.88 \pm 1.69\%$) and in deeper skin ($0.76 \pm 0.8\%$) compared with liposomes sized 191, 377 and 810 nm, which had 1.12-, 1.19-, and 1.83-fold lower concentration in stratum corneum and 4.68-, 7.29-, and 33.57-fold lower concentration in deep skin, respectively.⁴⁰ There is a lack of knowledge to understand the relationship between particle size and skin permeability. Further research is needed in this area. Moreover, zeta potential has an impact on the shelf stability of particle dispersions. If the zeta potential is close to 0, the particles will aggregate because they would have a low electrostatic repulsion. In general, the particles are considered as stable when the zeta potential is greater than 35 mV or lower than -35 mV.⁵³

Encapsulation Efficiency

Encapsulation efficiency (EE) is the amount of core material trapped into the microcapsule.⁵⁴ Since liposomes are able to carry hydrophobic and/or hydrophilic molecules,

the EE should be determined for all the compounds that are entrapped in the vesicle. EE is the difference in total amount of the compound added for the encapsulation and the amount of non-encapsulated compound divided by the amount of compound added for encapsulation (Equation 2). This EE is an indicator of the efficacy of the encapsulation process and the quality of the liposomes. It can be assessed by the following equation.^{55,56}

$$EE = \left(\frac{m1 - m2}{m1} \right) 100 \quad (2)$$

where EE= encapsulation efficiency, $m1$ = the total amount of loaded compound, and $m2$ = the amount of free compound in the liposome solution.

Encapsulation efficiency can be affected by the physical state of the molecule, Ong et al⁵⁰ showed that the EE of griseofulvin loaded in powder was lower ($31.8 \pm 0.9\%$) than griseofulvin dissolved in chloroform ($EE 97.9 \pm 0.3\%$)

Micro-Nano Structural Characterization

An important aspect when characterizing liposomes is the visualization of their morphology using microscopy. Available microscopy techniques are light, electron, and atomic-force microscopy.⁵⁷

Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) is the most employed method to characterize the ultrastructure of the nanoparticles, mainly shape, size, and internal structure. Using this method, liposomes can be characterized by three techniques, negative staining, freeze-fracture, and plunge freezing or cryo-TEM.^{58,59}

Negative staining is commonly used in TEM to increase contrast of lipids base nanoparticles, but it can also lead to the production of image artifacts when the water contributes to the shape of the particle.⁵⁹

Cryo-TEM is the most direct and precise method to characterize liposomal lamellarity, size, ultrastructure, and shape. With this technique, the particle can be visualized without staining.^{58,60}

Cryo-preserved samples can also be observed with more detail by fracturing techniques by which the perspective arrangement of the bilayers and the different phases can be observed.⁶¹

Confocal Scanning Microscopy

Confocal scanning microscopy has become attractive due to its superior image clarity. In the case of GUV, it is possible to visualize the internal structure of the lipid systems. Figure 5

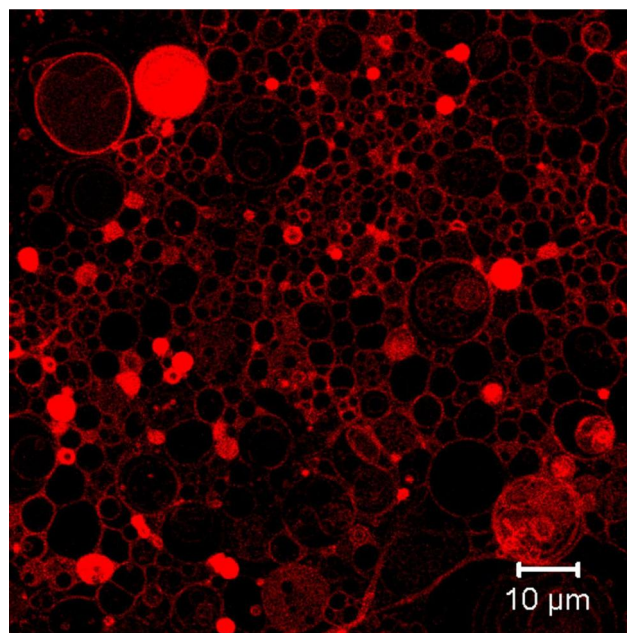


Figure 5 Fluorescent images of Nile red stained giant unilamellar liposomes obtained by confocal laser scanning microscopy (multiphoton confocal microscope LSM 710 NLO, Carl Zeiss).

shows GUV with Nile red staining obtained by the multiphoton confocal microscope LSM 710 NLO. Confocal microscopy can produce 3D images since this technique is able to scan z-axis, starting from the top of the vesicle to the bottom of the sample.⁵⁷ Confocal microscopy can be used for tracking the liposomes by fluorescence and determining the subcellular distribution of the particles. The localization of the liposomes can be studied using dyes specific to the respective subcellular compartment.⁶²

Image Analysis

Nanoparticles have different properties related to their size, shape, and size distribution. Manual measurements are limited by the large assessment variance between subjects analyzing the images, and their ability to separate particles in close contact with each other. The identification of the shape of the particles could be also a limitation when agglomerates exist. If these factors are controlled, the characteristics of particle size, orientation, and shape can be easily resolved.⁶³

Due to these problems, the improvement of image analysis has been of concern. Some computational tools are available for the purpose of image analysis. One available software, ImageJ, is not accurate for the analysis of nanoparticles assemblies.^{63,64} There are some other tools such as Particle Image Characterization Tool (PICT) coded in MATLAB R2012b.⁶⁴ This tool allows to carry out

statistical analysis of shape, size, and morphology.⁶⁴ Another software tool developed for nanoparticle image analysis is Nanoannotator. This new technology is able to identify particles and create automated boundaries between them with accuracy.⁶³

Physicochemical Properties of Liposomes

Particle size, PDI, zeta potential, EE, and D are properties and characteristics that are usually measured when designing liposomes. Table 1 summarizes the physical properties of liposomes prepared for skin delivery.

For instance, Petrilli et al¹³ reported an encapsulation efficiency of 0% in some liposomal preparations due to the incorporation of cholesterol into the liposomes (Table 1). The authors suggested that even when cholesterol produces bilayer stability, it displaces from the lipid bilayer, hydrophobic compounds, such as 5-fluorouracil, as confirmed by Hudiyant et al.⁶⁵ These authors found a constant decrease from 79.24% to 50.54% in the encapsulation efficiency of β -carotene as the concentration of cholesterol increased from 0% to 40%.

