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Review article

Nanostructured lipid carriers: A potential use for skin drug delivery systems

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ABSTRACT

Skin application of pharmaceutical products is one of the methods used for drug administration. The problem of limited drug penetration *via* topical application makes searching for safe drug carriers that will provide an expected therapeutic effect of utmost importance. Research into safe drug carriers began with liposome structures, paving the way for work with nanocarriers, which currently play a large role as drug vehicles. Nanostructured lipid carriers (NLC) consist of blended solid and liquid lipids (oils) dispersed in an aqueous solution containing a surfactant. These carriers have many advantages: good biocompatibility, low cytotoxicity, high drug content; they enhance a drug's stability and have many possibilities of application (oral, intravenous, pulmonary, ocular, dermal). The following article presents properties, methods of preparation and tests to assess the quality and toxicity of NLC. This analysis indicates the possibility of using NLC for dermal and transdermal drug application.

Contents

Introduction

The oral route is the most important and conventional method of drug administration. Unfortunately, oral drug delivery systems have many significant limitations, such as drug degradation in the gastrointestinal track (by enzymes, pH), pre-systemic metabolism or toxic side effects. One of the methods which could overcome problems associated with the oral route is transdermal drug delivery (TDD) [1,2]. Application of drugs in the liquid, semisolid or solid form to treat skin diseases has been used since the beginning of humanity. Later, the next goal was to use skin for transdermal drug delivery to provide the systemic therapy. The first transdermal therapeutic system was developed in 1981 in the form of a patch with scopolamine (Transdermal Scop®) for the treatment of motion sickness. Today, there is a great variety of transdermal products for drugs such as nitroglycerin, clonidine, fentanyl, nicotine, oestradiol, testosterone or diclofenac [3,4].

TDD has obvious advantages over other routes of delivery. It can improve compliance, especially for patients who do not tolerate oral dosage forms, including people who are unconscious or those with swallowing problems. It also allows for pain-free and safe

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administration of drugs, in addition to reducing the frequency of their application. Another advantage of TDD is assurance of more consistent serum drug levels, elimination of hepatic first-pass metabolism, avoidance of drug degradation in the gastrointestinal tract and reduction of side effects (e.g. gastrointestinal upsets) [1,5,6]. The main disadvantage of transdermal drug delivery concerns low permeability of skin, which limits drug penetration. Furthermore, some drugs and excipients used in transdermal formulations can cause skin irritation (erythema, itching, edema) or allergic reaction [7,8].

Problems with dermal and transdermal drug administration

Skin is the largest multilayered organ of the body, with a surface area of 1.7 - 2.0 m^2. Microscopically, skin consists of three layers: the epidermis (outer layer), the dermis (middle layer) and subcutaneous tissue (inner layer). The epidermis is a stratified and squamous membrane composed of several cell layers and it can be divided into the non-viable epidermis (stratum corneum, SC) and the viable epidermis. The SC consists of keratin-filled corneocytes that are anchored in a lipophilic matrix. The dermis is a thick (500–3000 μm) layer that contains blood vessels, lymph vessels, sweat glands and nerve endings. The most inner layer of the skin is a subcutaneous fatty layer that helps to regulate temperature and provides nutritional support and physical protection. The limited permeability of substances through the skin is due to the SC, which is a barrier mainly for hydrophilic drugs, while the viable epidermis is impermeable to highly lipophilic substances [2,9–12].

Drugs can penetrate human skin via two potential pathways: the transepidermal (across the SC) or the transappendegeal. The transepidermal pathway is the most important route for drug skin penetration. The SC route may take place as intra- or inter-cellular penetration. The intracellular route passes through corneocytes by partitioning into and out of cell membranes and allows hydrophilic substances to be transported. In intercellular transport, lipophilic drugs are transported around corneocytes in the lipid-rich extracellular regions. The transappendegeal route permits substances to be transported through sweat glands, across hair follicles and sebaceous glands. The surface of skin appendages is only 0.1–1.0% and it is assumed that the penetration of low molecular drugs via this route is very low, but it can play an important role in the early stage of skin permeation. Many research suggest that transappendegeal route is a useful pathway for skin permeation of macromolecules and nano-/microparticles [1,12–15] (Fig. 1).

Percutaneous absorption can be divided into three steps:

- penetration - release of drug from the vehicle/dosage form and passage into external layer of the skin;
- permeation - passage of the drug from one layer of the skin to the other;
- resorption - penetration of the drug into systemic circulation through the blood and lymph vessels within the dermis [10,16].

Despite the fact that skin is an attractive site for drug delivery, it constitutes a significant barrier to drug absorption 2]. Conventional TDD – drug permeation across the SC – is the passive process which complies with Fick's first law (equation 1). According to this law, the rate of substance absorption (flux, J) across the barrier is proportional to its concentration difference across the barrier. Fick's law is described by the following formula:

$$J = (DK_m / L)\, C_v \quad (1)$$

where: D is the diffusion coefficient of the drug in the SC, K_m is the partition coefficient between the SC and vehicle, L is the length of the diffusion pathway, and C_v is the concentration of the drug in the vehicle [2,11]. These four factors (D, K_m, L, C_v) control the kinetics of percutaneous drug absorption, while the partition coefficient (K_m) and the concentration of the drug in the vehicle (C_v) are highly dependent on vehicle properties. Many clinical reports have shown that the composition of the vehicle has a significant impact on the rate and extent of drug absorption. Development of a vehicle for transdermal drug administration requires consideration of two critical factors: drug solubility in the vehicle (C_v) and maximizing partitioning of the drug from the vehicle to the stratum corneum (K_m). The partition coefficient determines the drug's ability to move from the vehicle to the outer layer of the SC. This parameter is determined experimentally by measuring the partition of substances between octanol and water

