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Carrier Deformability in Drug Delivery

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Abstract: Deformability is a key property of drug carriers used to increase the mass penetration across the skin without disrupting the lipid barrier. Highly deformable vesicles proved to be more effective than conventional liposomes in delivering drugs into and across the mammalian skin upon topical non occlusive application. In the past five years, highly deformable vesicles have been used for local delivery of drugs on joint diseases, skin cancer, atopic dermatitis, would healing, psoriasis, scar treatment, fungal, bacteria and protozoa infections. Promising topical vaccination strategies rely also in this type of carriers. Here we provide an overview on the main structural and mechanical features of deformable vesicles, to finish with an extensive update on their latest preclinical applications.

Keywords: Topical drug delivery, highly deformable vesicles, skin cancer, topical vaccination.

1. INTRODUCTION

Topical drug delivery is an attractive route of administration that avoids the use of injectables or bypasses the gastrointestinal tract and first pass metabolism, when the skin itself (epidermis or dermis) is the target tissue [1]. Targeting the various layers of the skin becomes relevant in pathologies such as neoplasias, inflammatory disorders and microbial infections. In such cases, the topical route is a direct drugs pathway to those targets sited a few hundred of micrometers beneath the surface. Targeting the skin by topical route increases the biological potency of drugs, prolonging their effect, thereby reducing therapeutic dosage and the risk of adverse events. Transdermal or percutaneous delivery on the other hand, when subcutaneous tissue or systemic circulation is the target, is also an attractive alternative to systemic and oral routes of administration. The transdermal route may overcome the limitations of the oral route, including avoidance of drug degradation by the harsh gastrointestinal medium, avoidance of systemic first-pass metabolism and providing high compliance.

The skin is naturally organized to repel the entrance of foreign material [2]. Because of this, one most challenging task is developing drug delivery systems capable of entering the skin without disrupting such natural barrier. In this sense, a wide portfolio of therapeutic possibilities has recently been opened by new products based on soft nano-particulate drug delivery systems. Liposomes are the most popular and well studied of these systems, that have been largely employed on topical drug delivery since the early eighties [3]. Today, the problem of selectively accessing deep epidermal layers without entering the dermis or systemic circulation remains unsolved. Here we first will re visit the mechanisms responsible for the mass transport across the *stratum corneum* as a barrier to permeation and penetration. The main features of deformability, a unique elastomechanical property of drug carriers to increase the mass penetration across the skin, will be highlighted. Finally, we will provide an extensive update on the preclinical uses of highly deformable vesicles as drug carriers.

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2. OVERALL SKIN ORGANIZATION

The skin is a thin (normally less than ≤ 2mm thick, up to 16% of the body weight) and large (surface area of ~1.8m^2) human organ. Its permeability is 10^2-10^4 times lower than that of a blood capillary wall. The outer section of the skin is the epidermis, which physically separates the organism from its environment and serves as first line structural and functional defense against dehydration, chemical substances, physical insults and micro-organisms. The epidermis is made of avascular, stratified layers (in humans generally 0.02-0.2 mm, typically 50- 150 μm thin) of keratinocytes. Keratinocytes undergo a dramatic transformation as they differentiate and migrate outwards to replace cells that are shed from the body surface [4]. While basal cells of the '*stratum basale*' remain attached to an underlying matrix and proliferate, some of their daughter keratinocytes enter the spinous layer (or '*stratum spinosum*') through asymmetric mitoses, where they exit the cell cycle, grow larger and establish robust intercellular connections. Cells in the granular layer (the '*stratum granulosum'*) flatten and assemble a water impermeable cornified envelope underlying the plasma membrane. Finally, corneal layer (or '*stratum corneum*' SC) keratinocytes (corneocytes) release lysosomal enzymes to degrade major organelles, become completely squamous and are tightly crosslinked together to complete the cutaneous barrier [5]. Thus, the mature epidermis exhibits tissue-level polarization with asymmetric distribution of signaling activity, protein expression and cytoarchitectural organization that reflects the unique functions of its multiple layers [6]. The cell layers of the epidermis are mostly keratinocytes, together with a few number of Langerhans cells, melanocytes and Merkel cells. The skin is equipped with immunocompetent cells, which are, besides of keratinocytes, the Langerhans cells, subsets of T lymphocytes and strategically located lymph nodes constituting the skin-associated lymphoid tissue (SALT) [7]. The SC, as the outermost layer of the epidermis, is the primary barrier of the skin. Keratinocytes, besides of being responsible for establishing the physical barrier of the skin and guaranteeing the structural integrity of the epidermis, produce a wide range of cytokines upon activation by various stimuli [8]. These cytokines shape the local microenvironment to help maintain the appropriate balance of skin immune responses, and stimulate the maturation and migration of Langerhans cells.

The epidermis is connected by the dermo-epidermal union to the dermis, a normally up to 2 mm in thickness vascularized region

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divided in outer papillary and inner reticular dermis, which taken together are usually 5-20-times thicker than epidermis. The dermis contains only few cells, predominantly fibroblasts and adipocytes, and a lot of collagen (~70 % wt). The collagen bundles shelter and support glands, some immunologically active cells, nerves, plus dermal lymph and blood capillaries. The segmental area of cutaneous blood vessels peaks approx. 100-150 μm below skin surface, i.e. within the papillary dermis embracing the upper cutaneous blood plexus [9]. This cutaneous microvasculature represents the secondary skin barrier, by acting as a sink for the molecules that had diffused across the primary skin barrier. The total blood vessels area then decreases quasi-exponentially to a depth of approximately 0.75 mm. The papillary dermis contains relatively thin elastic elaunin fibers that are broadly perpendicular to skin surface. These fibers merge with the microfibrillar cascade (oxytalan fibers) and then intercalate into the dermal-epidermal junction. The collagen fibers in the reticular dermis are much thicker and largely parallel to skin surface. Dermal collagen thus forms a continuous, elastic network and imparts mechanical elasticity to the skin from the reticular and papillary dermis and up to the epidermis [10]. The dermis transitions into the hypodermis which together with the dermal fatty deposit helps to absorb mechanical shocks that might otherwise endanger skin vasculature and nerves. The same structures and deposits also contribute to skin elasticity. Hair, nails, sebaceous, sweat and apocrine glands are regarded as derivatives of skin

2.1. The Primary Barrier of the *Stratum corneum*

In humans, the SC is a transparent 5-20 μm thick layer, which consists of dead keratinocytes, the corneocytes, embedded in lipid regions (< 15% w) which are attached chemically to corneocyte envelope membranes [11, 12]. The corneocytes are flat, nearly co-planar 0.3-0.5 μm thick cells that are vertically stacked into penta to heptagonal columns. Each column contains between 15 and 22 cellular layers [13]. Each flat superficial corneocyte covers 1100 - 1200 μm^2 of the skin; deep in the epidermis, the cells are typically thicker and smaller, 700- 750 μm^2 [14]. In basal layer, for example, the average cell width is approximately four times smaller than in the SC [15]. The average number of corneocytes per unit area is ~$2x10^6$ cm^{-2}. The corneocytes are grouped together in clusters, 100 to 250 μm in diameter, separated by 10- to 25- μm-wide micro-anatomical skin-folds called canyons [16].These canyons occasionally extend down to depths comparable to that of the dermal-epidermal junction below the flat surface regions in porcine and human skin [17].The corneocytes are filled with keratin filaments, water and the natural moisturizing factor [18] and are surrounded by a densely crosslinked protein layer, the cell envelope. A monolayer of lipids is chemically linked to the cell envelope [19]. This lipid monolayer serves as an interface between the hydrophilic corneocytes and the lipophilic extracellular lipid matrix. Furthermore, corneodesmosomes interconnect the corneocytes and play an important role in the SC cohesion. The loss of cells from the SC is compensated by the cell growth in the *stratum basale*. In this way the thickness of the epidermis remains approximately constant [6]. Steep water concentration decline from around 75% in the viable epidermis to merely 10-30% at an air-exposed skin surface [20]. Skin surface pH is predominantly below neutral and typically slightly above 5 in humans [21].

3. BARRIERS TO MASS TRANSPORT I: THE ROLE OF CELLS, LIPIDS AND DIFFUSION ACROSS THE *STRATUM CORNEUM*

Excluding the water molecules which ordinarily evaporate at a daily rate of 0.4 mg h^{-1} cm^{-2} the skin is an unassailable barrier to mass transport [22]. The barrier function of the skin which is highest at ~50% of the SC thickness [23], has been thoroughly examined by the cosmetics and pharmaceutical technology. In the two fields, the products have to target skin sites after a -theoretically-controlled disruption of such barrier.

The mass transport across the skin is classically described in terms of permeation. The permeation of molecules is a process driven by molecular hydrophobicity, size (normally < 0.4 nm [24]), and the ability to interact with the other molecules, e.g. via hydrogen bond formation [25]. According to Cronin, the permeation across the skin is related to the octanol/water partition coefficient (K_{ow}) of a molecule, as depicted by the following equation:

$$\text{Log } K'_p = \log P - \log (D/d_{sc}) = 0.77 \log K_{ow} - 0.013 \text{ MW} - 2.3 \quad [n=107]$$

where K'_p is the permeability coefficient, P the SC partition coefficient, (D/d_{sc}) is the ratio of diffusion coefficient and barrier thickness and MW the molecular weight [26]. In the practice, the transdermal diffusion of large compounds falls below 1μg h^{-1} cm^{-2} when either MW > 328 for log K_{ow}=1 or else when permeant hydrophilicity exceeds log K_{OW}= -1.34 for MW = 50, always assuming 1 wt % drug-in-vehicle solubility limit. The mathematical expression of parameters governing permeation fits with a model where mass transport occurs across hypothetical pathways of ~ 0.5 nm pore width (the size of very small molecules) [27]. The permeation across the skin therefore, is well described as a process depending on a concentration gradient of small molecules.

The different types of intercellular unions bring cohesivity, compartmentalization and functionality to each strata of the epidermis and dermo-epidermis (Fig. **1**); their dysfunction being responsible for multiple, some of them lethal pathologies [6]. The transcellular diffusion across the stacked dead cells (leaving in normal mammalian skin an intercellular distance between 44-100 nm [28]) of the SC, is practically unimportant for transdermal drug transport: molecular diffusion occurs across the intercellular lipids [29, 30]. As seen above, the barrier confines molecules heavier than 400-500 Da to the skin surface, and its height decreases with permeants' lipophilicity [31]. Such lipophilic barrier is posed on the MW and distribution coefficient rather than on molecular size of permeants. The co-planar lipid multi-lamellae have for the most part 8 (and up to 10) individual layers that are laterally joined by thinner "linker regions" comprising 2, 4 or 6 lipidic layers [28]. The linker regions are both longer and more abundant at corneocytes periphery. It is believed the diffusion of lipidic and amphiphilic molecules occurs across discontinuities of the lipid barrier: the interlamellar regions in the SC, including linker regions, and the non-planar spaces between crystalline lipid lamellae and their adjacent cells outer membrane contain less ordered lipids and more flexible hydrophobic chains. Fluid lipids in skin barrier are crucially important for transepidermal diffusion as they secure the "free volume" needed for such molecules insertion and migration through intercellular lipid layers **(**Fig. **2**) [31]. Heavier molecules, with independence of their partition coefficient, are confined to skin surface. Large permeants need of sufficiently wide defects in the intercellular lipidic matrix to start diffusing through the lipids [29].