Most of the liposomes for skin delivery have small particle sizes, from 81.6 ± 6.5 to 143.4 ± 11.4 nm (Table 1). Particle size is a determining factor in skin permeation, which is particle size-dependent.⁶⁶ Although liposomes coated with chitosan with particles larger than 1 μ m have shown good permeation.⁶⁷

Liposomes, due their amphipathic nature, can improve the aqueous solubility of hydrophobic compounds.^{68,69} For instance, avequinone-B is a hydrophobic compound that aggregates forming conglomerates with a particle size of 4528 ± 595 nm giving a lower probability of cell internalization. However, liposomes encapsulating avequinone-B have a reduced particle size of 105.50 ± 3.68 nm which increases their bioavailability. PDI is also improved since liposomes have a PDI of 0.11 ± 0.04 , while for the agglomerates PDI was >1 .⁶⁹

Zeta potential is an important indicator of liposome stability and of probability of aggregation when the zeta potential is close to zero mV. For instance, zeta potential values far from zero mV showed more colloidal stability.⁷⁰ Liposomes loaded with a polar compound, ammonium glycyrrhizate, were found to have a zeta potential value of -22 ± 0.6 and encapsulation efficiency of 57.3 ± 3.7 .⁷¹

Other properties have also been measured. Tosato et al⁷² assessed the thermotropic behavior of lipid bilayers for

incorporating active compounds within a temperature ranging from -75°C to 75°C . The addition of 15 mg of resveratrol to deformable liposomes increased the enthalpy from 260 to 340 J/g suggesting that lipids in deformable liposomes have an orderly arrangement, such as the bilayer does not present major changes. The authors of this investigation found a similar behavior with ethosomes.

As indicated before, UDL was more deformable and elastic than conventional liposomes with better D values. Boakye et al⁷³ showed better D in UDL than conventional liposomes, 13.03 ± 0.71 and 9.86 ± 0.60 , respectively, indicating a better ability to squeeze through skin cells to deliver cargo.

The D value of UDL loaded with vismodegib was higher (D = 6152) than that of conventional liposomes (D = 2447) loaded with the same drug. D value was also improved by the incorporation of vismodegib. Empty UDL had lower D value (D = 4419) than loaded UDL (D = 6152), a similar behavior was shown with empty conventional liposomes which had deformability of 489-fold lower than loaded conventional liposomes.⁷⁴

Liposomes as Transporters

Liposomes as transporters were first described in 1964 by Alec Bangham.⁷⁵ Transporters can reduce the side effect of some drugs when loaded.^{47,76,77} There is also some evidence of possible dosage reduction when drugs are transported by liposomes.⁷⁸ These vesicles possess an important role in the delivery of substances into the skin, especially for lipophilic compounds.⁷⁹

Topical chemotherapy is becoming a promising strategy to avoid the side effects of conventional treatments. The development of low toxicity treatments, such as liposomes, has the advantage that the composition of these vesicles is closely similar to cell membranes, since they are elaborated with phospholipids and cholesterol.^{13,76} There is valuable scientific information regarding the use of liposomes for skin delivery of therapeutic compounds for treatment of skin cancer or for enhancing liposome permeability to improve drug delivery (Table 2). For example, Immunoliposomes loaded with 5-fluorouracil and cetuximab have been used against skin cancer cells A431. Drug-loaded transporter liposomes have also shown a potent cytotoxic effect against cancer cells after 120 hours of incubation.¹³

There are liposomes prepared for skin delivery (Table 2) that have encapsulated bioactive compounds that could be found in foods, such as curcumin (curcuma), naringin

Table 2 Liposomal Formulations for Skin Delivery of Different Compounds

Type	Encapsulated Compound	Aim	Outcomes	References
UDL	Oestradiol Concentration: 1 mg/mL	Evaluate Span 80 and Tween 20 as edge activators against Sodium Cholate for skin delivery in a human epidermal cadaver membrane.	Span 80 and Tween 80 are as effective as sodium cholate in terms of skin permeability if compared with a saturated aqueous solution.	[3]
UDL	Methotrexate Ratio: drug: lipid 1.5 w/w	Investigate the skin permeation potential of the chemotherapeutic encapsulated in the liposome. The skin used was abdominal male Sprague Dawley rats.	Skin permeation at 24h for UDL (470.3 $\mu\text{g}/\text{cm}^2$) was significant higher compared to the conventional liposomes (around 150 $\mu\text{g}/\text{cm}^2$), due to its deformability. Among the different vesicles that were prepared, PC: T80 ratio 7:3 w/w was more effective in skin permeation and deformability terms.	[25]
UDL	Psoralen, 5-methoxypsoralen, 8-methoxypsoralen Concentration: 5–15% respect to lipid weight.	Liposomes preparation Psoralen and derivatives-loaded to evaluate their efficacy in vitiligo treatment. B16F10 Melanocyte cells were used for cell uptake study.	UDL derivatives-loaded at a concentration of 2.5 μM shown stimulation of melanin and tyrosinase activity.	[38]
UDL	Naringin Concentration: 1–24 mg/mL	Formulate liposomes with different amounts of naringin, characterize them, and test their ability to diminish skin inflammation-induced in female CD-1 mice.	The treatment with UDL reaches an edema reduction of ~20% compared with the Naringin dispersion because the UDL promotes the internalization of the compound into epithelial cells.	[97]
UDL	Curcumin and STAT3 siRNA Concentration: Lipids: Curcumin ratio 5, 10 and 15:1 w/w. liposomes 10:1: STAT3 siRNA ratio 10:1	The co-delivery of curcumin and STAT3 siRNA by topical application for skin cancer treatment in epidermoid carcinoma cells (A431).	Cells treated with liposomes with both curcumin and STAT3 siRNA shows a bigger growth inhibition than the solutions or liposomes just with one of them. The nanoparticles suppressed STAT3 protein and induced apoptosis.	[94]
UDL	Pirfenidone Concentration: 5 mg/mL	Investigate the feasibility of load pirfenidone into liposomes for skin delivery, also enhance the permeation of the liposomes with a hydrogel with permeation enhancers. The tissue used for the permeation study was pig ear.	The permeation of pirfenidone loaded in liposomes was $251 \pm 4.2 \mu\text{g}/\text{cm}^2$, while free solution had a permeation $85.3 \pm 18.8 \mu\text{g}/\text{cm}^2$. When the vesicles are incorporated into a hydrogel with permeation enhancers such as isopropyl myristate and oleic acid the liposomes shown even more permeation ($344 \pm 28.8 \mu\text{g}/\text{cm}^2$).	[86]
CL	Curcumin	Measure the physical properties and skin permeability of curcumin-loaded liposomes using pig ear skin.	The melting temperature could be affected by the packing reduction of the bilayer when curcumin loading increases, even with that behavior, liposomes with the higher amount of curcumin have better permeability in the epidermis (retention of ~70%) and dermis (retention of 7%)	[87]
Ethosomes	Rhodamine B	Evaluate the percutaneous efficiency of a drug-loaded model and determine the mechanisms of skin permeation.	The permeation of loaded molecule in ethosomes is 2.8-fold higher than liposomes, and 2.5-fold higher than a mixture of ethanol and water, the authors suggest that the enhanced permeation is due to a synergistic mechanism among skin, ethanol, and phospholipids.	[28]

Abbreviations: CL, conventional liposomes; UDL, ultra-deformable liposome.