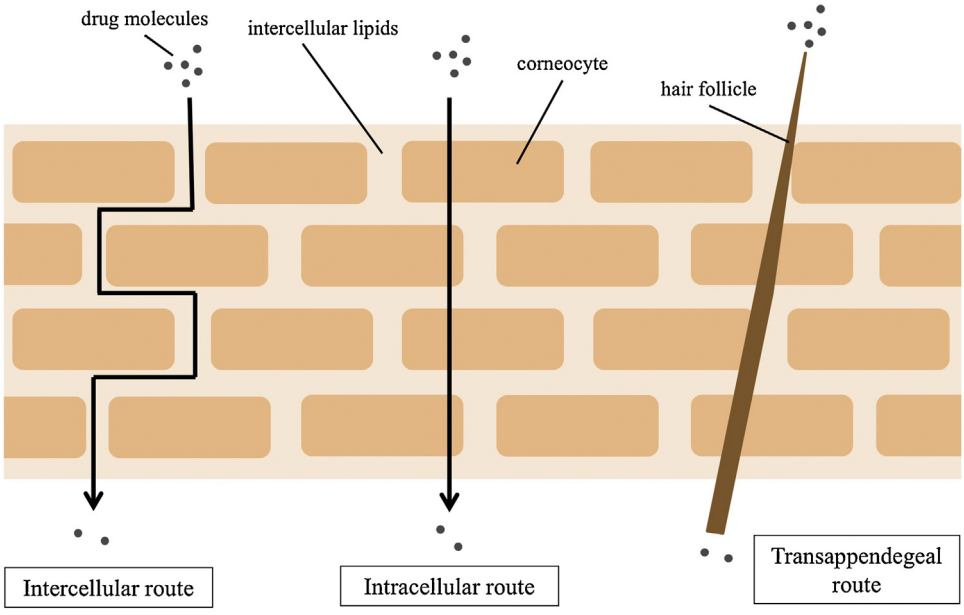

Fig. 1. Routes of drug penetration across the SC.

or lipid and water. Drugs that have intermediate partition coefficient values (log P octanol/water of 1–3) have adequate solubility in lipids of the SC and they are capable of diffusion. However, the diffusion coefficient (D) is related to low molecular size and the low melting point of a substance [2,10,11].

Many drugs applied to the skin are poorly absorbed because only a small fraction of the drug passes to the SC. The majority of the drug dose is removed from the skin surface as a result of exfoliation, sweating, wash-off, rub-off, adsorption into clothes and chemical or photochemical degradation. Drugs approved for TDD should possess appropriate physicochemical properties, such as low melting point (< 150 °C), low molecular weight (< 500 Da), moderate lipophilicity (log P 1–3) and high potency (total daily dose < 10 mg). However, there are many other factors that influence transdermal drug delivery. Skin features (age, diseases, hydration, blood flow, anatomic site of skin and in case of transfollicular permeation - hair density and follicle size) and components of TDD play very important role in drugs penetration through the skin. A variety of strategies are available to overcome low skin permeability, including chemical or physical methods [2,13,16,17]. Chemical methods include using chemical permeation enhancers, pro-drugs, and colloidal formulations, while physical methods refer to iontophoresis, electroporation, microporation, sonophoresis, needleless injection and magnetophoresis [17–20]. The simplest method to facilitate drug permeation through the skin is the application of chemical permeation enhancers. They are divided into many groups based on their chemical character (Table 1) and should be used with caution because they can induce the same mechanisms (disruption of ordered lipid bilayers of the SC or to corneocytes' structural organization) as substances that irritate the skin [10,21–23]. The action of permeation enhancers is not entirely limited to affecting the integrity of the SC; it also influences the structure of living structures (viable epidermis).

The safest permeation enhancer is water, which increases skin penetration of both hydrophilic and lipophilic drugs. Increased skin hydration affects a drug's solubility, modifies partitioning from vehicle to membrane and changes SC structure leading to more effective drug penetration [25].

Prausnitz et al. [4]proposed classification of TDD for three generations. The first generation includes small, lipophilic and uncharged molecules that penetrate through the skin by passive diffusion. Using chemical enhancers and methods such as ultrasound or iontophoresis is the second generation of transdermal products, whereby drug penetration occurs due to reversible disruption of the skin's outer layer. The third generation of TDD employs techniques such as usage of microneedles and electroporation to create temporary pores in the epidermis, which serve as pathways for passing drugs into cells. The most commercially available products belong to the first generation [6].

Lipid-based formulations as effective dermal vehicles for drugs

Nanotechnology is a modern and rapidly evolving trend in dermal and transdermal drug delivery which includes several forms of nanocarriers such as liposomes, nanoemulsions, nanocrystals, polymeric nanoparticles, lipid nanocarriers and dendrimers. Lipid nanocarriers show essential advantages over conventional drug forms and they are formulated with biodegradable, non-toxic and non-irritant lipids. The small size (from 40 to 800 nm) of lipid nanocarriers allows to adhere them to the lipid film of SC and to increase the number of drug molecules that penetrate into deeper layers of the skin. Moreover, they demonstrate the occlusion effect, which results in increased skin hydration, thereby improving the absorption of the drug [26–28].

