CHARLES UNIVERSITY

Faculty of Pharmacy in Hradec Králové

Department of Pharmaceutical Technology

Formulation, characterization, and skin application of Imiquimod loaded liposomes in combination with dendrimers.

Diploma thesis

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Hradec Králové 2022

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Consultant: Eleni Panoutsopoulou

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Abstrakt

Univerzita Karlova, Farmaceutická fakulta v Hradci Králové

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Školitel: Dr. Georgios Paraskevopoulos, Ph.D.

Konzultant: Eleni Panoutsopoulou

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Název diplomové práce: Formulace, charakterizace a kožní aplikace liposomů s imiquimodem v kombinací s dendrimery

Kožná bariéra tvorená v najvrchnejšej vrstve kože stratum corneum, spomedzi mnohých funkcií, obmedzuje prenikanie liečiv cez epidermis. Nanočastice, ako sú lipozómy a dendriméry, používané na (trans)dermálne podávanie liekov, ponúkajú riešenie, ako toto obmedzenie prekonať. Konvenčné lipozómy so zapuzdreným liečivom preukázali zvýšenú účinnosť a predĺžené uvoľňovanie liečiva pre lokálne dodávanie cez neporušenú kožu v porovnaní s voľným liečivom. Aby sa zvýšila účinnosť lipozómov, bola navrhnutá ich kombinácia s rôznymi systémami dodania.

V tejto štúdii bol Imiquimod naložený do lipozómov v prítomnosti rôznych generácií vlastne syntetizovaných dendrimérov tvoriacich nové pokročilé nanosystémy na dodávanie liečiv (aDDnSs). Po *ex vivo* permeačnom experimente na ľudskej koži sa zistilo, že nižšia generácia dendrimérov pôsobí synergicky s lipozómami a dodáva viac aktívnej látky do epidermis, pričom sa vyhýba nežiaducemu transdermálnemu prestupu.

Abstract

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The skin barrier formed in the uppermost skin layer by the *stratum corneum* - among many functions - restricts the penetration of drugs through the epidermis. Nanoparticles such as liposomes and dendrimers, used for (trans) dermal drug delivery, offer a solution how to overcome this limitation. Conventional liposomes with an encapsulated drug have proven increased effectiveness and prolonged drug release for local drug delivery across intact skin in comparison to free drug. In order to increase the effectiveness of liposomes, their combination with different delivery systems has been suggested.

In this study, Imiquimod was loaded to liposomes in the presence of different generations of in-house synthesized dendrimers forming novel advanced drug delivery nanosystems (aDDnSs). After an *ex vivo* permeation experiment on human skin, the lower generation of dendrimers was found to act synergistic with liposomes and delivered more active substance to the epidermis, while avoiding the undesired transdermal delivery.

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1. List of abbreviations

aDDnSs	advanced drug delivery nanosystems
DLS	dynamic light scattering
EE	encapsulation efficiency
GUV	giant unilamellar vesicles
IMQ	imiquimod
LUV	large unilamellar vesicles
MeOH	methanol
MLCRS	modulatory liposomal controlled release system
MLV	multilamellar vesicles
NPs	nanoparticles
PAMAM	polyamidoamine
PBS	phosphate-buffered saline
PDI	polydispersity index
PEG	polyethylene glycol
PET	physical enhancement technologies
PSC	particle size control
SB	*stratum basale*
SC	*stratum corneum*
SD	standard deviation
SG	*stratum granulosum*
SS	*stratum spinosum*
SUV	small unilamellar vesicles
Tc	transition temperature

2. Introduction and aim of the work

The skin barrier formed by the stratum corneum (SC) - among many functions - restricts the penetration of drugs through the epidermis. One way to circumvent this limitation is to use nanoparticles (NPs) for (trans) dermal drug delivery, such as liposomes and dendrimers. Although liposomes have proven to be very effective drug carriers that increase their penetration through the skin, there is a growing tendency to improve the characteristics of these formulations.

The main purpose of this work was to combine in-house developed dendritic molecules with liposomes and prepare advanced drug delivery nanosystems (aDDnSs) loaded with the lipophilic drug imiquimod (IMQ) and evaluate their ability for effective application on the skin. More specifically, the goal was to find out which combination of liposomes with different generations of dendrimers is the most suitable for topical application of the active substance. The most effective formulation would be the one which is able to deliver the maximum amount of IMQ within the epidermis while avoiding possible penetration of the active substance to the systemic circulation (transdermal delivery).