4. BARRIERS TO MASS TRANSPORT II: HYDROPHILIC PATHWAYS IN THE *STRATUM CORNEUM*

The skin contains also hydrophilic pathways, which description is far more complex than the lipids organized around the keratinocytes. These hydrophilic pathways have a role in the mass transport that is not mediated by chemical permeation enhancers (that solubilize and partially extract the least ideally organized lipids in the skin [32, 33]).

Hydrophilic regions are created by non-planarity of corneocytes outer membrane especially at the lateral cells junctions. Hydrophilic molecules diffuse predominantly "laterally", i.e. along surfaces of the water filled inter-lamellar spaces or through such volumes; polar molecules can also utilize the free space between a lamella and a corneocyte outer membrane to the same end [34]. Schätzlein and Cevc describe two types of hydrophilic penetration pathways across the normal mammal SC: one is a lowest resistance pathway, leads between clusters of corneocytes at the locations where such cellular

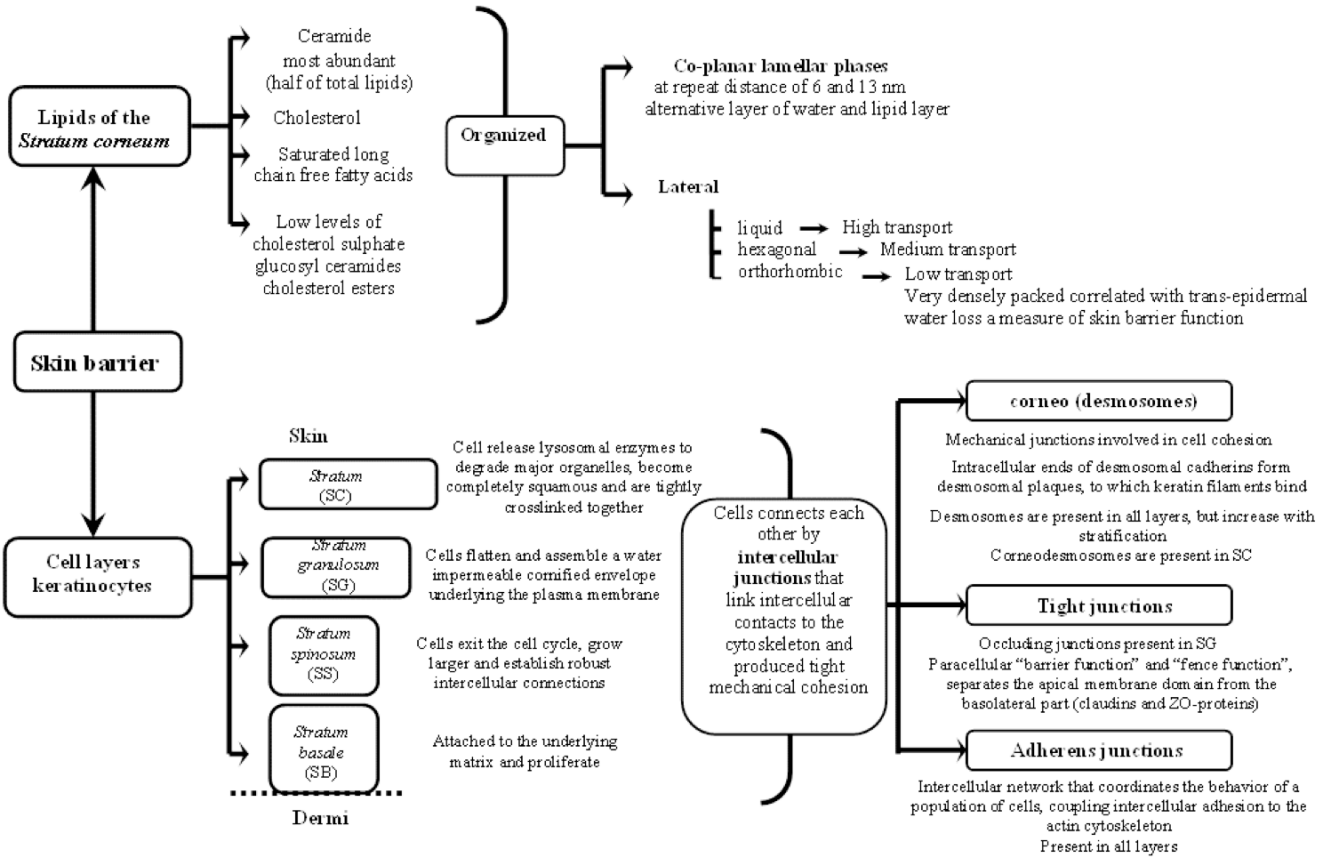

Fig. (1). Scheme of the structure of the skin functioning as a barrier.

groups show no lateral overlap. The other is the better sealed and more transport resistant, intra-cluster/inter-corneocyte pathway. Pathways of the latter kind lead between lipidic intercellular (multi)lamellae and/ or the adjacent corneocytes outer membrane [35]. Hydrophilic molecules/nanostructures therefore have the propensity to cross normal skin between corneocytes clusters or, if through a cluster, along the most curved cell plasma membranes. Despite the prevailing naturally occurring hydrophilic conduits through the SC are merely 0.5-10 nm wide [31], Cevc and Vierl recently depicted different types of hydrophilic pathways (excluding hair appendages) occurring at different depths, densities and widths within the SC [23]. For instance, ~ 3-8×10^4 nm width hydrophilic pathways within canyons, with a relative abundance of 10^4 cm^{-2} at the surface, that get thinner (~10^2 nm) towards the deep SC (Fig. **2**). Also << 5×10^4 nm width, ~10^8 cm^{-2} pathways within the inter-cells-cluster at the surface of the SC, having a width reduced at <10^2 nm in the deep SC. In such view, regions of poor cellular and intercellular lipid packing coincide with wrinkles on skin surface (intercellular "gorges") and are simultaneously the sites of lowest skin resistance to the transport of hydrophilic molecules/nanostructures.

Stimulated Raman scattering (SRS) microscopy is a label free methodology for acquiring three-dimensional (3-D) structural information with a high degree of molecular specificity in real time [36]**.** SRS imaging provided an improved understanding of the skin architecture first observed with two-photon microscopy [16]**.** In spontaneous Raman scattering, one laser beam at a frequency ϖp illuminates the sample and the signal is generated at the Stokes and anti-Stokes frequencies, ϖS and ϖAS, respectively, due to inelastic scattering. In SRS, two laser beams at ϖp and ϖS coincide on the sample. When the difference frequency, $\Delta\varpi = \varpi p - \varpi S$, also called the Raman shift, matches a particular molecular vibrational frequency Ω, amplification of the Raman signal is achieved by virtue of stimulated excitation. Consequently, the intensity of the Stokes beam, I_S, experiences a gain, ΔI_S (stimulated Raman gain, SRG), and the intensity of the pump beam, Ip, experiences a loss, ΔIp (stimulated Raman loss, SRL). In contrast, when $\Delta\varpi$ does not match any vibrational resonance, SRL and SRG cannot occur. Therefore, unlike coherent anti-Stokes Raman spectroscopy (CARS), SRL and SRG do not exhibit a nonresonant background or interference from autofluorescence. An additional advantage over CARS is the nearly linear dependence of the SRS signal with concentration. This allows direct interpretation of the SRS vibrational spectrum and obviates the need for post acquisition processing to visualize the chemical distribution. Skin tissue contains multiple components that produce strong Raman responses in the high wavenumber region of the vibrational spectrum. SRS imaging was recently utilized to probe the chemical nature of skin cluster and canyon microstructure, by visualizing the distributions of proteins, lipids, and water throughout the cell cluster and canyon regions of pig skin [17]. Image stacks were collected at three vibrational frequencies that approximately correspond to the Raman shifts of the three predominant skin chemical classes: ca. 3340 cm^{-1} (υOH water), 2950 cm^{-1} (υCH3 protein and lipid), 2850 cm^{-1} (υCH2 lipid). The spatial distribution of water across and through the skin layers was surveyed, identifying lipid-rich regions separating the water rich cell clusters. As described previously, corneocytes were observed to aggregate into clusters ranging from 100 to 250 μm wide and separated by invaginations 10 to 25 μm wide near the skin surface, the canyons [37, 38]. Nearly 28 μm below the surface, the canyons become progressively narrow. The transition from the SC into the viable epidermis was detected 18 μm below the surface.

Accordingly, lipid levels in all areas start to decline 15 μm below the skin surface. A significant accumulation of lipid material near the top of the canyons, in contrast to the relatively even distribution in the cell clusters, was observed, in agreement with previous descriptions [38]. Importantly, the water signal was found predominantly within the cellular areas (clusters) and not in the canyons, where may be present below the limit of detection. In healthy skin, the water content is ~30% in the SC, to sharply rise at the boundary between the SC and viable cells, eventually reaching a plateau of 60 to 70%. Such sigmoidal profile of water content as a function of depth observed by SRS, is in agreement with water profiles measured using *in vivo* confocal Raman spectroscopy in human skin [39].

Despite of the width, abundance and location of hydrophilic pathways is still controversial, entering these "leads" requires of molecules or nanostructures fitting the channels width (Fig. **2**). Alternatively, nanostructures larger than channels width may adjust their size to the physical constraints imposed by the pathway. In other words, to cross the skin across the hydrophilic pathways the nanostructures have to be "deformable".

5. NANOSTRUCTURES TO CROSS THE *STRATUM CORNEUM*: FLUIDITY VERSUS DEFORMABILITY

Conventional liposomes were described for the first time in 1964, by Alec Bangham as closed vesicles made of phospholipids, formed in an excess of water [40]. Up to the 70-80´s the liposomes were mostly regarded as bilayers models, of use limited to fundamental biophysical research. The principles of steric stabilization discovered in the early 90´s, and a better knowledge on the relationship between liposomal structural features and blood, provided a partial control on pharmacokinetics and biodistribution of liposomes, that led to a higher colloidal stability, drug/lipid ratio, drug retention in blood circulation, as well as a long shelf life [41]. Today, a growing number of liposomal therapeutic products have entered the pharmaceutical market [42, 43]. Liposomes are versatile drug carriers because they can incorporate hydrophilic drugs within the inner aqueous space, while hydrophobic and amphipathic drugs can partition in liposomal bilayers. Liposomal bilayers can also be tuned to make them selectively taken up by cells exposing specific ligands, or to be disrupted in a controlled fashion as a response to external stimulus [44]. Importantly, to take full advantage of such bilayer engineering, liposomes have to be administered by parenteral, preferably intravenous route. Routes other than parenteral such inhalatory, mucosal, topical, are much less popular, excepting in cosmetic uses. The reason lies in the poor colloidal stability of liposomes. Lipsomes, independently of their more or less fluid composition, are prone to coalesce and fuse when submitted to dry environments, such as the skin surface [45]. Because of this reason, the fused bilayers of liposomes widely used in cosmetics, are not drug carriers, but non-penetrating lipid depots trapped in the first layers of the SC.