Traditional model of lipid carriers are liposomes, which were discovered in 1960 [29]. As these formulations have many disadvantages, including short shelf life, poor stability, low encapsulation efficacy and cell interactions, in the years that followed, new lipid-based forms were developed [30,31]. In 1990, two scientists, professor R.H. Müller and professor M. Gasco, conducted their first research on lipid nanocarriers, which they called solid lipid nanoparticles (SLNs). Their research showed that SLNs can be an alternative to organic nanoparticles (e.g. PLGA nanoparticles) and traditional lipid-based formulations (e.g. emulsions, liposomes) [32–35]. SLNs are prepared from single solid lipids or their mixtures (triglycerides, fatty acids, steroids, waxes) at concentrations of 0.1% to 30%, dispersed in an aqueous phase and stabilized by a surfactant (0.5% to 5%). The lipid phase of an SLN is solid at body and room temperatures [30,35]. These lipid carriers minimize problems connected with traditional drug formulations and have some advantages, such as: ease of preparation, good biocompatibility, lower cytotoxicity, avoidance of organic solvents and wide possibilities of application. Due to the solid lipid matrix of SLNs, the sustained release of a drug can be provided. However, the main problems of SLNs are low drug loading and the possibility of drug expulsion during storage. This process takes place due to polymorphic transformations. During SLN fabrication, part of the particles crystalize and form a high energy modification (α or β'). During storage, these modifications can change to a more ordered (low energy) β form, in which the number of imperfections in the crystal lattice is small and the space to accommodate the drug is reduced, which leads to drug expulsion [35–38].

The second generation of lipid nanoformulations is nanostructured lipid carriers (NLC), developed in 1999 [39,40]. The first use of an NLC was related to retinol - a substance degraded by oxidizing agents and light [39]. Further studies have been carried out confirming the applicability of NLC as carriers for many drugs [41]. Drug molecules can be encapsulated within an NLC for different routes of administration: oral (e.g. etoposide, fenofibrate,

Table 1
Types of chemical permeation enhancers [11,24].

Chemical group	Examples
Alcohols	*ethanol, pentanol, benzyl alcohol, lauryl alcohol, propylene glycol, glycerol*
Fatty acids	*oleic acid, linoleic acid, valeric acid, lauric acid, capric acid*
Amines	*diethanolamine, triethanolamine*
Esters	*isopropyl palmitate, isopropyl myristate, ethyl acetate*
Amides	*1-dodecylazacycloheptane-2-one*
Hydrocarbons	*alkanes, squalene*
Surfactants	*sodium laureate, Brij®, Tween®, sodium cholate*
Terpenes	*limonene, carvone, menthone, nerolidol, farnesol*
Sulfoxides	*Dimethylsulfoxide*
Phospholipids	*Lecithin*
Urea and its derivatives	*1-dodecylurea, 1-dodecyl-3-methylurea*
Cyclodextrins	*2-hydroxypropyl-β-cyclodextrin*

lovastatin, spironolactone) [42–45], intravenous (e.g. artemether, bufadienolides, β-elemene) [46,47], pulmonary (e.g. celocoxib, dexamethasone, itraconazole, montelukast) [48–51] and ocular (e.g. cyclosporine A, flurbiprofen, ibuprofen, ofloxacin) [52–57]. Examples of drugs in the form of NLC intended for dermal and transdermal administration are shown in Tables 2 and 3, respectively.

Formulation of NLC and their potential application is also described in patents. Patent CN104784158 A ("PLGA (poly (lactic-co-(glycolic) acid) electrospinning fiber loaded with daidzein NLC as well as preparation method," 2015) [77] designs the electrospinning technique as method for NLC preparation containing daidzein (cardiovascular properties), while patent CN105769817 A ("Nanostructured lipid carrier with surface modified by TCS (thiolated chitosan) and preparation method of NLC", 2016) [78] discloses a novel synthetic method of NLC preparation with the surface modified by TCS. Positively charged surface of TCS modifies the negatively charged surface of NLC and accordingly, the mucosa adhesion, bioavailability and pharmaceutical efficacy of various drug delivery system are improved. NLC as carriers for substances in cosmetic products are also widely studied. Patent CN107349116 A ("Nanostructure lipid carrier coated with sunscreening agent and preparation method and application thereof", 2017) [79] describes method of preparation and characteristic of nanolipid carriers containing sunscreen agents, while patent CN105534724 A ("Resveratrol-coated nano solid lipid carrier and preparation method thereof", 2016) [80] concerns NLC with resveratrol. Poor biocompatibility and problems with resveratrol stability limit its application in cosmetics, and NLC seem to be promising carriers for this drug. The first formulations containing NLC - NanoRepair Q10® cream and NanoRepair Q10® serum (Dr. Rimpler GmbH, Germany) were introduced in the cosmetic market in 2005. Currently on the market there are more than 30 cosmetics containing NLC but no pharmaceutical products are available [39,81].

NLC are the mixture of solid and liquid lipids, dispersed in the aqueous phase with surfactants (Table 4). NLC are characterized by a less organized structure, which allows for higher drug loading capacity and more drug stability during storage. It has been reported that higher drug entrapment efficiency in NLC results from imperfections in NLC structure, higher space for drug loading and the presence of both solid and liquid lipids, in which many drugs exhibit better solubility [82–84]. The melting point of a lipid mixture in an NLC is lower than the melting point of pure solid lipids, but NLC remain solid at body temperature. In NLC, solid lipids are mixed with liquid lipids, most frequently in the ratio of 70:30 up to 99.9:0.1, whereas the surfactant concentration is in the range of 1.5–5.0%. NLC are designed to overcome SLN imperfections: they reduce the risk of drug expulsion during storage, minimize high water content in SLN dispersion and enable greater drug loading capacity [26,41,85].