3. Theoretical part

3.1. Introduction

The theoretical part of this thesis is divided in several subchapters. Initially, a general description of skin's basic characteristics that affect the transport of drugs into and through its different layers is given. Later, an introduction to NPs that are used for skin targeted drug distribution is presented with special attention to liposomes and dendrimers as permeation enhancers for skin application. Various preparation methods of liposomes are described together with their advantages and disadvantages, including their further treatment, such as extrusion and sonication. The different characteristics and use of dendrimers are also highlighted. Furthermore, basic information about the immunomodulatory action of IMQ is provided. The section is closing with a brief description of aDDnSs together with representative examples of targeted delivery.

3.2. Skin and its properties

Skin - except being the outermost protective part of human body - is also its largest organ. It consists of 3 layers; epidermis, dermis, and hypodermis. (1) Its complex and intricate structural parts are creating the first barrier and therefore human body's protection against pathogens, ultraviolet (UV) radiation, temperature extremes, bacteria, toxins, chemicals, or simple mechanical damage. (1) (2) Human skin contains a tangled network of blood vessels serving as a cooling or heating system for the body depending on the situation. Moreover, they play an important role not just in the regulation of blood pressure. Blood flow is controlled by the function of certain sphincter-like vessels. Lymph vessels, which are forming a mesh in the skin, contribute to the skin related immunologic responses by their consistent lymphatic drainage. (3)

3.2.1. Skin structure

Skin is a part of the integumentary system - together with its appendages such as nails, hair, and certain glands – which can reach a total surface of $2m^2$. (2) Numerous stratified squamous layers of highly differentiated keratinocytes are forming the upper skin layer (epidermis). These cells steadily proliferate and consecutively create *stratum basale* (SB), *stratum spinosum* (SS), *stratum granulosum* (SG), and *stratum corneum* (SC) as the main layers of the skin which are depicted in Figure 1. (4) The inner-most part, (SB), mainly contains cells which are expressing the proliferative protein keratin 14. These cells are tangled with minor populations of melanocytes, leukocytes, and sensory Merkel cells. The cells of SB secrete components of the extracellular matrix that subsequently form a separative structure called the basement membranes. (5)

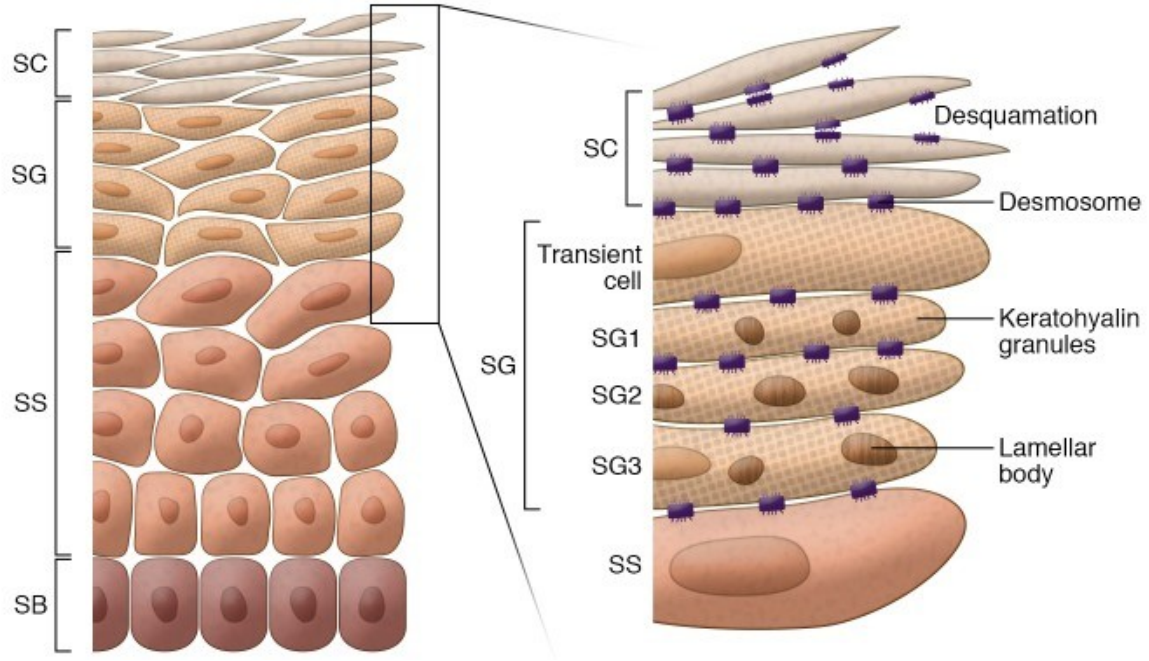

Figure 1- Structure of epidermis (4)