In the 90´, two types of vesicles emerged almost simultaneously, showing to be efficient at crossing the SC instead of being stacked in the surface: the highly fluid and the highly deformable vesicles. The two of them however, enter the skin employing different mechanisms worth to be differentiated.

5.1. Highly Fluid Vesicles: Ethosomes

Ethosomes are fluid vesicles prepared from phospholipids, a high proportion of the permeation enhancer ethanol and water; typically 2-5% phosphatidylcholine (PC), 20-45% ethanol and water to 100% w/w [46, 47]. Ethanol, even at low concentrations, binds to the lipid polar heads and increases the fluidity of the liquid crystalline state [48, 49]. By employing phosphorous-31 nuclear magnetic resonance spectroscopy ($NMR^{31}P$), the polar head group motions of PC were found to be restricted and anisotropic, and the existence of bilayers in the 20%-45% ethanol range was confirmed. By the paramagnetic-ion NMR technique using Pr^{+3} as a shift reagent, the vesicularity and permeability of phospholipid dispersions in 30%-45% ethanol was revealed, showing that, between 20% and 30% ethanol, PC forms bilayers in the form of closed vesicles. Transmission electron microscopy of ethosomes composed of PC/ethanol (2/30%) was shown to contain multilamellar vesicles, with lamellas extended to the core of the vesicles [50]. As the ethanol content approaches 45%, a small soluble fraction of phospholipids is mixed with the closed vesicles.

A unique property of ethosomes is the possibility of controlling their size as a function of the ethanol content, with no need for *ad hoc* equipment. For example, at 2% PC, the size of ~200 nm diameter vesicles can be reduced to half as the ethanol concentration is increased from 20% to 45%. Conversely, the ethosome size has a limited dependence on the PC concentration. An eight-fold increase in PC concentration from 0.5% to 4% resulted in a two fold increase in ethosome size (120 nm to 250 nm). Moreover, the high ethanol content is responsible for the negative Z potential in comparison to liposomes in the absence of ethanol. Due to the high negative Z potential, ethosomes has higher colloidal stability than its liposomal counterparts. On the other hand, compared to liposomal bilayers the phospholipids in ethosomes are packed less tightly and the membrane presents a higher permeability for hydrophilic/ionic solutes. The ethosomes therefore are not well suited to entrap hydrophilic solutes [51].

Ethosomes have a high content on permeation enhancers, which is responsible for mediating the disruption of the lipid barrier by which small hydrophobic molecules permeate. After topical application, the permeation enhancement from ethosomes is much greater than would be expected from ethanol alone, suggesting a synergic mechanism between ethanol, vesicles, and skin lipids. Ethosomes are more effective permeation enhancers than ethanol, aqueous ethanol, or ethanolic phospholipid solutions. It is hypothesized that ethosomes might act as enhancers of drug permeation and as drug carriers through the SC. The ethanol may increase the solubility of the drug in the vesicle, disturb the organization of the SC lipid bilayer, and enhance its lipid fluidity. The subsequent mixing of phospholipids with SC lipids of the intercellular layers was observed to enhance the permeability of the skin [52]. The rapid ethanol permeation across the skin, solvent 'drag', may carry drugs into the tissue as ethanol traverses. Ethanol is a highly volatile constituent that might extract some of the lipid fraction from within the SC, with the consequence of improved drug flux through the skin when used at high concentrations for prolonged times [53]. In other words, ethosomes disrupt the lipophilic barrier of the skin. Remarkably, the entrapment efficiency (EE) of hydrophobic/amphipathic solutes by ethosomes is significantly high because of its solubilization on multilayers and hydroethanolic inner compartments. Liposomes lacking ethanol would only solubilize hydrophobic/amphipathic solutes within phospholipid bilayers [54]. Finally, ethosomes can be successfully applied under occlusive conditions, which allows for the employment of patches. In spite of the high ethanol concentration, the average size and size distribution of ethosomes remains constant for at least 2 years at room temperature [55].

5.2. The First Highly Deformable Vesicles: Transfersomes

Transfersomes are highly deformable (a synonym for elasticity/flexibility) unilamellar vesicles, of nearly 100 nm diameter. The first generation of transfersomes was made of phospholipids combined with edge activators (EA). EA are single chain surfactants of high radius of curvature and mobility [56]. In a typical 85: 15 phospholipids/EA weight ratio transfersome composition, the EA proportion is close to that required for membrane solubilization, so as the lipid bilayers are destabilized and the bilayer deformability is increased. Common EA are sodium cholate, sodium deoxycholate, Span 80, Tween 20 and 80 and dipotassium glycyrrhizinate. The

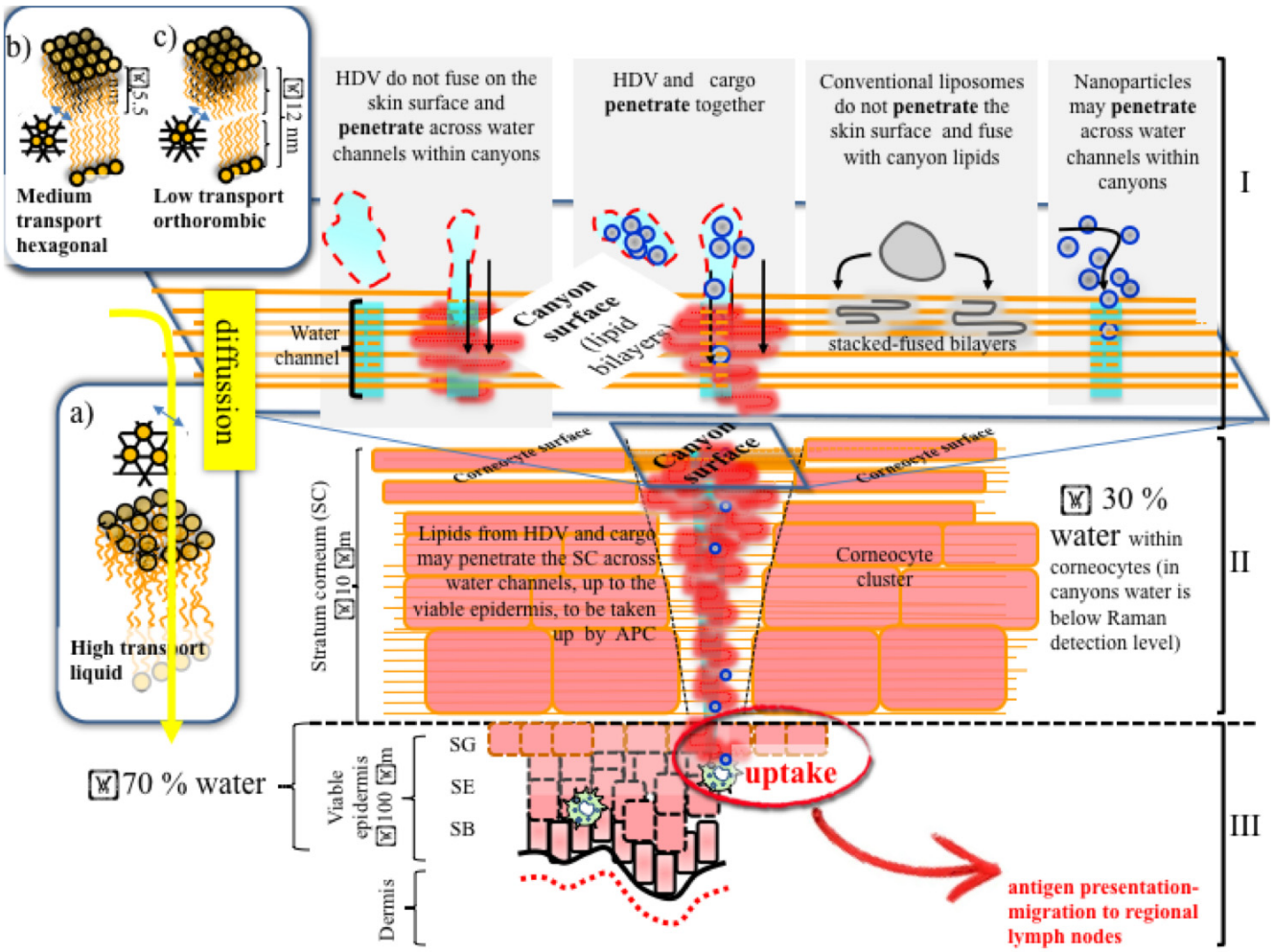

Fig. (2). Scheme depicting the main structural sections of the skin: SC, viable epidermis and dermis, and barriers to permeation-penetration (not a scale):

(I) The diffusive pathway across the lipids ( ) of the SC is mediated by disordered bilayers, represented by the X-ray diffraction pattern corresponding to the lateral packing in liquid phase of bilayers shown in a) (is ~0.46 nm) . Bilayers b) and c) with more organized lateral packing (0.41 and 0.41-0.37 nm, respectively), not involved in diffusion across the skin. The interaction between HDV, conventional liposomes, hydrophilic solutes and lipids at the SC surface is also represented. HDV and hydrophilic solutes penetrate across hydrophilic leads in the canyons in-between corneocyte clusters.

(II) A scheme of HDV and associated cargo crossing an hydrophilic channel in the SC, according to [35, 76]. The colloidal structure of HDV is lost below the surface (HDV lipid bilayers:). The associated cargo penetrates along with the lipid bilayers. No endocytic uptake of HDV, liposomes or nanoparticles occurs at I and II since the SC is made of dead corneocytes.

(III) Below 10 µm depth, endocytic uptake of material penetrating across hydrophilic channels accessing the viable epidermis may occur. Epidermis: *Stratum basale* (SB), *Stratum spinosum* (SS), *Stratum granulosum* (SG).

(Dermo epidermal basal membrane:)

(Reticular capillary plexus in the dermis:)

second-generation of transfersomes was made of at least one bilayer forming lipid, typically phosphatidylcholine having fluid acyl chains, and at least two more polar lipophilic substances, e.g. one surfactant and one surfactant mimicking drug [27]. Transfersomes are said to penetrate (and not to permeate) the SC by mechanisms dependent on structural adaptability but independent of any particular composition. As discussed above, permeation is a term describing material transport across a barrier depending on a concentration gradient following Fick's law of diffusion, with the trans-barrier transport linearly correlated with the permeant's diffusivity and distribution coefficient [54] and the *in vitro* permeation broadly resembling the *in vivo* permeation [57]. Lack of such proportionalities or correlations indicates a non-diffusive mechanism of transport. Clearly, the mechanism mediating skin interaction is of key importance to classify the fluid vesicles as structures completely different from transfersomes [23]. Controversies over the classification of deformable or fluid vesicles arise when the mechanism of skin interaction is poorly defined [27].