(citrus species such as grapefruit) and epigallocatechin-3-gallate (green tea), among others (Figure 6).

Liposomal Transdermal Drug Delivery Systems

Drug delivery mechanisms utilizing transdermal administration are regarded as best alternative to parenteral and oral methods due to its underlying advantages, such as avoiding the first-pass metabolism and encouraging patient compliance.²⁵ However, it encounters skin permeation disadvantages since it can only accept a limited type of drugs due to the effect of the stratum corneum layer, which can only stretch to a thickness of 40 μm .⁸⁰ Notable mechanisms adopted to increase skin permeation include electroporation, permeation enhancement, iontophoresis, microneedles, nanocarriers, and jet injectors.²⁵

Skin Delivery

Skin delivery of active compounds could be transdermal or topical. Transdermal occurs when an applied molecule crosses the skin layers until the bloodstream with systemic effects; meanwhile, topical has a localized action on the skin with minimal systemic absorption. This last type of delivery is important for pathologies located on the skin, such as skin cancer.^{81,82}

Transdermal delivery can improve the bioavailability and safety of drugs by reducing drug dose and application frequency due to a longer duration in the skin maintaining plasma levels due their low release rate. It also reduces side effects and avoids the first-pass metabolism in the liver compared to conventional oral delivery systems. High molecular weight compounds usually exhibit relatively low transdermal delivery efficacy, mainly because of the molecular weight, hydrophobicity, and charged state.⁸²

In addition to charge, particle size can be a major factor affecting transdermal delivery of the liposomes. Polyethylene glycosylated anionic liposomes with a diameter from 31 to 41 nm are able to cross the stratum corneum but particles larger than 105 nm can not reach deeper skin layers beyond the stratum corneum.⁶⁷

Skin Permeation

There are two routes to permeate the skin. Dermal appendage through sebaceous glands and hair follicles, and transdermal route through the intact stratum corneum, which occurs in two ways: intracellular, through lipoidal pathway channels, and transcellular, through keratinocytes (Figure 1A–C).^{80,81,83} The

passage of the molecules through stratum corneum is mainly by passive diffusion.^{80,83} However, one of the limitations of transdermal delivery is that macromolecules and hydrophilic compounds cannot penetrate through the stratum corneum.⁴⁷

Peralta et al⁸⁴ reported that ultra-deformable liposomes (UDL), with an average particle size of 100 nm, function effectively as enhancers of skin penetration. In addition, permeation of the liposomes depends on the molecule that was incorporated; a large insoluble molecule (amphotericin B) permeates less than a small soluble molecule (indole). In this case, the concentration of the molecules on the dermis was 22.2% and 0.9%, respectively.

In addition, Dar et al⁸⁵ showed that UDL improved the penetration across the skin of the drug-loaded, increasing deposits of sodium stibogluconate (SSG) and ketoconazole in the skin from 3.8% and 6.1% to 23.1% and 21.3%, respectively. The total area permeated when utilizing UDL loaded with SSG increased from 39.42 ± 7.43 to 211.63 ± 18.51 $\mu\text{g}/\text{cm}^2$.

Liposomes prepared with pirfenidone (5-methyl-1-phenylpyridin-2[H-1]-one) showed more permeation in porcine ear skin than the free drug when using a hydrogel prepared with oleic acid and isopropyl myristate as permeation enhancers.⁸⁶ Campani et al⁸⁷ used the same tissue to measure the permeation of curcumin loaded into liposomes. They show that liposomes with 15% w/w mass ratio of curcumin: phospholipids, increased retention of curcumin in almost 7% in dermis compared with a lower mass ratio (0.1% w/w of curcumin: phospholipids) which had a retention <1%.⁸⁷

Edge Activators

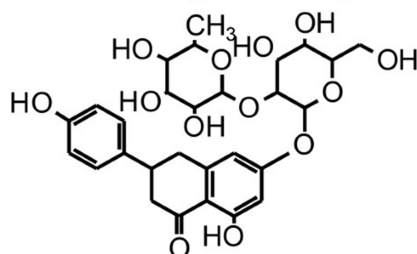
The use of chemical enhancers facilitates the permeation of large molecules through the stratum corneum. Common permeation enhancers include terpenes, glycols, surfactants, fatty acids, and pyrrolidones.⁸⁸

EA are chemical enhancers used to reach deeper skin layers by a hydration gradient. EA are generally single-chain surfactants with a high radius of curvature that allows more deformability of the liposomes, increasing permeability of the intact vesicle.^{89–91} Some of the compounds used as EA are Tween 80, Tween 20, Span 80, SC, and dipotassium glycyrrhizinate (Figure 7).²⁴ EA allow the vesicles to pass across pore sizes smaller than the vesicle itself by the relocation of EA on the lipid bilayer to the zones of higher curvature. This relocation avoids the disassembly of the liposomes while permeating through the skin.^{85,90}

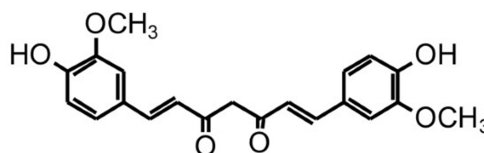
Lin et al⁸⁹ prepared UDL and found that cationic-UDL prepared with 11.33 mg/mL of Tween 20 had a higher

Naringin (C₂₇H₃₂O₁₄)