NLC can protect drugs from chemical degradation, simultaneously enhancing the stability of drugs sensitive to light, oxidation, and hydrolysis and enabling modulation of drug release (Fig. 2). Furthermore, the small size of NLC provides close contact to the stratum corneum and leads to reduction of corneocyte packing, which in consequence can facilitate drug penetration to deeper skin layers [41,85,86]. After application, NLC produce a mono-layered lipid film on the skin, causing an occlusion effect. They prevent water evaporation from the skin, lead to increased skin hydration, and thus facilitate drug permeation through the SC. In addition, the presence of surfactants in NLC causes changes in skin structure and plays the role of skin permeability agents [41,87] (Fig. 3). NLC with particles smaller than 400 nm and containing at least 35% high crystallinity lipid have the most effective occlusion effect – 15-folds more occlusive than microparticles [85,88,89].

Drug penetration after topical application of an NLC is conditioned by many factors, such as the type and concentration of lipids and surfactants, drug localization in the NLC and the NLC production method. NLC change intercellular packing as a result of reduced corneocyte arrangement and extended inter-corneocyte gaps. The mechanism of drug release from an NLC consists of diffusion and lipid particle degradation in the body [41,85]. Drug release is affected by alteration from a highly disorganized lipid structure to one with more organized stable modifications. It is ideal to achieve controlled release from an NLC that is triggered by an impulse after application. Impulses for designing skin preparations with NLC can be temperature increase or water evaporation leading to changes in the lipid structure and, in consequence, to drug release. A drug release profile might be modified according to composition of the lipid matrix, surfactant concentration and production parameters (time, pressure, temperature) [37,93,94].

Table 2
Drugs encapsulated within NLC for dermal application.

Drug	NLC composition	Outcome	Ref.
Artemether	Gelucire 43/01/Transcutol/Phospholipon 85G	*Ex vivo* study showed at 46 h about 26% cumulative amount of artemether had permeated through skin irrespective of dose and served as a reservoir to gradually control drug release over an extended period of time.	[58]
Benzocaine	Compritol 888 ATO/Miglyol 812/Lutrol F68	The test of antinociceptive effect in mice showed that NLC containing benzocaine was an effective drug reservoir and provide prolonged anaesthetic effect.	[59]
Coenzyme Q_{10}	Cetyl palmitate/Miglyol 812/Tegocare 450	The test showed fast release of coenzyme Q10 from NLC during 3 h and prolonged release afterwards. Q10-NLC showed 10.11 times greater drug uptake in epidermis in comparison with Q10 emulsion.	[60,61]
Econazole	Precirol/Squalene/Pluronic F68/Lecithin	NLC showed higher permeability and thermodynamic stability for dermal delivery in fungal skin diseases.	[62]
Methotrexate	Witepsol S51/Oleic acid/Tween 60 or 80	The *in vitro* test showed that NLC increase drug skin penetration.	[63]
Quercetin	Glyceryl monostearate/Stearic acid/Medium chain triglycerides/d-α-Tocopherul polyethylene glycol 1000 succinate	NLC showed increased drug retention in epidermis and dermis and higher anti-oxidant and anti-inflammatory effects	[64]
Progesterone	Tristearin/Miglyol 812/Poloxamer 188	NLC constituted of tristearin with caprylic/capric triglyceride showed slower release of progesterone than nanoparticles constituted of pure tristearin. The *in vivo* study, based on tape stripping indicated decrease of progesterone in stratum corneum within 6 h, suggesting an interaction between nanoparticle and skin lipids.	[65]
Spironolactone	Compritol 888 ATO/Olive oil/Transcutol/ Tween 80	Confocal laser scanning microscopy confirmed the possibility of localized NLC delivery into the hair follicles.	[66]
Vitamin E	Precirol ATO 5/Vitamin E/Tween 80	NLC containing witamin E was adequate for skin application and was characterized by stability, biocompatibility, non-toxicity and improving skin hydration.	[67]

Table 3
Drugs encapsulated within NLC for transdermal application.

Drug	NLC composition	Outcome	Ref.
Flurbiprofen	Stearic acid/Soybean oil or Olive oil or Castor oil/Lecithin/Tween 20	NLC showed biphasic and significantly prolonged drug release through excised rat skin compared with the commercial gel. DSC indicated that NLC penetrate into skin follicles and accumulate in dermis. In the study of carrageenan-induced rat paw edema NLC with flurbiprofen showed quick onset and sustained anti-inflammatory effect within 24 h	[68]
Ibuprofen	Witepsol E85/Miglyol812/Lutrol F68	NLC demonstrated higher drug permeation than from the suspension. The ibuprofen permeation through the excised human skin was significantly higher from NLC gel than from control gel.	[69]
Lansoprazol	Glyceryl monostearate/ Stearylamine/ Sodium dodecyl sulfate/Pluronic F68	Transdermal application of NLC hydrogel sustained drug release and maintained drug concentration in blood for longer period of time with compared to intravenous administration. NLC was accumulated in the skin and ensured drug penetration through the skin continuously to main constant drug concentration in the blood.	[70]
Lornoxicam	Compritol 888 ATO/Lanette/Oleic acid/Pluronic F68	NLC showed higher drug penetration through rat skin compared with control gel.	[71]
Meloxicam	Cetyl palmitate/Caprylic acid/ Tween 80/Propylene glycol	NLC gel containing meloxicam enhanced the skin permeation and deposition of drug into dermis in comparison with control (meloxicam gel).	[72]
Nebivolol	Glyceryl monostearate/Oleic acid/ Span 80/Cremophor EL	*Ex-vitro* skin permeation study indicated that NLC hydrogel provided sustained release of nebivolol.	[73]
Nitrendipine	Dynasan 114/Lecithin/Caprylic/ capric triglycerides/Tween 80	NLC gel showed antihypertensive activity *in vivo* from the first hour compared to oral administration.	[74]
Sildenafil	Cetyl palmitate/Glicerol monolinoleate/ Span 85/Propylene glycol	NLC formulation demonstrated significantly enhanced drug release compared with its suspensions. NLC allowed to higher initial release of sildenafil from NLC followed by controlled release (gives possibility for faster onset and longer sildenafil duration).	[75]
Simvastatin + Olanzapine	Tripalmitin/Oleic acid/Tween 80	NLC presented better *in vivo* performance compared with the control gel. Penetration enhancers such as limonene and ethanol showed synergistic permeation enhancement for drugs.	[76]