The mass transport mediated by transfersomes is neither mediated by fluidification nor fusion with the SC lipids. More specifically, penetration describes the transport of vesicles as non-fragmented bilayers through a semipermeable barrier with pores smaller than the vesicular diameter under a driving force that can be hydrotactic, which vanishes after skin occlusion. The penetration occurs across hydrophilic pathways; instead, the diffusion or permeation occurs across the lipid filling. Different from diffusion, the

penetration is independent of a concentration gradient and partition coefficient of the material. Cevc, in its seminal review discusses and extensively illustrates the reasons why diffusion cannot mediate the transport of particulate material across the skin [27].

Transfersomes differ from conventional liposomes (made of phospholipids with or without cholesterol) and from ethosomes, in their high and stress-dependent adaptability (high elasticity) compared to the higher bilayer stiffness of the two others [58]. A generally accepted explanation to bilayer deformability, is that as a response to a mechanical stress (such as passing across pores of size smaller than the vesicle diameter), the EA are demixed from the lipid bilayer and displaced to relocate in the zones of higher curvature/stress. Phospholipids on the other hand, enrich the bilayer regions of smaller curvature. Such rearrangements diminish the membrane elastic energy. The average elastic energy of deformable bilayers is on the order of thermal energy ($\kappa \sim kT$) and locally may be much smaller than this, while for conventional liposomes κ is known to be $\geq 20kT$ [59].

Unlike conventional liposomes, Transfersomes change shape and volume at minimal energetic cost [54, 60]. Transfersomes possess high bilayer permeability than conventional liposomes and can adapt their local bilayer composition in response to ambiental stress. Because of this, transfersomes are said to behave as "molecular devices" [27]. In the first (includes phospholipid or phospholipid-surfactant blends) and second-generation (synergistic phospholipid-amphipat or drug mixtures) of these vesicles, such rearrangement took place within or across the mixed lipid bilayer. In the third-generation ((non-phospholipid) amphipat combinations with appropriate effective tail(s) cross-section), water-soluble molecule redistribution near a bilayer surface is sufficient to increase bilayer flexibility. Hydrophilic modulators of bilayer properties can increase vesicle adaptability, stability, and/or drug payload. Examples of this could be novel antifungal preparations, surfactant- or phospholipid-free nonsteroidal anti-inflammatory drug (NSAID) preparations, phospholipid free drug-carriers, etc. This includes bioactive hydrophilic and highly adaptable composite vesicles for drugless treatment of non-severe pain and inflammation involving physical mechanisms of action [60, 61].

5.3. Other Highly Deformable Vesicles

Several other novel highly deformable vesicles (HDV) followed the launching of the first generation of transfersomes; amongst them are the nonionic surfactants elastic vesicles, the invasomes, the elastic noisomes and the menthosomes.

Nonionic surfactants elastic vesicles are made of a bilayer-forming surfactant (sucrose laurate ester, L-595) and a micelle-forming surfactant (octaoxyethylene laurate ester, PEG-8-L) acting as EA, plus sulfosuccinate as stabilizer, typically at 50/50/5 molar ratio [62].

Invasomes are vesicles made of phospholipids such as lysophosphatidylcholine, terpenes and ethanol (10: 0.5-1: 3.3 % w/v) [63]. Flexibility of the bilayer membrane is mainly due to the lyso PC acting as an EA. Ethanol is a good penetration enhancer while terpenes have also shown potential to increase the penetration of many drugs by disrupting the tight lipid packing of the SC.

Elastic niosomes are vesicles made of non-ionic surfactants (Span, Tween and Brij) and sodium cholate as an EA [64]. Vesicles composed by Span and Tween 80 as EA (Span 60: Tween 80 at 80: 20 w/w) prepared by the ether injection are called spanlastics [65].

Menthosomes are vesicles made of phospholipids, cholesterol, menthol and cetylpyridinium chloride (0.77: 0.04: 0.10: 0.07 w/v). Cetylpyridinium, a cationic surfactant, and menthol acts as penetration enhancers. Menthol however, was reported to affect the lipid arrangement in the SC [66].

Despite polymeric nanocapsules are not vesicular carriers, their promising use as carriers in cosmetics and therapeutics makes worth to highlight a recent report on nanocapsules deformability. Briefly, polymeric nanocapsules are capsules of a shell of biocompatible and biodegradable polymer, such as poly(ε-caprolactone, poly(lactide) or poly(lactide-*co*-glycolide, that surrounds an internal oil phase (made of liquid lipid, or mixture of liquid lipids, or liquid and solid lipids) [67]. While the wall polymer has a great influence in the control of drug release, the oily core composition was found to affect the rigidity of the nanocapsules. The elastomechanic-behavior of nanocapsules was reported to be tuned by dispersing a solid component (sorbitan monostearate, Span 60) in the oily phase core [68]. The impact on *in vitro-in vivo* skin penetration of nanocapsules having Span 60 as an elastic modulator is shown in section 8.1.2.

6. METHODS TO ASSESS DEFORMABILITY

Different to conventional liposomes, a full structural HDV characterization requires of a bilayer elasticity determination. Such parameter can be estimated by methods based on different principles: the Van der Bergh method [62], (rendering a deformability value *D)*, and the Young´s modulus (E) determination. *D* is a parameter inversely proportional to E and is calculated as $D = J\,(rv/rp)^2$,where J is the rate of vesicles penetration through a permeability barrier, rv is the size of vesicles measured after extrusion and rp is the pore size of the barrier. To measure J, vesicles are extruded through two stacked 50 nm (rp) membranes at 0.8 MPa. Extruded volume per minute is collected along 15 min, phospholipid are quantified in each fraction, and J is calculated as the area under the curve of the plot of phospholipid recovered as a function of time. Conventional liposomes would clog up the pores and the extrudate would not contain liposomes. In contrast HDV were shown to pass across the pores, suggesting the latter had flexible membranes that allowed them to adapt their size to fit the pore diameter. *D* is inversely proportional to the size of vesicles after extrusion, meaning the mean size of flexible vesicles remain constant upon deformation. The resulting *D* is therefore a relative, not an absolute parameter. As such it is useful for comparing deformability of different liposomal formulations and to determine whether or not the partitioning of (mostly hydrophobic) drugs within the lipid bilayer, would modify the *D* value.

The Young´s modulus (E) or longitudinal elastic modulus is the ratio between the increased tension applied by traction (in the zone of elastic behavior of the material) ($d\sigma$) and the resultant increased relative deformation ($d\varepsilon$) (E: $d\sigma/d\varepsilon$). E is a measure of a material rigidity, the higher the Young´s modulus, the stiffer the material. *D* and E thus, are inversely related.

Atomic force microscopy (AFM) is a well suited technique for studying structural features of liposomes. It enables the simultaneous analysis of shape and mechanical properties of the bilayers in the nanoscale. The technique allows picking up surface topographical images with a space resolution close to 1 Å and force vs distance curves with a detection limit close to 10^{-12} N. Young's modulus can be obtained from AFM force curves by fitting data with an adequate model of contact. The most popular is the Hertz model [69]: $F = 2E\tan\alpha\ \delta^2/\pi(1-\nu^2)$; where F is force, E is the Young Modulus, α is the half opening angle of the conical indenter (53º; based in geometrical characteristics of the tip and scanning electron microscopy observations), δ is the indentation and ν is the Poisson radius which for soft biological samples is assumed to be 0.5. Using this approach our research group showed that E values of HDV were around 3 folds lower than that of conventional liposomes made of soybean phosphatidylcholine (430 ± 137 vs 1119 ± 242 kPa) [70]. This technique was also used to measure the E value of polymeric nanocapsules. The results showed that E value is increased when the solid lipid Span 60 is added to the oil core (E= 0.364 MPa for nanocapsules vs E = 0.537 MPa for lipid core nanocapules [68].

Physicochemical and colloidal stability are also relevant structural parameters of vesicle population. It is well known that EA destabilizes and increases permeability of lipid bilayers. The presence of EA in vesicles leads to a leakage of aqueous content of HDV over time [71]. Because of this, the long-term stability of HDV is one major drawback.

7. MECHANISM OF HDV-MEDIATED DRUG PENETRATION

Two potential mechanism for drugs carried by HDV penetration are currently proposed: i) a carrier mediated mechanism, where HDV carries drug molecules into the skin and ii) a mechanism where HDV act as penetration enhancers; in there vesicles enter the SC and subsequently modify the intercellular lipid lamellae facilitating the penetration of free drug molecules into and across the SC.

In the first mechanism, the driving force for HDV penetration into skin is the so called "xerophobia", the tendency of phospholipids to avoid dry surroundings [72, 73]. To stay fully swollen, HDV move towards the more hydrated layers of skin (epidermis and dermis), driven by the hydration gradient across the layers (from 10-30% at the air-exposed skin surface to 75% in the viable epidermis). Conventional liposomes dehydrate and fuse in the dry surface of the SC, and only accumulate in the upper layers of the SC, not beyond the first micron depth. In contrast, HDV applied under non-occlusive conditions (since occlusion eliminates the hydration gradient), penetrate the intact skin because of its high deformability combined with the tendency of phospholipids to move towards more hydrated zones [58]. Hydrophobic dyes carried by HDV have been detected several microns depth below the skin surface, and probably traces of lipid material access the viable epidermis. Despite HDV were speculated to access the blood in intact form carrying insulin, poorly reproducible results were reported [74]. The penetration of transfersomes was proposed to occur through hydrophilic intercellular channels of the SC [35]. Remarkably, as skin structural features were studied on detail, the involvement of a xerophobia-driven mechanism to explain the locomotion from surface to deep skin layers of transfersomes was challenged. For instance, it was shown the water gradient across the skin may not be linear but sigmoidal [39]. Even in fully hydrated state the water content in the lowest SC layers close to the viable epidermis is much lower than in central regions of the SC [75]. According to this, deformable vesicles would be stacked within the SC and the gradient dissipation beyond the lowest layers of the SC would not be of help for penetration. Also, hydrophilic pathways on the intact SC cover only a tiny (< 0.001%) fraction of the normal skin surface and most of them are 10-200 times narrower than the regular size of HDV (~100 nm). Thus, the HDV locomotion across these channels would be rather difficult, in spite of their high deformability.