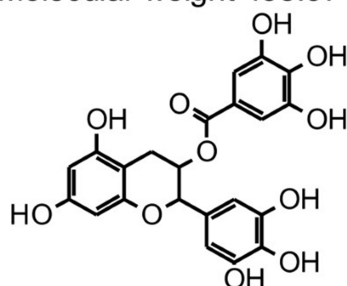
Molecular weight 580.4 g/mol

**Curcumin** (C₂₁H₂₀O₆)

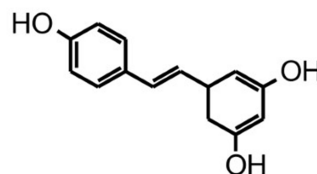
Molecular weight 368.38 g/mol

**Epigallocatechin-3-gallate**(C₂₂H₁₈O₁₁)

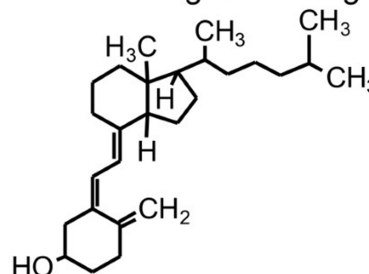
Molecular weight 458.37 g/mol

**Resveratrol** (C₁₄H₁₂O₃)

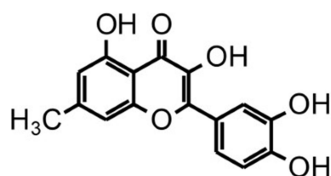
Molecular weight 228.24 g/mol

**Vitamin D₃ (Cholecalciferol)**(C₂₇H₄₄O)

Molecular weight 384.64 g/mol

**Quercetin** (C₁₅H₁₀O₇)

Molecular weight 302.24 g/mol

**Figure 6** Chemical structures of bioactive compounds that have been incorporated into liposomes.

deformability index (D) (135.08 ± 5.74) than liposomes without EA (55.71 ± 1.67). In addition, the permeability coefficient was higher in cationic-UDL ($3.54 \pm 0.09 \mu\text{g}/\text{cm}^2/\text{h}$) than in conventional liposomes ($1.29 \pm 0.10 \mu\text{g}/\text{cm}^2/\text{h}$). Perez et al⁹⁰ found that increasing the amount of EA while preparing the liposomes, promoted the increase of D. For instance, liposomes without EA had lower D (765 ± 267) than the two different concentrations of Tween 80 as EA. The concentration of phospholipid, Tween 80 ratios 43:28.5 w/w had a higher D (6938 ± 635) than the ratio 43:21.5 w/w (3923 ± 44).

Cell Uptake

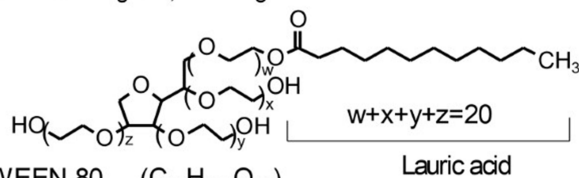
Nanoparticles can access the cell through different pathways, such as clathrin, caveolin mediated endocytosis,

micropinocytosis, and phagocytosis. However, the most common is endocytosis.^{92,93} Jose et al⁹⁴ demonstrated that cell uptake of liposomes is through clathrin and caveolae-mediated pathways using endocytosis inhibitors in A431 human epidermoid carcinoma cells. Fluorescence microscopy was used for determining cell uptake of liposomes loaded with curcumin applied to B16F10 mouse melanoma cells incubated from 30 to 120 min. This study showed that cell uptake increases proportionally with incubation time.⁹⁵

Cell uptake was analyzed by Petrilli et al⁹⁶ with confocal microscopy and flow cytometry in cell lines A431 and B16F10 treated with liposomes and immunoliposomes loaded with 3,3'-dioctadecyloxycarbocyanine perchlorate. They showed that immunoliposomes are easier to capture by the

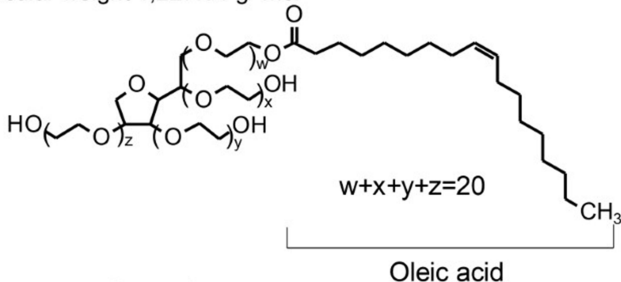
TWEEN 20 ($C_{58}H_{114}O_{26}$)

Molecular weight 1,309.65 g/mol



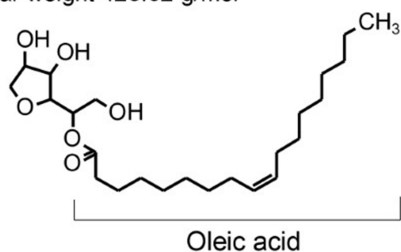
TWEEN 80 ($C_{64}H_{124}O_{26}$)

Molecular weight 1,227.51 g/mol



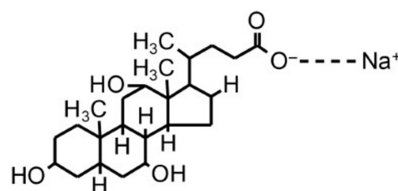
SPAN 80 ($C_{24}H_{44}O_6$)

Molecular weight 428.62 g/mol



Sodium Cholate ($C_{24}H_{39}NaO_5$)

Molecular weight 430.6 g/mol



Dipotassium glycyrrhizinate ($C_{42}H_{60}K_2O_{16}$)

Molecular weight 899.11 g/mol

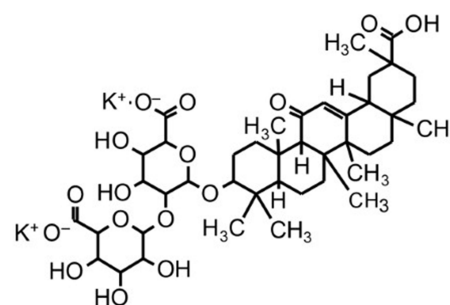


Figure 7 Chemical structures of most used edge activators in the preparation of liposomes.

cells than simple liposomes. The internalization of immunoliposomes in A431 cells was 75%, and for B16F10 cells only 6%.