Table 4
Excipients used in NLC for topical application [26,41].

Excipients	Chemical name	Trade name examples
Solid lipids	*Beeswax*	–
	Carnauba wax	–
	Cetyl alcohol	Lorol® C16
	Cetyl palmitate	Cutina® CP, Crodamol™ CP
	Glyceryl dibehanate	Compritol® 888 ATO
	Glyceryl monostearate	Imwitor ® 900, Geleol™
	Glyceryl palmitostearate	Precitol ATO® 5
	Glyceryl trimyristate	Dynasan® 114
	Glyceryl tripalmitate	Dynasan® 116
	Glyceryl tristearate	Dynasan® 118
	PEG-8 Beeswax	Apifil®
	Stearic acid	–
	Stearyl alcohol	–
Liquid lipids	*Caprylic/Capric trigliceride*	Miglyol ® 812, Miglyol® 810, Labrafac™, Softisan® 378
		–
	Castor oil	–
	Sesame oil	–
	Squalene	–
	Oleic acid	Capmul® MCM
	Glyceryl caprylate/Caprate	Gelucire® 44/14
	Lauroyl polyoxylglycerides	
Surfactants	*Poloxamers*	Pluronic ® F68
	Polysorbates	Tween® 20, Tween® 80
	Tyloxapol	–
	Sodium cholate	–
	Phosphatidylcholine	Phospholipon® 80/H
	Soybean lecithin	Epikuron™200
	PEG-40 Hydrogenated castor oil	Cremophor® RH 40
	Caprylocaproyl macrogol-8 glycerides	
	Sorbitan laurate	Labrasol®
	Sorbitan oleate	Span® 20
	Macrogol-15-hydroxystearate	Span® 80
		Solutol® HS 15, Kolliphor® HS 15

Formulation of NLC

The most popular method of NLC production is high pressure homogenization (HPH), which is divided into hot and cold techniques. This method is especially common for large-scale production of nanocarriers and has many advantages, such as easy scale up and short production time. In addition, it does not use organic solvents. In the hot method, a hot surfactant solution is added to a mixture of melted lipids (being melted around 5–10 °C above melting point) containing a given drug, using a high-speed stirring. The obtained emulsion is homogenized under high pressure (generally at 500 or 800 bar) to create hot nanoemulsion, then cooled to room temperature, forming an NLC [26,41,95,96]. In cold homogenization, the melted lipid containing active substances is cooled down (using liquid nitrogen or ice). Then, the obtained mass is crushed and ground. The prepared microparticles

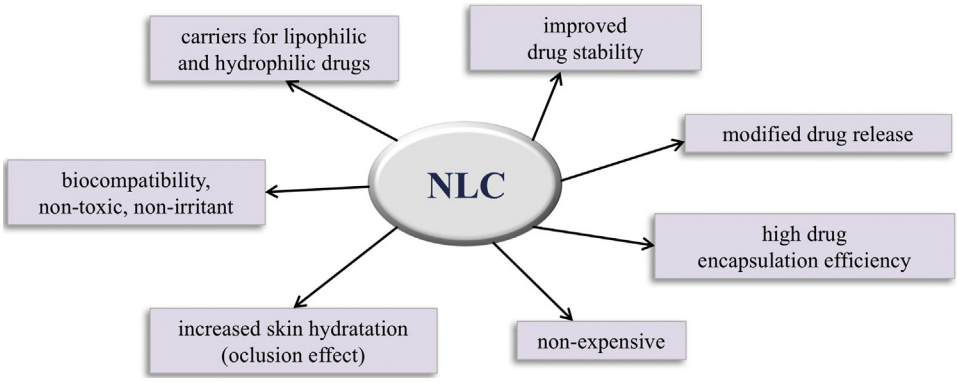

Fig. 2. Advantages of nanostructured lipid carriers (NLC).

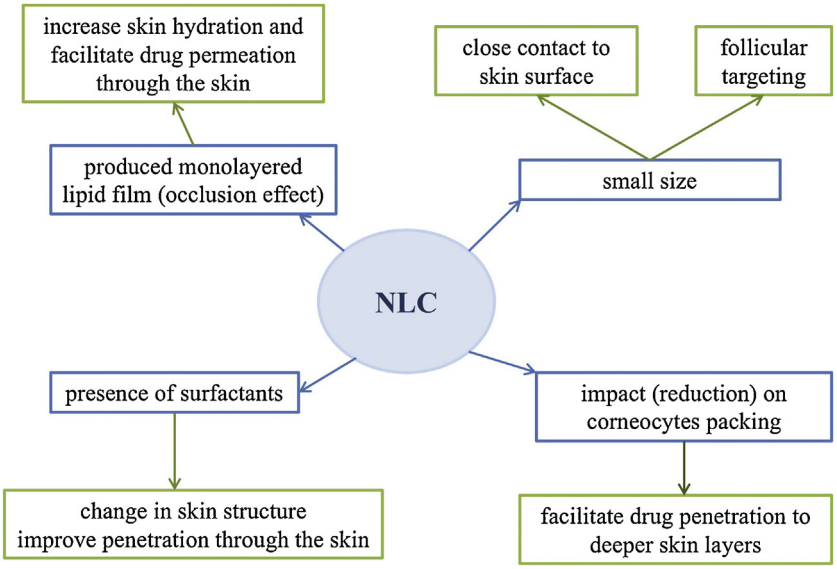

Fig. 3. Effect of NLC interaction on skin associated with improved drug penetration [90–92].