On the other hand, structural changes in the SC following topical application of HDV and intact HDV have been visualized within the SC lipid lamellar regions, but no intact HDV were detected in the deepest layers of the SC [76]. Ordinary colocalization analysis of two-color fluorescently labeled liposomes into skin layers using confocal microscopy, neither provide substantial information about liposome interaction with skin nor on the persistence of the colloidal structure of liposomes after penetration. Recently, structural tissue features measured at different depths (by multiphoton excitation fluorescence microscopy imaging) combined with stacks of two-dimensional maps of the fluorophore's diffusion coefficient within the tissue (by Raster image correlation spectroscopy) were used to study the skin penetration of HDV [77]. These techniques can determine whether two different fluorescent probes (one in the bilayer and the other in the inner aqueous space), diffuse together. A high cross correlation between the two signals is expected, if the liposomes are intact and the hydrophilic content is retained with the vesicle. Both in HDV and in conventional liposomes, the absence of cross-correlation for the two labels was shown, indicating that below the skin surface, the labels did not diffuse together. This suggested that penetration of intact vesicles is highly compromised by the skin barrier, and that the structural integrity of the vesicles is lost across the SC. The low water activity existing in the SC region may affect the water entropic component (hydrophobic effect), causing the vesicles lose their structural integrity and hydrophilic content (Fig. **2**).

On the other hand, recent experimental evidence has challenged the parameter elasticity as the paramount factor affecting the penetration of lipophilic and hydrophilic drugs within HDV. For instance, no differences were registered on the flux of the lipophilic drug ketoprofen loaded in nonionic surfactant elastic vesicles or in rigid non-deformable vesicles, across human skin. Moreover, the flux of ketoprofen loaded in HDV was similar to that of drug in solution [78]. On the other hand, the flux of the hydrophilic dye calcein loaded in HDV and invasomes across human skin, was not linearly correlated with vesicle elasticity [79].

Regarding the second mechanism, several studies support the fact that surfactants present in HDV act as penetration enhancers [80]. Besides, terpenes of invasomes interact with intercellular lipids perturbing their lamellar packing, resulting in increased drug solubility in the terpene-treated SC and skin penetration enhancement.

Overall, the current experimental evidence suggests that the two mechanisms may play a role in the enhanced skin drug delivery by HDV. According to the physico-chemical properties of the drug, one of the two mechanisms might predominate. Hydrophilic drugs for instance, would penetrate driven by the second mechanism, while hydrophobic drugs would employ the first one. The penetration of hydrophilic drugs is limited by their partitioning in the lipophilic SC. Because of this, the penetration enhancing effect seems to play a more important role for hydrophilic than for lipophilic drugs (as occurring with many penetration enhancers). Hydrophilic drugs therefore, might not be necessarily entrapped in vesicles in order to penetrate. Instead, the penetration of hydrophobic drugs is limited by its partition between SC and epidermis, which is less lipophilic. In this case the optimal skin penetration is achieved with drugs loaded within vesicles [80]. Closely related to this subject is the mechanism of drug release from vesicles. Entrapment might result in slow transdermal flux and sub-optimum permeation. Once released, its diffusion in skin must occur; because of this the fate of released drug is dependent on its solubility in the tissue.

The dose-per-area plays a relevant role in penetration depth of the carried drug: while a low dose-per-area favors the drug retention in the skin, an increased total applied drug dose and an increased dose-per-area were shown to promote the systemic drug availability [81].

Summarizing, factors such as drug electric charge and hydrophilic/hydrophobic balance, drug loading, vesicle elasticity, mode of application (occlusive or non-occlusive) and dosage, strongly influence the drug disposition and distribution across the skin. However, systematic physicochemical and pharmacokinetic studies are still needed to define the precise effect of these factors and unravel the mode of action of HDV.

In the following sections we will provide an overview on the latest studies employing HDV for topical/transdermal drug delivery.

8. TARGETS FOR HDV PENETRATION: SKIN, MUSCLE AND BLOOD.

A considerable amount of data on preclinical settings where HDV were used to target the epidermis, joints, the underlining muscle; also aiming systemic delivery to target blood circulation, is currently available. Local pathologies span from joint diseases, skin cancer, atopic dermatitis, would healing, psoriasis, scar treatment,

Table I. Pre-clinical application of HDV.

Disease/Drug	Composition and structural properties (mean size, Z potential, EE)	Main results
Joint disease		
Meloxicam 351.4 Da [90]	SPC: chol: cetylpyridinium chloride (0.77: 0.04: 0.10 w/v; HDV) Same composition with menthol (0.07 w/v; menthosomes) 60-100 nm, 43-49 mV, EE 65-88%	*In vitro* permeation in hairless mice skin showed a steady-state flux of drug ranked as follows: menthosomes ≅ HDV> conventional liposomes > suspension Drug deposition in the skin ranked as follows: conventional liposomes> menthosomes> HDV> suspension
Skin câncer		
5-ALA [94]	(DOTAP or EPC or EPC: DOPA (5: 1 molar ratio)): Tween 20 (5.6: 1 weight ratio), for cationic cHDV), neutral and anionic HDV (cHDV, nHDV and aHDV), respectively 96-108 nm, cHDV 31 mV , nHDV -1.7 mV; aHDV -15 mV	*In vitro* permeation in hairless mice skin of 5-ALA in cHDV was 2-fold compared with aHDV and nHDV, and 8-fold compared with solution. 5-ALA-induced PpIX accumulation in the viable skin following 4 h of topical application of all vesicles in hairless mouse; the highest amount was observed after the treatment with cHDV.
Temoporfin [95, 96]	SPC: ethanol: cinole (10: 3.3: 1 % w/v).	Invasomes led to significantly slower increase in tumor size compared to mice without any treatment and mice treated with photoirradiation.
Resveratrol 228 Da and 5-fluorouraci 130 Da [97]	SPC: Nachol (88 mg: 12 mg) 120 nm; -25-30 mV; EE: 97% for resveratrol and 42% for 5-fluorouracil	Flux on human skin for resveratrol was 7.84 $\mu g/cm^2 h^{-1}$ for HDV and 1.39 $\mu g/cm^2 h^{-1}$ for solution, and for 5-FU, was 5.54 µg/cm2 h^{-1} and 0.67 for HDV and solution, respectively.
Doxorubicin 543.5 Da [99]	SPC film was hydrated with PBS containing NaDChol, HA-GMS and drug 251 nm; EE: 61%	Flux on mouse dorsal skin for doxorubicin was 1.35 $\mu g/cm^2 h^{-1}$ for HDV and 0.46 $\mu g/cm^2 h^{-1}$ for solution Confocal fluorescent images after topical application on Wistar rats showed that HDV deliver drug into dermis and lymph nodes.
Wound healing, psoriasis and scar		
Curcumin 368.4 Da [107]	SPC: Tween 80. 203 nm; -33 mV; EE: 96.5 %	*In vivo* skin deposition on Wistar rats showed higher drug deposition in hyaluronic acid core HDV (4.62 % of the applied dose) compared to HDV (0.92 %), after 24 h. No drug was detected in the skin from plain gel formulations.
Asiaticoside 959.1 Da [108]	SPC: Nachol 110 nm; -17.3 mV; EE 55 %	HDV increased permeation of drug 10.6-fold with respect to the aqueous solution on human skin.
Papain 23.5 KDa [109]	Tween 61: chol: Nachol (1: 1: 0.1 molar ratio) 520 nm; -46.2 mV	*In vitro* permeation on rat skin showed accumulation of drug in the whole skin and in the receiving solution, for drug in elastic niosomes of 3.10 and 2.24; 10.08 and 4.92; 4.86 and 7.38 times more than that from non-elastic niosomes, PLGA nanospheres and solution, respectively. Elastic niosomes reduced hypertrophic scars in the rabbit ears 10.20, 2.73 and 2.31 times than gel base, gel containing free drug, and gel containing drug loaded in non-elastic niosomes, respectively
Calcipotriol 412.6 Da [110]	DSPC: NaChol: PEG2000-DSPE (89-84: 11: 0.5, 1 and 5 mol %) 93.1-95.4 nm; -4 mV -14 mV	No difference in the amount of drug in the pig skin delivered with PEGylated and non-PEGylated HDV, were observed. Drug was most located in SC (18.7 %), 7% in viable epidermis and 5 % in dermis, less than 2% of drug was found in the receptor fluid.

(Table 1) Contd….

Disease/Drug	Composition and structural properties (mean size, Z potential, EE)	Main results
Fungal infections		
Terbinafine 291.4 Da [111]	PC: SDC (0.5: 0.083% w/v; HDL) PC: ethanol (0.5% (w/v): 45% (v/v); ethosomes) 0.5% (w/v) PC dissolved in 45% (ethanol to propylene glycol 7: 3 w/w; binary ethosomes). PBS was added into the PC hydro alcoholic solution	Amount of drug in mouse skin from ethosomes, binary ethosomes HDV and conventional liposomes was 42.44; 50.7, 52.4 and 33.5 μg/cm^2 respectively
Miconazole nitrate 416.1 Da [112]	PC: surfactant (75-95%: 5-25% w/w). Surfactants: NaDchol, Span 60, Span 80, Tween 80 160-186 nm; EE 70-91%	Transdermal flux on rat skin was highest for Span 80. HDV transferred 6-7.5 μg/h.cm^2
Amphotericin B 924 Da [113]	SPC: Tween 80 (1: 0.3 molar ratio) 107 nm; -3 mV; EE 75%	Upon 1 h of incubation, 33 ± 2 % and 7 ± 2 % of the administered dose of drug in HDV was found in the *SC* and viable epidermis, respectively.
Ketoconazole 531.44 Da [114]	Span 60: Tween 80 (80: 20 w/w)	*Ex vivo* corneal permeability *In vivo* safety studies and distribution
Clotrimazole (344.8 Da) Metronidazole (171.1 Da) [115]	EPC: NaDchol (70: 30 w/w) with propylene glycol	Deformability was 6.27 and 4.97 for clotrimazole and metronidazole HDV respectively. Metronidazole and clotrimazole released from HDV in gel were found to be 13.1 and 50.4 mg/h in the first 4 hours, respectively.
Microbial infections		
Ciprofloxacin 331.4 Da [116]	EPC: Nachol (5: 1 molar ratio)	HDV showed higher amount of drug permeated and deposited than commercial product on ear skin and tympanic membrane The AUC 0-24 for *in-vivo* deposition on tympanic membrane of rabbits for HDV was 2.56 folds higher than for commercial drops.
Leishmaniasis		
Paromomycin [118]	SPC: Nachol: ethanol (20: 2: 5 w/w) 200 nm; -14 mV; EE: 60%	HDV showed higher percentage of drug retention in the skin compared to the cream on mice skin *ex vivo.* HDV (twice a day for 4 weeks started at week 4 after infection) induced significantly smaller lesion size and lower spleen parasite burden than control groups and free drug cream, in BALB/c mice infected with *L. major*
Zn phthalocyanine [119, 120]	SPC: Nachol (6: 1 w/w) 100 nm; -36.7 mV; EE: 85% SPC: Nachol: archeal lipids (3: 1: 3 w: w) (HDA) 130 nm; −35 mV	HDV showed 100% anti-promastigote and 80% anti-amastigote activity against *L. braziliensis* after 15 min sunlight irradiation (15 J/cm^2). Free phtalocyanine showed 20% of anti-promastigote and antiamastigote activity. HDA showed 100 % anti-amastigote activity at 0.2 J/cm^2
Hypertension		
Valsartan [129]	SPC: NaDchol (85: 15 w/w) 130 nm; EE: 86 %	HDV showed maximum flux over conventional liposomes (626.6 vs 18.5 μg/cm^2/h) on abdominal wistar rat skin *ex vivo.* HDV gradually decrease blood pressure, with the maximum effect observed at 6 h, and controlled blood pressure up to 48 h. Oral valsartan controlled the hypertension at 3 h, then blood pressure rise gradually up to 48 h.