Advantages and Disadvantages of the Use of Liposomes

Because liposome composition is like biological membranes, they can reduce the toxicity of the drugs. This type of vesicle improves bioavailability and solubility of loaded compounds and prevents their interaction with other molecules.⁹⁷

In skincare, liposomes can reduce the transepidermal water loss, supply lipids, and help to avoid dry skin by providing water to the stratum corneum.²¹

The degradation of the liposomes takes place when the hydrocarbonated chains hydrolyse the ester bond to glycerol, and by peroxidation of unsaturated chains. This latter effect depends on the kind of fatty acid contained in the phospholipids used for liposome preparation. Both could lead to short-chain lipids, which will form soluble derivatives decreasing the quality of the liposomes.¹⁹ Also,

some physical factors can affect the shelf life of liposomes. They include aggregation/flocculation and fusion/coalescence, size, and drug loss.¹⁹

Toxicological Aspects in the Use of Liposomes

There is some potential health risk when using nanomaterials. Some particle components may promote inflammation, cytotoxicity or genotoxicity in lungs after airway exposure^{98–100} and may also promote allergic sensitization.¹⁰¹ Therefore, it is imperative to conduct toxicological evaluations when using newly developed liposomes. Knudsen et al¹⁰² used rats to determine the potential toxicity of liposomes by conducting a systemic nanoparticle administration by intravenous injection to mimic the delivery route of a drug carrier using liposomes prepared as described by Gjetting et al.¹⁰³ Patients exposed to liposome treatment (Doxil[®]), usually receive between 148 mg and 1176 mg of the lipid vehicle, resulting in a range of 30 mg/L - 235 mg/L of lipids in blood. In this study, rats were given either 1.35, 3.38 or 13.5 mg of

lipid nanocarriers per rat, which equals 135, 337.5 and 1350 mg/L in blood, respectively. After the last of three intravenous injections of 100 mg/kg every other day, they observed little effect of the liposome treatment. However, genotoxicity observed as DNA damage in lungs and spleen, and gene expression of cytokines in lungs, spleen and liver were elevated. It was suggested that the particles cross the cell membrane due to their cationic surface charge, thus interfering with the intracellular space. Oxidative stress leading to inflammation and consequently DNA damage was also suggested. Therefore, there are opportunities to improve the properties of the liposomes to lower their potential toxicity.

To prevent toxicity of some chemotherapeutic drugs such as doxorubicin, liposome encapsulation has been an option. In addition, liposomes facilitate drug distribution to tissues with abnormal blood vessels and results in higher drug accumulation within the tumor compared with normal tissue. It was suggested that the use of liposomes decreased the incidence of cardiac and hematological toxicity.¹⁰⁴

The safety profile of liposomes has suggested a potential use for topical therapy of psoriasis. To treat psoriasis, nanoformulations are promising for better penetration, targeted delivery, and efficacy. In addition, a significant reduction in the level of central psoriatic cytokines, such as IL-17, IL-22, and tumor necrosis factor- α has been reported. It was concluded that the use of nanoformulations for novel drug delivery may provide highly competent and low toxic treatment modalities.¹⁰⁵

In an interesting approach to treat atopic dermatitis using siRNA, Ibaraki et al¹⁰⁶ tested a flexible liposome combination with the peptide AT1002 (seq: Phe-Cys-Ile-Gly-Arg-Leu) in a concentration of 100 $\mu\text{g}/10 \mu\text{L}$ of liposomes that opens tight junctions for better delivery by phosphorylating the structural protein ZO-1. One important finding was the enhanced intradermal penetration of the material without causing toxicity due to the action of the AT1002 and the flexibility of the liposome.

More studies are needed to establish the full genotoxic potential, in apparently low systemic toxicity. On the other hand, an in vitro study exposing hepatocytes to cationic liposomes (0.116 mg/mL, 24 hours), detected alterations in energy and lipid metabolism, and in proteins involved in stress response. Although this was a good omics exercise, in vivo studies are needed to better understand the toxicological behavior of liposomes.¹⁰⁷

Applications: Reduction of Inflammation and Treatment of Melanoma

Liposomal formulations have been developed for multiple purposes (Tables 2 and 3). UDL was prepared with curcumin (1:10 lipid: curcumin ratio) for the treatment of melanoma. It was shown that liposomes loaded with both, curcumin and complex STAT3-small interfering RNA, injected into the melanoma tumor decreased tumor volume from $3225 \pm 558 \text{ mm}^3$ to $1316 \pm 419 \text{ mm}^3$, and its weight from $4.61 \pm 0.49 \text{ g}$ to $1.59 \pm 0.69 \text{ g}$. The iontophoretic application showed a decrease in the tumor volume and weight, $726 \pm 128 \text{ mm}^3$ and $0.88 \pm 0.10 \text{ g}$, respectively.⁹⁵ In terms of curcumin penetration, a study showed that after incubation of liposomes in the skin for 24 hours, 2% to 7% of curcumin was present in the dermis.⁸⁷

In the case of skin cancer treatment, immunoliposomes were loaded with 5-fluorouracil and cetuximab and applied to cancer cells A431. Drug-loaded transporter showed a potent cytotoxic effect after 120 hours of incubation, with an IC_{50} of 7.2 nM and 0.7 μM for 5-fluorouracil and cetuximab, respectively.¹³

Temperature-sensible liposomes were prepared with phospholipids with T_c higher than 37°C , which allows control of drug release on the desired site by applying an external thermal source. These vesicles were prepared with lysophospholipids, phospholipids, and sorbitan monooleate as EA ratio: 70:15:15. Using a lipid drug (tamoxifen: imatinib) at a ratio of 3:10 showed a high encapsulation efficiency (<70%) and a T_c of 39.4°C . The liposomes were tested in cell lines MCF-7 and MDA-MB-231.¹⁰⁸

Patents related to liposomes and skin are regarding mostly for cosmetic products aimed to care for the skin (Table 3). There were not recent patents directed to liposomes and skin cancer. Patent No. US 20090232881A1¹⁰⁹ is aimed to prepare liposomes for UV blocking by loading zinc oxide, iron oxide, silica, titanium dioxide, ultrafine titanium dioxide in either of its two forms, water-dispersible titanium dioxide or oil-dispersible titanium dioxide.