are dispersed in a cold surfactant solution and then passed through a high-pressure homogenizer at room temperature. The disadvantages of the hot HPH method include the use of high temperatures and pressures and large concentrations of surfactants. Lipid formulations prepared by this method are often characterized according to burst release. In order to achieve prolonged release and to reduce the burst effect, some modifications are used (e.g. low temperature - cold homogenization, lower surfactant content) [35,39,97].

Other methods of NLC preparation include the microemulsion technique [98,99], solvent diffusion method [100,101], ultrasonication [30,102,103] and membrane contactor process [104,105]. The solvent diffusion technique is often used as an alternative to HPH and is very easy, does not require special equipment and allows sustained drug release to be obtained. However, the main drawbacks of this method are the use of organic solvent and lack of large scale production. In the solvent diffusion method, lipids and drugs are dissolved in an organic solvent (acetone, ethanol) and the prepared organic solution is then quickly dispersed into water under mechanical agitation at 70 °C. During the evaporation process (heating the pre-emulsion), the organic solvent is removed. The obtained pre-emulsion is then cooled at room temperature and dispersion of nanoparticles is achieved [106–108].

In the preparation process of NLC, their aqueous dispersions are obtained. In order to ensure stability of aqueous NLC dispersions, adding a preservative agent (e.g. phenoxyethanol with ethylhexylglycerin or parabens, propylene glycol, pentylene glycol) is recommended [41,109]. To obtain the solid form of an NLC, water from the dispersion is removed using either the freeze-drying or spray-drying method. Preparation of the final formulation for skin application includes incorporation of an aqueous NLC dispersion or solid NLC in preformed topical products (e.g. creams, gels). Another method is the addition of viscosity enhancers (e.g. Carbopol, chitosan, cellulose derivatives) to an aqueous dispersion of an NLC to obtain a gel [35,41,109].

In the NLC, a drug can be incorporated between the fatty acid chains or lipid layers or in imperfections of the lipid matrix (amorphous structure) (Fig. 4) [35,84]. Type I, referred to as an "imperfect type," is characterized by a low content of liquid lipids in relation to the solid phase. In the first step, o/w nanoemulsion is prepared from a mixture of blended liquid and solid lipids. Then, the nanoemulsion is cooled to room temperature and a highly disordered matrix is created as a result of the crystallization process. In the imperfect matrix, there are gaps between the triglyceride fatty acid chains in the crystal, which allows the drug capacity to be increased. Type II is created with a specific mixture of lipids (e.g. hydroxyoctacosanylhydroxystearate with

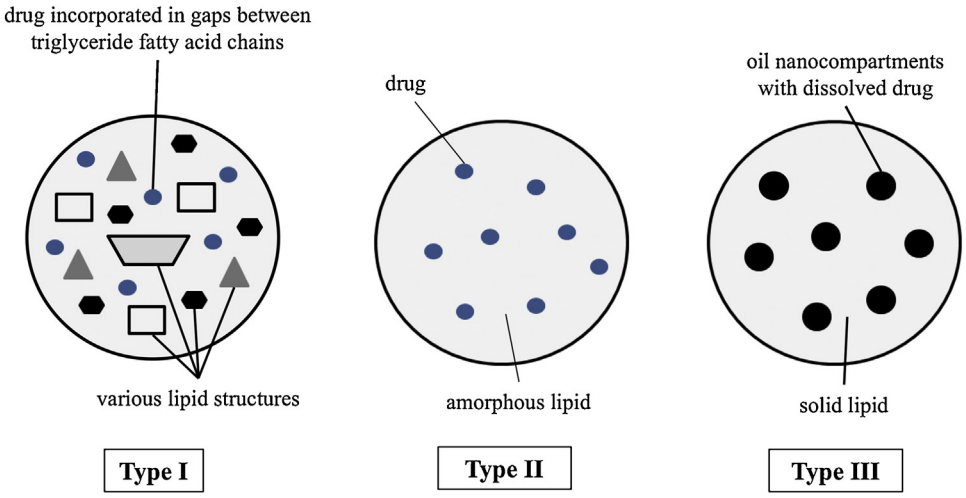

Fig. 4. Different types of NLC: I – imperfect type, II –- amorphous type, III – multiple type (modified according to 112,113).

isopropylmyristate) which forms lipid matrix in an amorphous state. Type III, referred to as a "multiple type," contains a high concentration of liquid lipids and is especially useful when solid lipids are characterized by poor drug solubility, and the addition of a greater amount of liquid oil improves drug solubility and provides higher drug loading [110–113].

Quality evaluation of NLC

Preliminary studies are needed in order to select appropriate liquid and solid lipids and emulsifying agents. It is important to use lipids that provide the highest drug solubility and enable a stable NLC to be obtained (no lipid phase separation) [114]. When the composition of NLC is being designed, an important issue is qualitative and quantitative selection of lipids and surfactants. NLC composition and methods of production affect properties such as particle size, stability, drug loading and drug release profile [30]. The crucial features estimated for NLC are presented in Table 5.