(Table 1) Contd….

Disease/Drug	Composition and structural properties (mean size, Z potential, EE)	Main results
Felodipine [130]	EPC: Span 80 (95: 5 w/w) 76 nm; -49.8 mV; EE: 85.14 %	HDV showed highest cumulative drug permeation (94.9 %), skin deposition (8.1 %) and transdermal flux (23.7 μg/h/cm^2) on rat skin *ex vivo*. The maximal permeation from UDV in gel was 2.6 times higher as compared to free drug gel at 24 h Cmax for UDV in gel was 8.05 ng/ml, for oral drug was 2.31 ng/ml in 6.0 and 4.85 h (tmax) respectively, in rats. $AUC_{0-\infty}$ was 162.26 and 45.27 ng.h/ml for UDV in gel and oral formulation, respectively

fungal, bacteria and protozoa infections. Preclinical determinations include recent nanovaccination strategies. Hypertension was the only pathology that required blood access from topical route. A recent review has covered most of these experimental approaches up to 2014 [82]. In this section an extensive landscape embracing previous and new applications of HDV is provided, Table **1** shows formulation details and main results obtained.

8.1. Local Targets: Skin and Underlining Skeletal Muscle And Joints

8.1.1. Anti-Inflammatory Drugs Against Joint Diseases

Nonsteroidal anti-inflammatory drugs (NSAID) are generally orally administered and cause mucosal injury throughout the gastrointestinal tract. Their use is associated with nearly 4 fold increased risk of serious upper gastrointestinal complications. Despite topical NSAIDs is commonly used for chronic musculoskeletal pain in adults, such as rheumatoid arthritis, the only indication is for chronic pain caused by osteoarthritis.

The delivery of NSAID to skeletal muscle and joints requires of local drug retention and blood avoidance. Most NSAID molecules diffuse across the SC and into deeper skin regions rather well, through the intercellular lipid matrix of the skin, because of their suitable lipid-water partition coefficient and low MW. However, classic topical formulations (cream and gel) have limited local action because of the fast clearance from the skin by the capillary plexus underlying the skin surface, which leaves too little of the drug in the target organs [83, 84].

First studies made in 2001 by Cevc, showed that transfersomes enabled to modify the route of administration, reducing the therapeutic dose, and achieving regioselective delivery to deep subcutaneous tissue of the poorly water soluble NSAIDs diclofenac and ketoprofen (Transfenac® and Diractin®, respectively, from IDEA AG, Muenchen, Germany) [85]. Transfenac® showed longer effect and reached ten times higher concentrations in the tissues under the skin of mice, rats, and pigs, as compared to diclofenac in a commercial hydrogel. Due to the size of the transfersomes (~100 nm), diclofenac within the HDVs is cleared less efficiently by the dermal capillary plexus than the free drug. HDV are too bulky to penetrate through the nonfenestrated capillary of the skin. It was observed, on the other hand, that the AUC for Diractin® gel in the peripheral deep muscle exceeds the AUC for a conventional gel by 35 fold [56, 57]. Two Phase III clinical studies showed that Diractin® was superior to oral placebo for the treatment of osteoarthritis of the knee and was comparable with an oral NSAID without gastrointestinal complications [60, 61].

Between 2011 and 2014 the poor water soluble NSAID piroxicam [86], lornoxicam [87] and diclofenac [88], as well as the hydrophilic ketorolac [89], were loaded into HDV and *ex vivo* permeation measured. Studies showed an increased drug penetration in comparison with free drugs or conventional liposomes when formulated in HDV. The muscle and blood drug concentration however, were not determined. Piroxicam loaded in HDV showed better anti-inflammatory activity in carrageenan-induced paw edema model compared with the free drug. This model however, is far from reproducing the anatomopathological context of osteoarthritis or rheumatoid arthritis, mainly because of the absence of a capsular barrier. Conventional formulations would reduce the symptoms of osteoarthritis arising from periarticular structures, partly by local delivery and partly by intracapsular access of drug delivered to blood. The superiority of HDV for drug delivery against this disease remains to be shown.

Recently, the low water soluble NSAID meloxicam was incorporated in menthosomes, HDV and conventional liposomes and *in vitro* skin penetration using hairless mice skin was evaluated [90]. Despite menthosomes showed higher elasticity than HDV (152 vs 82 mg/s cm^2), no significant difference between both formulations was observed in the cumulative amount per area and skin permeation flux of meloxicam. Both menthosomes and HDV showed higher flux of meloxicam than conventional liposomes.

8.1.2. Skin Cancer

Non-melanoma skin cancer is the most common type of cancer in Caucasian populations, with squamous cell carcinoma and basal cell carcinoma accounting for the majority of cases [91]. Surgical removal is the standard therapy, but it may cause morbidity in high risk individuals and have negative cosmetic outcomes. Topical treatment has to act locally, on epidermal keratinocytes or its appendages and epidermal basal cells where squamous cell carcinoma and the basal cell carcinoma locate, respectively. A minimal systemic access of topically delivered drugs is desirable.

Photodynamic Therapy

Photodynamic therapy is based on the use of a photosensitizer that produces reactive oxygen species upon light excitation in the presence of oxygen. Topical photodynamic therapy with 5-aminolevulinic acid (5-ALA) has been used for the treatment of various dermatological and pilosebaceous unit-related diseases, such as actinic keratoses, psoriasis, Bowen's disease, superficial skin carcinomas and acne. 5-ALA is a precursor of the photosensitizer protoporphyrin IX (PpIX), formed *in vivo* after the exogenous application of 5-ALA [92]. PpIX activated by a suitable wavelength generates singlet oxygen, by a cascade of reactions. The cytotoxicity of singlet oxygen on tumors occurs by two pathways: destruction of tumor cells by necrosis or apoptosis and the failure of tumor vascularization by a decline in oxygen-carrying blood [93]. For efficient topical application of 5-ALA, accumulation of 5-ALA in the epidermis and dermis with minimal systemic uptake is desired since conversion of 5-ALA into PpIX occurs preferentially in epidermis. However, 5-ALA is a hydrophilic zwitterion molecule of poor skin penetration. Recently, 5-ALA has been loaded in cationic, anionic and neutral HDV in order to improve its skin delivery [94]. Cationic HDV showed higher stability in terms of particle size and higher 5-ALA encapsulation than neutral and anionic HDV. Cati-

onic HDV also showed higher permeability, and could deliver 5-ALA into deep skin tissue after topical application. Moreover, 5-ALA loaded in cationic HDV was retained by a longer time period, and induced higher amount of PpIX in viable skin than in neutral or anionic HDV. The plasma levels of 5-ALA were below detection limit, 4 h after non-occlusive application *in vivo*.

Temoporfin is a highly hydrophobic second-generation photosensitizer with low percutaneous penetration. Intravenous application of temoporfin has shown to be effective in the photodynamic therapy of early or recurrent oral carcinomas, in the palliative treatment of refractory oral carcinomas, and in the treatment of primary non melanoma skin cancer of the head and neck. Temoporfin loaded into invasomes containing 1% (w/v) cineole showed enhancement of skin penetration and although tested on a not a suitable model of non melanoma skin cancer, it slowed down the tumor growth in mice bearing a subcutaneously implanted human colorectal tumor cells HT29, as compared with non treated animals [95, 96].

Chemotherapy

Oxidative stress is a key component in the multistage carcinogenic process. Endogenous antioxidants prevent ROS-mediated injury. Many experiments showed that a supplement of antioxidant drug during anticancer therapy may reduce adverse reactions and improve the potential for success in terms of tumor response. It has recently been demonstrated that resveratrol, a polyphenol largely used as antioxidant, synergistically promotes 5-fluorouracil mediated apoptosis of cancer cells irrespective of p53. Recently, it was shown that co-encapsulation of the hydrophilic 5-fluorouracil and the hydrophobic resveratrol within HDV increased 8.3-fold and 6.2-fold, the percutaneous permeation of both drugs, respectively [97]. Additionally, the co-encapsulation of both drugs improved their *in vitro* anticancer activity on skin cancer cells (SK-MEL-28 and Colo-38) as compared to the free drug and the single entrapped agents.

Retinyl palmitate oil is the most stable form of vitamin A that plays an important role in cellular differentiation and carcinogenesis prevention and it is largely employed in anti-ageing formulations. Polymeric nanocapsules with an oily core of retinyl palmitate and Span 60 and a polymeric wall of poly(D,L-lactide) (PLA) were prepared by the pre-formed polymer interfacial deposition method [98]. These nanocapsules have shown to be deformable by measuring the permeation of the particles through two superposed membranes of smaller pore diameters that size of nanocapsules. The nanocapsules permeated through deep layers into human skin. The higher amount of retinyl palmitate was found in the dermis plus the epidermis. Retinyl palmitate also reached the receptor chamber. Permeation profile of the nanocapsules with Nile blue conjugated to PLA showed that nanocapsules were distributed uniformly, suggesting that the permeation mechanism through skin is intercellular.

Targeting to Lymphatics by the Topical Route: Tumor Metastasis Therapy

Tumor metastasis accounts for 90% of cancer-associated deaths and is almost inaccessible by chemotherapy, surgical operation or radiotherapy. Before distant spread, metastasized cancers have to invade the lymphatic system as the first step in their progression. Lymph node metastasis plays a major role in cancer staging and prognosis. Effective control of the disease state of the lymphatics draining tumor is crucial to treatment. The lymphatic system begins in the dermis with initial lymphatic vessels and blind-end lymphatic capillaries. These anatomical characteristics endow transdermal administration with promising ability in facilitating lymphatic adsorption in dermis. Because hyaluronic acid has receptors (LYVE-1) on lymphatic endothelial cells, a hyaluronic acid modified HDV was prepared to deliver the anthracycline doxorubicin to lymphatics through the transdermal route [99]. Hyaluronic acid was modified with glycerol-α-monostearate (HA-GMS) to be incorporated into the membrane of HDV. The *in vitro* accumulative transdermal penetration of doxorubicin loaded into hyaluronic acid HDV was 3 times higher than in solution. Moreover, doxorubicin loaded into hyaluronic acid HDV led to significantly higher accumulation in lymph nodes *in vivo* than doxorubicin loaded in naked HDV. Doxorubicin-hyaluronic acid HDV led to about 10 fold stronger doxorubicin intensity than doxorubicin-HDV in cervical lymph nodes. The uptake of hyaluronic acid-HDV by MCF-7 breast tumor cells was reported to be 9 times higher than that of naked HDV.