Inflammation

Inflammation is highlighted for its role in defense against pathogens. It has also an important role in tissue reparation, regeneration, and remodeling. Slight inflammation is essential for tissue homeostasis.¹¹⁰ Inflammation is present as a response to cell death or microbial/viral infection due

Table 3 Patents for Liposome Formulation in Skincare

Patent	Country	Aim of Patent	Entrapped Compounds	Highlights	Inventor	Assignee
US8,846,611 B2, 2014 ¹³⁴	United States	To provide a formulation for topical administration for improving skin conditions with human growth hormone as an active ingredient.	Human growth hormone	The liposomes are prepared with soybean or egg yolk lecithin as the lipidic phase, the vesicles are can be used in cosmetics or pharmaceutical products.	Kyun Oh, Dahl; Jung Baik, Sang; Young Lee, Kyung	Regeron Inc
US2012/0201871A1, 2012 ²³	United States	To provide a method to produce small liposomes with components that enhance skin permeation.	Water	The composition of the liposome includes phospholipids, oil-rich essential, fatty acid, behenic acid, oleic acid, skin lipids, and butter-rich in linoleic and linolenic acid.	Kyeong-Nam, Yu	Professional Compounding Centers of America, LTD.
US2009/0232881A1, 2009 ⁰⁹	United States	To achieve an enhanced delivery of skin lightening or UV blocking agents to the skin.	A skin-lightening agent such as niacinamide and a UV-blocker, as 2-ethylhexyl-p-methoxycinnamate or butylmethoxydibenzoylmethane	The loaded compound was loaded in the range of 0.1 to 40% by weight of liposomes, which were prepared with soybean lecithin; mono-, di- or tri-ester of glycerol	Bandyopadhyay, Prasan; Bandyopadhyay, Punam	
WO2009/094238A1, 2009 ¹³⁵	International	The development of a vesicle with exceptional cosmetic, oral, and dermatological properties.	Propose the inclusion of water-soluble ingredients.	The lipidic phase is conformed by fatty acid esters and olivates.	Rosemarie, Lorenzo; Nadya, Lawrence	IGI Laboratories, Inc
WO2007/041627A1, 2007 ¹³⁶	International	To prepare liposomes capable to entrap hyaluronic acid, collagen, and other skincare agents to improve the condition of the skin.	Hyaluronic acid, collagen, and other skincare agents.	The liposomes are used in a day cream.	Mark A. Pinsky	
WO2006/014035A1, 2006 ¹³⁷	International	To prepare a multilayered liposome that entrapped active substances	Physiological active substances such as peptides, proteins, nucleic acids, synthetic compounds, natural extracts, sugars, vitamins, and inorganic compounds.	The liposome is prepared with: squalane, sterols, ceramide, neutral lipids, fatty acids, and lecithin as a lipidic phase.	Deok-Hoon, Park; Jong-Sung, Lee; Kwang-Sun, Jung	Biospectrum Inc.
US2006/0292244A1, 2006 ¹³⁸	United States	The use of liposomes carrying PVP-iodine for acne treatment.	An antiseptic compound such as PVP-Iodine	The antiseptic agent concentration could be between 0.1 and 10 g in 100 g of preparation, which contains 1–5 g of liposomes. The formulations can be emulsions, dispersions, suspensions, solutions, gels, ointments, waxes, spray, lotions, among others.	Reimer, Karen; Fleischer, Wolfgang; Hopp, Michel	

US2004/0180082A1, 2004 ¹³⁹	United States	To provide a method for submicrometric liposome preparation containing high concentrations of triterpenoid	Triterpenoid with one or more acid groups such as ursolic acid, <i>Centella asiatica</i> extract, etcetera.	Liposomes are prepared preferably with soybean or egg lecithin. The content of triterpenoid is 0.5–2.5% by weight of total liposome. The liposomes could be incorporated into skincare products such as cosmetic, pharmaceutical or quasi-pharmaceutical.	Hyung-Seok, Kang; Gae-Won, Nam; Sang-Hoon, Han; Ih-Seop, Chang	Amorepacific Corporation
US2002/0128222A1, 2002 ¹⁴⁰	United States	To create a wound coverage material with liposomes.	Genes encoding growth factors for wound healing.	Cationic liposomes containing cholesterol. The concentration of gene-encoding growth factor is 2.2 µg/mL	Herdon, David N; Perez-Polo, Jose R; Barrow, Robert E	Research Development Foundation
US6,365,145B1, 2002 ¹⁴¹	United States	To provide a method for hair removal using a liposomal composition	Photosensitizers such as phthalocyanines, porphyrins purpurins, psoralens, bergapten, angelicins, chlorins, and flavins.	The liposomes are prepared with egg yolk lecithin and cholesterol.	Ben-Hur, Ehud; Zuk, Maria M; Wai-Shun, Chan	New York Blood Center Inc

to stimulation of tissue regeneration and fight against pathogens. However, excessive inflammation can lead to different pathologies as metaplasia, fibrosis, and cancer. Up to 20% of human cancers are related to chronic inflammation.¹¹¹ Chronic inflammation destroys the tissue parenchyma, which can result in tumorigenesis.¹¹²

Factors as infection, tissue damage, activation of oncogenes, and loss of tumor suppressors can activate the inflammatory response in tumors.¹¹³ Almost all tumors are characterized by an inflammatory tumor microenvironment composed by inflammatory cells and mediators.^{110,111,114} Inflammatory cells and their products participate in tumor incidence and progression. Some of the inflammatory cells include lymphocytes, dendritic cells, neutrophils, immature myeloid cells, mast cells, eosinophils, and tumor-associated macrophages (TAM), which are the most abundant immunosuppressive leukocytes in the tumor microenvironment.^{113,115–118} These inflammatory cells have been founded in melanoma, squamous cell carcinoma, and basal cell carcinoma.¹¹⁶

Since macrophages are the most abundant myeloid cells in cancer, these cells are the most important target for therapies that modulate myeloid cells in the tumor microenvironment.^{112,119} There are two types of macrophages: M1 classically activated macrophages, which have a role in inflammation, cytotoxic effector cells in Th1 immune responses. M2 alternatively activated macrophages are involved on immunosuppression, scavenging of debris, and tissue repair.¹²⁰ However, they can adopt M1- or M2-like activation profiles.¹²⁰ TAM exhibit an M2-like profile, they promote angiogenesis, tumor-cell progression, metastasis, and suppress activated T cells, such as B7 family, B7-homolog family including programmed death-ligand 1. Macrophages control the memory of CD4⁺ and CD8⁺ T-cell responses, producing chemokines, growth factors, also inflammatory cytokines (IL-1, tumor necrosis factor α , IL-6), regulatory cytokines (IL-4, IL-13 IL-10, transforming growth factor β), and T-cell-polarizing (IL-12, IL-1) cytokines.^{117,119}

Piaggio et al¹¹⁸ developed clodronate-loaded liposomes, which is a macrophages-depleting drug. The vesicles were tested in RAW 264.7 murine macrophages cells, B16F10 murine melanoma cells, and FIBRO/293 human skin fibroblasts cells. The formulations were able to decrease cell viability of RAW 264.7 in a dose- and time-dependent manner. The dosage with the highest inhibition for these cells was 25 µM in 24 hours (55%) and 48 hours (80%) of treatment. B16F10 was inhibited only 20% after

48 hours with a concentration of 25 μM . When the liposomes with DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate) and the clodronate in a concentration of 200 nM were applied to a murine model, they showed that the tumor decreased from 668.9 mm^3 to 154.7 mm^3 .