Particle size, polydispersity index (PDI) and zeta potential are examined using the dynamic light scattering technique. Before analysis, samples are appropriately diluted with double distilled water to a suitable concentration, at room temperature [115,116]. Zeta potential is an important parameter for assessing the repulsion of NLC particles and refers to the assessment of non-aggregation and physical stability. Higher zeta potential (values greater than + 30 mV and less than −30 mV) increases the stability of NLC during storage, and the addition of surfactants allows for the electrosteric stabilization of NLC. The ratio of solid to liquid lipids might affect the interactions among NLC components, which influences different mean sizes [117–119].

The morphology and structural properties of NLC can be evaluated by scanning electron microscopy, dynamic light scattering, transmission electron microscopy or atomic force microscopy (AFM) [26,120]. AFM is widely used to evaluate the size, shape and morphology of NLC. X-ray diffraction and differential scanning calorimetry provide information about crystallinity and polymorphism. Confocal laser scanning microscopy (CLSM) allows interactions with cells to be assessed, while nuclear magnetic resonance (NMR) provides information about the size and the qualitative nature of an NLC. The solid-state properties of NLC and physicochemical modifications of drugs incorporated in lipid nanoparticles can be examined by DSC, X-ray diffraction, hot stage microscopy, Raman spectroscopy, and Fourier-transform infrared spectroscopy [70,93]. Drug encapsulated in NLC can be in different physical states – crystalline or amorphous form. To evaluate the physical state of drug in NLC, many techniques are used, e.g. differential scanning calorimetry (DSC) method. Analysis of drug peak observed at DSC thermograms allows to determine whether the substance exists in a crystalline form (presence of endothermic peak) and in an amorphous state or in molecularly dispersed structure in a lipid matrix [121,122].

Drug loading (DL%) and entrapment efficiency (EE%) are assessed by measurement of free drug concentration in the external aqueous phase, obtained by the centrifugation method.

Table 5
Characterization of NLC.

Parameter	Test method
Zeta potential	Dynamic light scattering (DLS)
Drug loading and entrapment efficiency	Ultracentrifugation and quantitative analysis by spectrophotometric or high-performance liquid chromatography method
Morphology (size and shape)	Scanning electron microscopy (SEM), transmission electron microscopy (TEM), photon correlation spectroscopy (PCS)
Degree of crystallinity and lipid modification	Differential scanning calorimetry (DSC), X-ray diffraction (XRD)
In vitro drug release	Franz diffusion cell/dialysis bag method/USP 2 apparatus
In vitro skin occlusion test	Determine the water loss from beakers covered by cellulose membrane with NLC applied
Ex vivo skin penetration study	Franz diffusion cell with human skin or Wistar rat abdominal skin
Skin irritation study	*In vivo* tests using animal models and *in vitro* tests (cytotoxicity) using cell cultures (HFF cell line, RAW 264.7, HaCaT, L-929, BALB/c-3T3 cells) or reconstructed human epithelium
Stability	Measurement of particle size, polydispersity index and zeta potential

Calculations are made on the basis of formulas (W – weight of drug [in mg]) [69]:

$$DL\% = W_{initial\ drug} - W_{free\ drug} / W_{lipid} \times 100\%$$

$$EE\% = W_{initial\ drug} - W_{free\ drug} / W_{initial\ drug} \times 100\%$$

where: $W_{initial\ drug}$ is the weight of the total drug added to the NLC, $W_{free\ drug}$ is the weight of free drug detected in the supernatant layer, and W_{lipid} is the weight of the lipid used in the preparation process. In this method, an NLC dispersion is placed in the upper chamber of a centrifuge tube matched with an ultrafilter and centrifuged at high revolutions per minute, the exact value of which is selected based on the size of particles. Smaller particle size requires higher revolutions per minute of centrifugation. The obtained supernatant is further examined to determine drug content [69,84,123].

Drug release from NLC formulations is affected by many factors such as particle size, type of lipids or surfactants, and the type and concentration of a drug. Drug release can be evaluated using various equipment and acceptor fluids (dissolution mediums) and under various test conditions (temperature, mixing speed) [70,85,93]. The most frequently used model in the dissolution test is the vertical diffusion cell – Franz cell. Another simple method uses a dialysis bag – a dialysis sac made of cellulose membrane is placed in a beaker filled with the dissolution medium. The *in vitro* release test might also be conducted in a USP type 2 paddle dissolution apparatus (Table 6) [69,119,124].

The specific method used to assess the NLC occlusion effect is an occlusion test. Beakers are filled with water, covered with a cellulose membrane and sealed. NLC samples are applied to the membrane surface and stored at 32 °C and 50–55% relative humidity for 48 h. At appointed times (e.g. after 6, 24 and 48 h), the beakers are weighed to determine the water loss due to evaporation. The reference sample is a beaker covered with a membrane without the NLC. The occlusion factor (F) can be calculated according to the following equation:

$$F = (A\text{-}B\ /\ A) \times 100$$

where: A is water loss without the NLC (reference sample), B is water loss with the NLC. The F value of 0 indicates no occlusive effect and F = 100 exhibits maximum occlusion property [90,126].

Ex vivo skin permeation can be conducted in Franz diffusion cells, using animal skin (e.g. porcine ear skin, rat abdominal skin). Samples are placed onto the skin and maintained at 32 °C for 24 h or 48 h, using a phosphate buffer with a pH of 7.4 as the diffusion medium. The next stage of the study is to determine the amount of drug in the receptor medium, in the SC and in deeper skin layers (epidermis and dermis, E + D). The SC is removed by taping and stripping and the remaining skin is fractioned into small pieces. Then, the SC and parts of the skin (E + D) are extracted using an organic solvent. After the samples undergo filtration, the amount of drug is analyzed according to an appropriate method (spectrophotometry or high-performance liquid chromatography) [115,125].