8.1.3. Atopic Dermatitis, Wound Healing, Psoriasis and Scar Treatment

The main goal of topical treatments for these pathologies is the local drug action. Drugs so delivered have to enter neither the blood nor accessing underlying skeletal muscle.

Atopic dermatitis is a chronic inflammatory skin disease associated with cutaneous hyperreactivity to environmental triggers. Principal features of atopic dermatitis include barrier dysfunction, excessive transepidermal water losses, secretion of immunoglobulin E (IgE), epithelial cell hyperplasia, fibrosis, infiltration of inflammatory cells into the dermis and epidermis, and secretion of TH2 cytokines. The major clinical symptoms of atopic dermatitis are pruritic and chronic eczematous skin lesions that are distinguished by infiltration of inflammatory cells. Anti-inflammatory treatment based on topical glucocorticoids and topical calcineurin antagonists (tacrolimus and pimecrolimus) is used for the management of exacerbation and, more recently, for proactive therapy in selected cases [100]. However, the prolonged use of glucocorticoids at high doses causes a variety of systemic and local adverse effects, such as atrophy and telangiectasia, at the skin level.

Between 2012 and 2013, taxifolin [101], tacrolimus [102], glycyrrhetinic acid [103] and cetirizine [104], were loaded in HDVs and their activities were tested on chronic allergic contact dermatitis murine models. Overall, it was showed a higher drug skin penetration and local *in vivo* efficacy for drugs loaded into HDV, as compared to commercial creams. However, none of these experimental approaches quantified the drug in blood and muscle after repeated applications. Further studies are needed to assess its realistic therapeutic usefulness in humans.

The healing process involves different overlapped phases: inflammation (inducing haemostasis and clot formation), fibroplasia and neovascularization, generation of granulation tissue, re-epithelialization, and finally the formation of new extracellular matrix and tissue remodeling [105]. In minor wound damages, full restoration of the integrity of tissues structure and barrier function occurs. However, in severe cases such as large skin injuries, second and third degree burns, several factors are involved and prolong the healing time [106]. The longer the time for spontaneous healing process, the worse is the obtained result. Edema, inflammation, hypertrophic scars and unsightly pigmentation changes are likely to be associated with such complicated cases.

Curcumin has been reported as a promising wound healing agent when used topically. Despite its effectiveness, curcumin dermal delivery is handicapped by hydrophobicity, high metabolism and poor skin permeation. On the other hand, hyaluronic acid has wound healing properties and unique viscoelastic properties. Hyaluronic acid gel-core HDV loaded with curcumin were prepared in order to combine positive effect of HDV in enhancing skin penetration with the reported stability of gel-core liposomes against degradation [107]. Hyaluronic acid gel-core HDV showed five folds higher skin deposition compared to conventional HDV. Hyaluronic acid gel-core HDV treated burn wounds showed almost normal skin with no scar confirmed by histological analysis.

Asiaticoside is a triterpene glycoside from *Centella asiatica*, commonly used in wound healing and scar formation in post-surgery events. Asiaticoside promotes fibroblast proliferation and

collagen and glycosaminoglycan synthesis. High intracellular delivery of asiaticoside and high stimulation of collagen biosynthesis on primary human dermal fibroblasts with low cytotoxicity was shown for asiaticoside-loaded HDV [108]. HDV provided the greatest *in vitro* skin permeation of asiaticoside and favored an increase in *in vivo* collagen biosynthesis compared with aqueous solution.

Papain is a protease enzyme from *Carica papaya* latex which is widely used for scar treatment from its collagenolytic activity. Papain was incorporated in elastic niosomes and PLGA nanospheres and compared its penetration and scar reduction in rabbit ear model [109]. Elastic niosomes in carbopol gel exhibited the highest papain amount both in the whole skin and the receiver solution in comparison to gel containing papain loaded in non-elastic niosomes, in PLGA nanospheres and in solution. After 28 days of application, gel containing papain loaded in elastic niosomes exhibited highest reduction of hypertrophic scars on rabbits' ears. The numbers of collagen fibers and the height of the scars treated with gel containing papain loaded in elastic niosomes were significantly decreased compared with the control group.

Calcipotriol, a hydrophobic vitamin D3 analogue, is commonly used for topical treatment of psoriasis. The pharmacological target site is the D-vitamin receptor expressed by keratinocytes present in the lower epidermis, where calcipotriol inhibits keratinocytes proliferation and differentiation. The effect of incorporating poly(ethylene glycol)-distearoylphosphoethanolamine (PEG-DSPE) on colloidal stability of HDV and the ability to deliver calcipotriol into the skin was recently reported [110]. In contrast to previous studies, authors did not observe any increase in the deformability of the PEGylated liposomes upon addition of sodium cholate. Inclusion of 0.5, 1 and 5 mol% PEG-DSPE in the membrane enhanced the colloidal stability of the liposomes without compromising the delivery of calcipotriol into excised pig skin. Small (100 nm diameter) vesicles enhanced calcipotriol penetration as compared to large multilamellar vesicles, indicating that the liposomes to some extent migrate as intact vesicles into the SC. However, calcipotriol (^{3}H-calcipotriol) penetrated the skin better than the lipid component of the liposomes (^{14}C-DSPC), suggesting that at least a fraction of the drug is released from the liposomes during skin migration.

8.1.4. Antifungal Treatment

Superficial fungal infections, i.e. those confined to the SC, squamous mucosa or cornea can be benefited by treatments relying on topical drug delivery. The effectiveness of the topical antifungal treatment depends on the penetration of drugs through the skin. However, most antifungal agents have poor skin penetration and it is not efficacious because deep seated fungal infections are difficult to treat with conventional topical formulations.

Terbinafine hydrochloride is a highly hydrophobic antifungal drug, mainly effective on the dermatophyte group of fungi. As a 1% cream or powder, it is used as topical medication for superficial skin infections such as jock itch (*Tinea cruris*), athlete´s footh (*Tinea pedis*), and other types of ringworm (*Tinea corporis*). Oral 250-mg tablet are often prescribed for the treatment of onychomycosis, however most frequent adverse effects are gastrointestinal disturbances such as nausea, diarrhea, and mild abdominal pain. Topical administration can avoid its adverse effects. Recently, the skin permeation of terbinafine hydrochloride loaded in HDV, ethosomes and binary ethosomes (ethosomes containing propylene glycol) was determined [111]. The skin deposition of drug from binary ethosomes, ethosomes, and HDV was 9.88, 3.34 and 2.52 times higher than that of conventional liposomes, respectively. It was observed that binary ethosomes carry drug more efficiently across the skin barrier and into the systemic blood circulation than ethosomes and HDV. HDV made drug easiest to accumulate in the skin, while ethosomes improved drug delivery with greater improvement in skin permeation than improvement in skin deposition

Miconazole nitrate is a widely used antifungal agent, commonly applied topically to the skin or to mucous membrane. HDV prepared with Span 80 as EA improved skin permeation through rat skin of miconazole nitrate compared with conventional liposomes [112]. *In vivo* efficacy on cutaneous candidiases in rats showed that 88.9% of animals treated with HDV and 44.5% and 33.4%, of animals treated with conventional liposomes and drug solution respectively, became culture negative. Fast recovery from fungal infection was noted in case of HDV treatment.

Amphotericin B is also a widely used poor water soluble antifungal agent. In a recent approach, amphotericin B was reported to be loaded in HDV [113]. HDV prepared with Tween 80 as EA showed the highest deformability and AmB incorporation compared with other EA. Fungal strains (*albicans* and non-*albicans Candida* ATCC strains and clinical isolates of *C. albicans*) were more sensitive that mammal cells to amphotericin loaded in HDV. Minimum inhibitory concentration values for amphotericin loaded in HDV were 5-24 and 24-50 times lower than IC50 for J774 and HaCaT cells, respectively. Remarkably, upon 1 h of incubation on human skin, the total accumulation of amphotericin in skin was 40 times higher when applied as HDV than as the commercial liposomal amphotericin B (AmBisome).

Fungal infections of the retina are among the most devastating ocular infections. Topical is the preferred route for drug administration, primarily because of better patient compliance and cost affordability. However, topical application is associated with many other complications, such as extensive precorneal drug loss by high tear fluid turnover, non-productive absorption, drainage through the nasolacrimal duct, impermeability of the corneal epithelium, transient precorneal residence time and metabolism of the drug by anterior segment enzymes. In this context, the lipophilic ketoconazole was loaded into elastic niosomes to target the posterior segment of the eye [114]. Elastic niosomes showed 2 times better corneal permeation in comparison to conventional niosomes. Elastic niosomes results safe in terms of cytotoxicity and genotoxicity on normal human gingival fibroblast, acute dermal/eye irritation/corrosion and chronic eye irritation/corrosion tests. Fluorescent elastic niosomes labeled with 6-carboxyfluorescein when applied topically to the rabbit eye were observed intact in vitreous and the internal eye tissues 2 h post application.

The vaginal route of drug administration is often used for the local treatment of microbial infections, such as bacterial vaginosis and vulvovaginal candidosis. Vulvovaginal candidosis is a mucocutaneous infection caused by yeast of the species *Candida*, and 70-90% of cases involve *C. albicans*. Vulvovaginal candidosis is the second most frequent medical disorder of the reproductive system in women of childbearing age. Besides, bacterial vaginosis is the most common vaginal infection, accounting for 40-50% of cases of vaginal infection in women of childbearing age. Local administration of the water soluble metronidazole for bacterial vaginosis and the low water soluble clotrimazole for vulvovaginal candidosis have been favored to avoid the numerous side effects caused by systemic drug administration. However, topical vaginal drug delivery has some limitations including leakage, messiness and a short residence time. Clotrimazole and metronidazole within HDV containing propylene glycol were evaluated as a strategy to improve the treatment of vaginal microbial infections in which deeper epithelial layers of the vagina are affected [115]. HDV containing metronidazole showed 8-fold higher elasticity than conventional liposomes. Liposomes containing clotrimazole in contrast, were slightly less flexible than HDV with metronidazole, but still retained a remarkable level of elasticity. Prolonged release of both drugs from the HDV after their incorporation in a carbopol hydrogel compared to the free drugs in hydrogel was found, which should be enough to provide adequate antimicrobial effect.

8.1.5. Antibiotic Ototopic

The typical management of acute otitis media usually includes an oral course of a broad spectrum antibiotic. Ototopically delivered antibiotic has been limited to those patients with perforated ear drums or tympanostomy tubes, since the SC layer in the tympanic membrane presents a barrier for trans-tympanic diffusion. *However, local noninvasive antibiotic delivery across an intact tympanic membrane to the middle ear may be considered as a promising alternative to systemic antibiotics for the treatment of acute otitis media.* Ciprofloxacin is a moderate lipophilic synthetic fluoroquinolone antibiotic that has been used for systemic treatment of otitis media in adults and it was approved for topical treatment of otorrhea in children with tympanostomy tubes. Ciprofloxacin was loaded into HDV to enhance delivery to the middle ear across an intact tympanic membrane [116]. The optimal HDV formulation exhibited enhanced *ex-vivo* drug flux through ear skin and tympanic membrane compared with the commercial product (Ciprocin® drops). Greater extent of drug deposition in the tympanic membrane of albino rabbits relative to Ciprocin® was demonstrated.