Skin cancer-related inflammation was treated with liposomes loaded with resveratrol (2.5 mg) and quercetin (12 mg) applied on the skin of mice. Inflammation was induced by TPA (12-O-tetradecanoylphorbol 13-acetate). Liposomes showed a good response against inflammation, reducing 50% oedema. This was because the active compounds internalized on fibroblasts potentiating the anti-ROS activity, resulting in tissue repair and wound healing.⁷⁹

Naringin-loaded UDL reduced skin oedema in $\sim 20\%$. The inhibition of myeloperoxidase (MPO), as inflammation marker was tested, showing that UDL with naringin concentration of 9 mg/mL had the highest inhibition ($86.75 \pm 4.93\%$), while betamethasone cream only had an inhibition of $12.70 \pm 1.81\%$.⁹⁷

Oedema and MPO were also inhibited in female CD-1 mice when 100 μL of liposomes prepared with 100 mg of curcumin, and/or 10 to 50 mg of sodium hyaluronate were topically applied. Liposomes with curcumin and sodium hyaluronate had an inhibition of oedema of $81 \pm 2\%$ and MPO of $82 \pm 3\%$, while liposomes with curcumin inhibited $80 \pm 3\%$ and 67 ± 3 , respectively.¹²¹

In addition, liposomes hydrated with 100 mg/mL of budesonide disodium phosphate decreased the expression of the pro-angiogenic factors by 50% to 70% in tumors induced by inoculation of 1×10^6 B16F10 melanoma cells in C57B1/6 mice. The main factors inhibited were granulocyte-colony stimulating factor, granulocyte-macrophage-colony stimulating factor, monocyte-colony stimulating factor, insulin growth factor II, IL-1 β , IL-12p40, Fas ligand, basic fibroblast growth factor, vascular endothelial growth factor, and by 75% to 100% of IL-1 α , IL-6, IL-9, tumor necrosis factor α , monocyte chemoattractant protein-1, and leptin. Pro-angiogenic factors are related to favoring angiogenesis and tumor-associated inflammation.¹²²

On an in vivo study, a solution of ammonium glycyrrhizate (AG) was loaded into ethosomes and applied to eight human healthy volunteers pretreated with 0.2% w/v methyl nicotinate solution for erythema development in forearm. Results showed that the anti-inflammatory activity of AG increased while incorporated into ethosomes by decreasing the erythematosus index 15-fold in 1 hour, and 30-fold after 2 to 6 hours compared to control.⁷¹

Melanoma

Melanoma or malignant melanoma is a tumor that occurs more frequently in the skin and less in the eye, meninges, and mucous membranes, such as the mouth or genital region. In men, it appears 51% on the trunk and 15% on the legs; in women, 24% of the time appears on the trunk and 38% on the legs.¹²³

The melanoma progression model includes 5 stages as follows (Figure 2).¹²⁴

1. Congenital and common acquired nevi from normal melanocytes.
 - (I) Dysplastic nevi are characterized by atypical morphologic and structural features (stages IA and IB).
 - (II) Micro invasive malignant melanoma or radial growth phase involves intraepidermal proliferation and pagetoid spread (stages IIA, IIB and IIC).
 - (III) Invasive malignant melanoma or vertical growth phase. In this stage, melanoma penetrates the underlying dermis and metastasized (poor prognosis).
 - (IV) Metastatic melanoma is characterized by metastases to other areas of the skin and distant organs.

One study reports that 46% of patients on stage III present a BRAF gene mutation.¹²⁵ In this sense, several pathways of melanomagenesis have been described, such as mitogen-activated-protein-kinase (MAPK), phosphoinositide-3-kinase (PI3K), cyclin-dependent kinase inhibitor 2A (CDKN2A) (Figure 8).^{124,126,127} From the last one, there are two main alterations, the inactivation of p16^{CDKN2A} and amplification of CCND1, the first genetic (gene mutation or chromosomal rearrangement), or epigenetic (promoters region methylation) mechanisms. Other common mutations are located on NRAS and BRAF genes which belong to MAPK.^{124,126–128} The most predominant mutation in melanoma is the replacement of valine for glutamic acid at codon 600.^{127,128} This mutation is dependent on RAF/MEK/ERK signaling¹²⁹ and over-stimuli cell proliferation leading to a prolonged cell survival.¹²³

Melanoma Treatment

The treatment includes surgical therapies that involve the excision of the tumors or nevi which could be from minimal margins on stage 0, to 3 cm margins. The thickness