Transdermal drug administration requires the risk assessment of skin irritation. An irritant's effect on the skin might present as either irritant contact dermatitis (ICD) – an inflammatory response caused by skin contact with weak irritants, or as allergic contact dermatitis (ACD) – a delayed response to a specific allergen. The symptoms of ICD usually include erythema and/or necrotic burns, whereas ACD reactions appear as erythema, edema and vesiculation [24,130,131]. The dissociation constant (pKa) of a drug can change the physiological pH of the skin and, consequently, cause irritation. Drugs that possess a pKa value less than 4 and greater than 8 are characterized by the irritant effect on the skin [132–135]. Drugs that have shown moderate or severe irritant effects in animal studies are excluded from use in skin preparations. The preservation of the physiological pH of the skin (5.4–5.9) is an important element of the skin barrier function. A number of compounds (solvents, drugs) can lead to an increase in surface pH, thus causing skin irritation. Preparations at pH 9 and above can cause swelling of the SC and increased transepidermal water loss, which in turn leads to disruption of the skin's protective functions [136].

The evaluation of NLC safety for dermal use requires toxicity studies including *in vivo* and *in vitro* tests. Before 2009, the *Draize test* was the *in vivo* method officially accepted by the OECD (Organisation for Economic Co-operation and Development) [137]. According to the *Draize test*, skin irritation estimation is conducted mainly with albino rabbits. The method includes applying a test substance on a small area of rabbit skin. Scoring of skin irritation, including erythema and edema, is performed after 1, 24, 48 and 78 h and assessed on a scale of 0–4 [138]. Value 0 indicates no erythema or edema, 1 – very slight erythema and edema, 2 – well-defined erythema and slight edema, 3 – moderate and 4 – severe erythema and edema [16]. The *Draize test* is one of the most criticized animal tests due to the large variation in test results and specific differences between human and rabbit skin. Alternatives to the *Draize test* are methods using cell-based assays or reconstructed human epithelium (RhE) models [139,140]. The first method – cell-based assays – applies various cell lines, most often the HFF cell line (human foreskin fibroblast), the RAW 264.7 cell line (murine macrophages), HaCaT cells (human keratinocytes), L-929 cells (mouse fibroblast), and BALB/c-3T3

Table 6
Review of *in vitro* drug release tests using NLC.

Method	Dissolution medium	Temperature [°C]	Mixing speed [rpm]	Final time of sample withdraw [hours]	Ref.
Vertical diffusion cell (Franz diffusion cell with cellulose membrane)	phosphate buffer pH 7.4	32	–	48	[115] [a]
	PBS pH 7.4	32	700	6	[123]
	PBS pH 7.4	32	300	48	[125]
	acetate buffer pH 6.0	32	–	24	[126]
	phosphate buffer pH 6.8	37	–	6	[121] [a]
Dialysis bag	methanol:PBS pH 6.4 (3:7)	37	–	24	[96] [a]
	methanol	37	50	10	[116]
	PBS pH 7.4	37	60 [b]	24	[127]
	water or 1% SDS	25	200	72	[122]
USP type 2 paddle dissolution apparatus	phosphate buffer pH 7.4	32	25	8	[128] [a]
	0.5% SLS (500 ml)	37	100	8	[129]

PBS – phosphate buffer saline, SDS/SLS - sodium dodecyl sulfate, rpm - revolutions per minute.
[a] research conducted for gel containing NLC.
[b] speed of mixing expressed as strokes/minute.

cells (mouse embryonic fibroblasts). In cell lines, the most commonly used parameters are assessment of cell viability using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction test or membrane integrity test (e.g. neutral red uptake assay, lactate dehydrogenase release) [141–146]. The second method – RhE models – are the most advanced *in vitro* skin irritation tests and they include such models as: EpiSkin™, EpiDerm™, SkinEthic™, LabCyte EpiModel24, and Keraskin™. RhE models are made of non-transformed primary human keratinocytes, which mimic the human epidermis. In these models, cell damage is assessed by measuring viability with the MTT test [139,147,148]. In cytotoxicity tests and animal skin irritation studies, it has been shown that formulations containing NLC are either non-irritants or poor irritants to the skin. Moreover, an increase in the safety of NLC preparations (e.g. NLC based gels) has been demonstrated compared to traditional drug forms (marketed gel) [115,121,141–143,149].

Conclusions

Applying drugs to the skin has become an evolutive method for treatment of local and systemic diseases. Replacing conventional dermatological drug forms (ointments, gels) with nanolipids has the main aim of obtaining small particle size and increased penetration of drugs through the skin. The modern form of lipid nanocarriers are nanostructured lipid carriers (NLC). NLC are used particularly for drugs that dissolve poorly in water. The process of obtaining an effective form of NLC requires conducting many experiments. Important criteria include selection of lipid mixture, concentration of surfactants and use of a proper method of fabrication. NLC composition and the process by which NLC are prepared affect the properties of the final product (particle size, stability, release). This article has aimed to provide an overview of the most important and necessary tests to assess NLC properties. While designing NLC formulations, safety is an important aspect. Thus, the article also contains a general description of toxicity methods. Upon careful consideration of the issues discussed, one can conclude that NLC seem to be promising drug carriers for transdermal application.

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Conflict of interest

The authors declare no conflicts of interest.

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