8.1.6. Anti-Leishmanial Drugs to Macrophages of the Skin

Leishmaniasis is a neglected zoonosis caused by the intracellular protozoa of the *Leishmania* genus, transmitted by the bite of a sandfly vector. Its multiple clinical manifestations include the lethal visceral form, the mostly benign cutaneous form and the more severe and chronic mucocutaneous leishmaniasis. Once injected into the skin by the bite of sandfly, *Leishmania* promastigotes invade local phagocytic host cells, wherein the promastigotes transform into amastigotes, which survive in the harsh environment of phagolysosomes. After a brief period during which amastigotes multiply, promastigotes are released in a cell burst, and skin macrophages and dendritic cells including Langerhans cells are colonized, together with lymph nodes and mucosal cells. The intracellular location of amastigotes within phagolysosomes is the main structural and phenomenological barrier that leishmanicidal drugs have to overcome. The recommended treatment for cutaneous and mucocutaneous leishmaniasis is the parenteral administration of pentavalent antimonial compounds (sodium stibogluconate or *N*-methylglucamine, daily dose for 3 weeks) [117]. This extensive treatment is sufficient to heal the symptoms of up to 77% cases but causes significant adverse events. This drawback, together with the growing resistance to pentavalent antimonials, underscores the need for new therapeutic strategies against cutaneous and mucocutaneous leishmaniasis.

Paromomycin is used in clinical trials for both cutaneous and visceral leishmaniasis since the 1960s. However, its high water solubility and oligosaccharide nature makes its penetration through the SC difficult. Topical application of paromomycin in HDV was reported to cause a significant reduction in lesion sizes and lowered the parasite burden in spleen in *L. major*-infected mice, as compared to paromomycin cream. In vitro drug permeation across stripped skin of paromomycin in HDV was enhanced and the in vivo activity in *L. major*-infected mice was improved, as compared to free drug [118].

Amphotericin B is also used as second line treatment of leishmaniasis, but its high molecular weight and amphoteric nature hinders its adequate cutaneous penetration. Amphotericin loaded in HDV increased skin permeation of drug and showed 100 and 75 % anti-promastigote and anti-amastigote activity on *L. braziliensis* at 1.25 μg/ml [113].

Photodynamic therapy is an attractive therapeutic alternative that could be used to switch from parenteral to topical administration of leishmanicidal agents. Because of the accessibility of skin to irradiation from laser or incoherent light sources, photodynamic therapy has been used with variable outcomes, in experimental and clinical settings against cutaneous leishmaniasis. To increase the delivery of photosensitizer molecules to infected macrophages, recently, the photosensitizer Zn phthalocyanine (ZnPc) was loaded within HDV [119]. It was shown that the activity against intracellular amastigotes of *L. braziliensis* of ZnPc was significantly increased when ZnPc was loaded in HDV. Later, the antiamastigote activity of an improved HDV formulation containing total polar lipids extracted from the hyperhalophile archaea *Halorubrum tebenquichense* was tested (UDA). The new formulation was more extensively taken up by macrophages than HDV lacking archaeolipids, and it was observed to eliminate intracellular *L. braziliensis* amastigotes, without reducing the viability of host cells, keratinocytes, and bone marrow-derived dendritic cells, when irradiated at 0.2 J/cm^2 [120].

8.1.7. Nanovaccination

HDV were also used as topical adjuvants because of their capacity of transporting antigens across the outermost skin layer up to the antigen presenting cells underlying the SC.

The first report of 1998, showed that topically aplied HDV containing Gap junction proteins on mice produced titles of serum specific IgG antigens that were similar to the titles obtained upon intramuscular administration [121]. Later, it was shown that HDV containing a plasmid that codifies the hepatitis B surface small protein produced higher serum IgG titles than the produced with conventional liposomes and equals than the intramuscular administration using alumin (universal adjuvant) [122-124]. More recently, it was shown that upon topical application on mice of HDV contaning ovalbumin (OVA, MW 42.7 KDa) elicited a strong immune response similar to that of OVA solution administrated by subcutaneous injection with alumin [125]. Co-administration of imiquimod achieved significantly higher levels of anti-OVA IgG. *In vivo*, the fluorescence-labeled liposome was detected in hair-follicle ducts, indicating that liposome can penetrate the skin barrier through the hair follicles.

Our group has prepared highly deformable archaeosomes (HDA), nanovesicles made of SPC, sodium cholate and polar lipids from the hyperhalophile archaebacteria *Halorubrum tebenquichense* (3: 1: 3 w: w). These lipids are sn2,3 ether linked saturated archaeolipids, that make vesicles more resistant against chemical, physical and enzymatic challenges than HDV. The combination of deformability with a much more pronounced uptake by phagocytic cells than HDV, make the HDA a promising platform for drug targeted delivery and topical nanovaccination. Topical application on mice of HDA containing OVA produced an IgG2a-biased anti-OVA specific immune systemic response ten folds higher than the produced by OVA within HDL [126]. We observed also that both HDA and HDV could bring OVA across the pathways between keratinocyte clusters known as canyons of human intact skin, at least up to nearly 100 μm depth. A rough measure based on the fluorescence of alexa fluor 647 labeled OVA within both type of deformable vesicles, indicates an intercluster canyons thickness oscillating between 50 -100 μm [127]. Afterwards, an outsized cargo (mixed micelles made of total antigens from *L. braziliensis* promastigotes detergent solubilized with sodium cholate) was formulated within HDA and HDL [70]. Topical application of HDA and HDV containing the antigens raised a serum antigen-specific IgG immune response. These results suggested that the deformable vesicles were capable of efficiently bringing the outsized cargo (nearly 4 folds larger than OVA) towards the antigen presenting cells, probably across the intercellular canyons of the skin. Remarkably however, the IgG2a titers induced by HDA were sustained at levels ten folds higher than those from HDL, which faded within 3 weeks after the first dose.

Recently, it was shown that mice immunised topically with 80 μg tetanus toxoid (MW 150 kDa) plus monophosphoryl lipid A in HDV were protected against a lethal toxin dose for at least 6 months [128]. In contrast, the mice immunised with a similar lipid A free formulation were paralysed for 96 h post-challenge, but re-

cover 5 days thereafter. The immune response to the epicutaneously administration consists chiefly of circulating IgG_1 and IgG_{2b} antibody isotypes, indicative of a specific Th2 cellular response bias. Immunisations by subcutaneous injections elicit mainly IgG_1 and IgG_{2b} as well as IgG_{2a} antibody isotypes, indicative of a mixed Th1/Th2 response. The higher IgG2a concentration measured in the injected animals is regulated by IFN-γ concentration in the animal spleen. IL-10 secretion by splenocytes may moreover affect animal's protection against the normally lethal tetanus toxin challenge via immuno-suppression.

8.2. Antihypertensive Drugs Targeted to Blood Circulation

Hypertension is a chronic disease, with a high chance of causing death, which requires oral long-term treatment with antihypertensive drugs. A transdermal delivery patch of the antihypertensive drug, clonidine, has already been marketed. However, only antihypertensive drugs capable of permeating the skin can be delivered by the transdermal route. Two lipophilic low-MW drugs, valsartan [129] and felodipine [130], were recently loaded in HDV and the activity and pharmacokinetics of these formulations were determined. Valsartan in HDV formulated in a carbopol gel showed better antihypertensive activity in comparison with placebo and conventional liposomes. Valsartan in HDV was released gradually, which resulted in prolonged control of hypertension, with a maximum effect observed at 6 hours and lasting up to 48 hours. On the other hand, topical administration of felodipine in HDV in contrast to oral delivery provided relatively constant, sustained blood concentration with minimal plasma fluctuation with rapid and prolonged peak time. The relative bioavailability of felodipine in HDV was found to be 358% versus that after oral administration.

9. CONCLUSION

A wide range of drugs (mainly lipophilic and low-MW drugs) has been loaded in HDVs aiming to enhance their penetration across the SC and to target diseases sited at different skin depths. Very often, however, a high *in vitro* drug penetration across rat skin is the single experimental evidence presented as a succeeding proof of concept about reaching a depth-specific skin target. Data of therapeutic levels at the target site and, very importantly, of blood levels of the drug (particularly after repeated applications during extended treatments, to discard the probability of toxicity of drugs having serious systemic side effects) have to complement the *in vitro* or *in vivo* penetration assessment, which alone, is insufficient to predict therapeutic activity. Exhaustive studies of *in vivo* drug distribution as a function of HDV structure are lacking. Increasing the bilayer deformability may not be sufficient to meet the complex task of selectively targeting different sites of the skin such as epidermis/dermis, subcutaneous muscle, or blood circulation by the same formulation. Clearly a more rational framework to improve the performance of HDVs is yet to be developed. Probably specifically tailored liposomal bilayers combined with specific drugs and dosage regimens, will be of help. In the last two years, new analytical tools allowed to chase the pathways followed by carried drugs and by carriers across the skin. More sophisticated interpretations of the bilayer mechanics rendered a more realistic picture on the fate and therapeutic potential of HDV. Currently, a single anti-inflammatory highly deformable vesicle formulation has reached Phase III clinical trials, but no commercial product based on this technology is yet available. Novelty and the appearance of subsequent regulatory hurdles is one potential reason for their delayed clinical implementation. As already mentioned, the other is the lack of a rationale under the specific dosage and structural needs to target completely different sites placed at distant levels from each other.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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AUTHOR´S CONTRIBUTION

Professor E.L. Romero planned the conceptual content of the manuscript, which included the section titles, their specific order, the Fig. **2** and the conclusions. Professor M.J. Morilla performed the bibliographic search under such guidelines, prepared the Table and Figure 1 and was focused in describing specific applications of deformable carriers to each target disease.

ABBREVIATIONS

AUC	=	area under the curve
Chol	=	cholesterol
*C*max	=	maximum (or peak) serum concentration
DOTAP methyl-sulfate	=	dioleoyloxypropyl-N,N,N-trimethylammonium
DOPA	=	dioleoylphosphatidic acid
EPC	=	phosphatidylcholine from egg yolk
EE	=	encapsulation efficiency
HDV	=	highly deformable vesicles
HAD	=	highly deformable archeosomes
NaChol	=	sodium cholate
NaDchol	=	sodium deoxycholate
PBS	=	phosphate buffer saline
SC	=	*stratum corneum*
SPC	=	soybean phosphatidylcholine
Tmax	=	the time at which the C_{max} is observed
T80	=	Tween 80

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