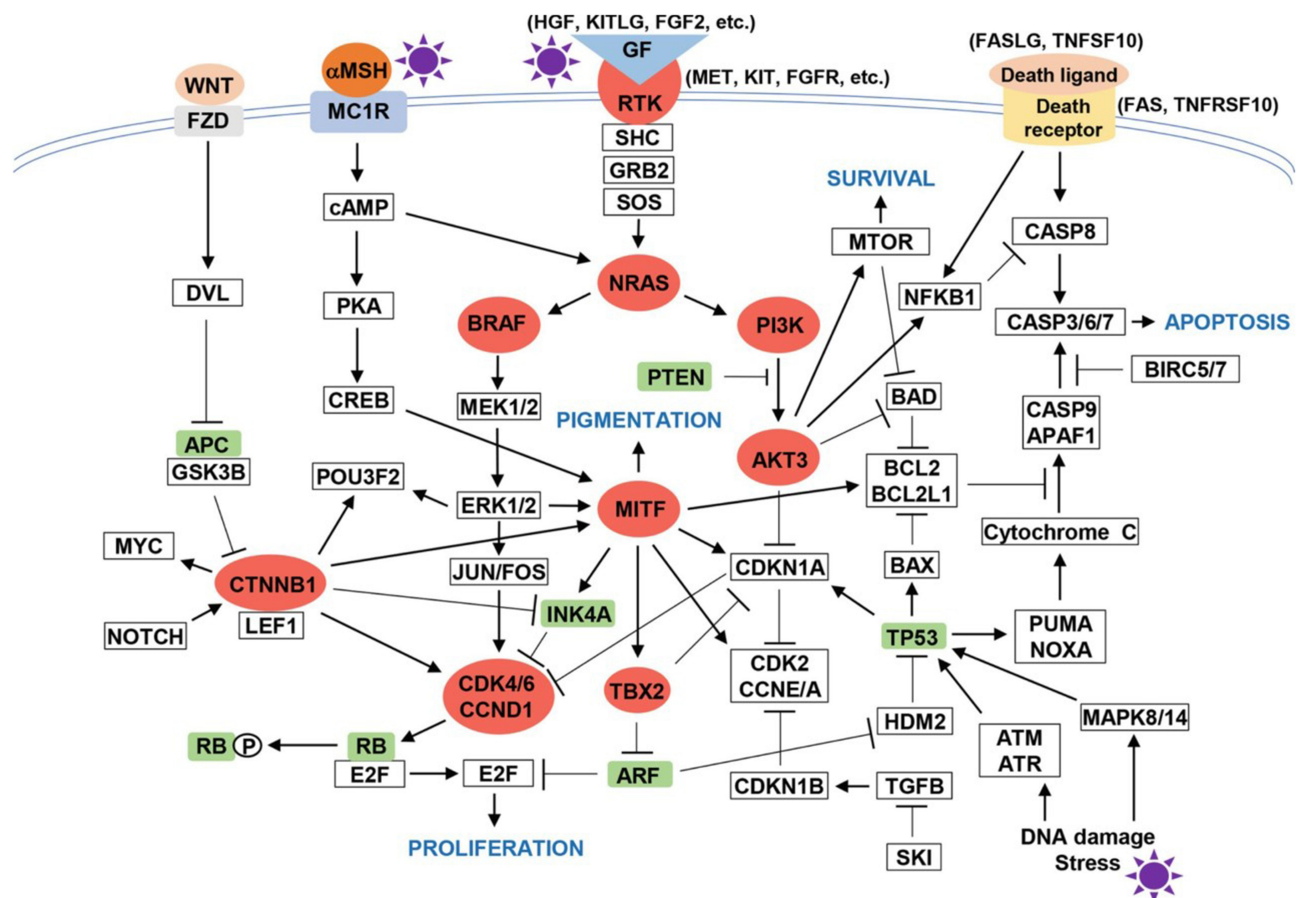


Figure 8 Melanogenesis pathway.

Notes: Reprinted with permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Springer eBook, Zaidi MR, Fisher DE, Rizos H, et al. *Biology of melanocytes and primary melanoma*. In: Balch CM, Atkins MB, Garbe C, editors. *Cutaneous melanoma*. Cham: Springer International Publishing; 2020:3–40.¹²⁴

will depend on the tumor stage and sentinel lymph nodes dissection.¹³⁰ Surgery becomes the primary treatment for patients who have melanoma in stages I to IIIB.¹³¹

Several treatments approved depend on the tumor location, stage, and genetic profile. In addition to surgical treatment, other therapy options, such as chemotherapy, radiotherapy, photodynamic therapy, immunotherapy, and targeted therapy are available.^{131,132}

The surgical treatment should be followed by other pharmacological therapy due to the high risk of cancer recurrence.^{130,132} Some available therapies are focused on BRAF inhibition alone or combined with MEK inhibitors.^{130,133} The available BRAF inhibitors are vemurafenib, encorafenib, and dabrafenib. The available MEK inhibitors are trametinib and cobimetinib.¹³⁰ Nevertheless, a combination of both inhibitors seems to have better results.^{130,133} Some of the side effects involve rash, cutaneous squamous cell carcinoma, fatigue, alopecia, among others.¹²⁹

Recently, messenger RNA (mRNA) has been incorporated as a new melanoma treatment. It has some advantages as mRNA is not included in the genome, which precludes problems associated with gene therapy. Also, it is directly translated in the cytoplasm and is capable to activate immune response. However, it can produce long-term effects. Nevertheless, some limitations need to be resolved such as making the industrial scale cost efficient and the sensibility to degradation through manipulation which means low in vivo stability.¹³²

Some treatments that are proposed involve the use of nanocarriers as liposomes. The topical treatment of melanoma with BRAF-targeted small interfering RNA (siRNA) trapped into UDL, was tested in UACC-903 human melanoma cells. Cells treated with liposomes with BRAF-siRNA showed a decrease in expression of BRAF protein.¹⁵ In another study of liposome-based treatment, liposomes with Tumor Necrosis Factor-related apoptosis inducing-ligand (TRAIL) and paclitaxel trapped and

a peptide that binds to $\alpha_v\beta_3$ integrin receptors attached were tested in female C57BL/6 mice and female SD rats. The results showed that animals treated with liposomes had a tumor reduction of 95.3% and 93.8% compared with those treated with saline.¹⁶ Liposomes loaded with kinases involved in cell cycle progression inhibitors and an apoptosis regulator (Bcl-xl) targeted siRNA were tested in B16 mouse melanoma cell line. These nanoparticles increased the G2/M fraction from 11.8% to 79% compared with the control. In C57 mice, it produced a reduction of 34% in the weight of the tumor with respect to the control.¹⁷

Conclusions and Future Perspectives

Methods of liposome production are still practically the same since their original development. Their physicochemical properties are affected by their components. Inclusion of molecules, such as cholesterol, into the lipid bilayer, affects the encapsulation efficiency of hydrophobic molecules. Also, incorporation of EA enhances the flexibility of the vesicle allowing its passage through a pore size smaller than the liposome itself preserving the integrity of the vesicle. Liposomes are a good option for the incorporation in skin therapy since they can decrease the side effects of skin cancer treatment. The evidence suggests that liposomes seem to be a promising nanocarriers with low toxicity, nevertheless more studies are needed to assess their genotoxic potential by using genotoxic tests and in vivo studies testing anionic and cationic liposomes. The incorporation of EA in the liposomes (UDL) entails an increase in skin permeability and, in some cases, drug penetration. The encapsulation of bioactive molecules such as curcumin, or naringin into liposomes gives another perspective for the usage of these nanoparticles for skin delivery because the vesicles potentiate the outcomes of the treatments. Studies about the relation between particle size and the permeation of the liposomes need to be performed, as well as the mechanisms of skin cells absorption. Research is still needed on the incorporation of bioactive peptides into liposomes for skin cancer treatment. Since cancer therapy produces several side effects, an interesting perspective for liposomes in skin therapy is the targeting of those vesicles by incorporating antibodies; for instance, the development of immunoliposomes targeting skin cancer or other diseases.

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Disclosure

The authors report no conflicts of interest for this work.

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