Basement membranes connect epidermis and dermis. Their major constituents are collagen, non-collagenous glycoproteins, and a set of distinct proteoglycans. (6) These membranes have a gate-keeping role that includes controlling cell traffic and diffusion of bioactive molecules. Furthermore, they play a key role in binding several

various cytokines and future release of growth factors during different skin damages (injury, inflammation, etc). (7)

3.2.2. Keratinocytes

Keratinocytes are epidermis' most significant cells, and they are found in all layers of epidermis. They have an ectodermal origin and differ from melanocytes and Langerhans cells in the epidermis by having intercellular bridges, larger size and ample cytoplasm. Corneocytes, which are also present in the skin, differ from keratinocytes since they are not able to produce keratin. (8) Adhering junctions between keratinocytes are called desmosomes in a similar manner like corneodesmosomes connect enucleated corneocytes. Thus, desmosomes and corneodesmosomes are adhering junctions which provide strength for the epidermal structure. (9)

Epidermal keratinocytes can undergo two processes; the first pathway, called differentiation, is present in healthy skin when keratinocytes differentiate from the basal layer through squamous, granular, and cornified layers. That process may be altered by vitamins, such as retinoic acid and vitamin D3. Expressions of specific keratin genes serve as markers for basal *versus* differentiating cells. In case of any pathologic condition, such as psoriasis or an epidermal injury, an alternative pathway called activation starts to initiate itself. The main differences between normal skin and psoriatic plague histology are that psoriatic plagues have a thickened SC while the granular layer is missing or altered. In addition, the SS is expanded creating club-shaped rete ridges. (Figure 2). (10)

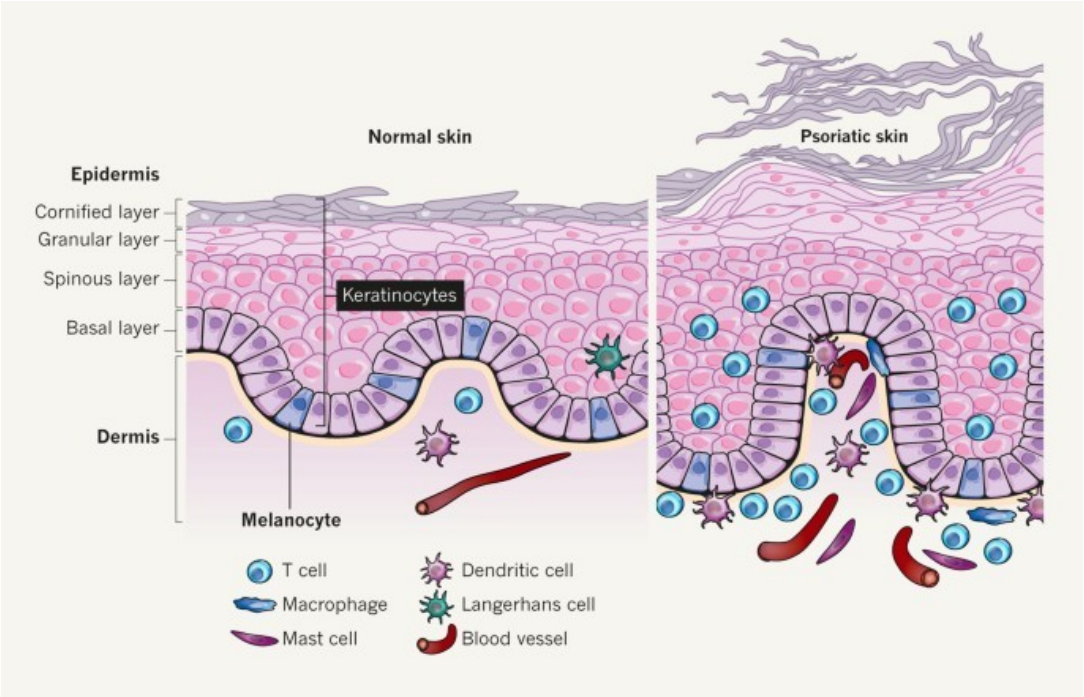

Figure 2 Normal vs psoriatic skin. In comparison to normal skin, psoriatic plaques have a thickened compact SC (11)

Growth factors and cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), transforming growth factor α and β, interferon-γ (IFN-γ) affect prominently the activation process. (12)

3.3. Delivery systems used for skin drug delivery

The uppermost part of epidermis, SC, serves as a barrier that restricts effective tissue permeation and consequently limits the extent to which the skin can be adequately used to deliver molecules topically, to the underlying tissues, or systematically for therapeutical purposes. (13)

A drug is passively delivered through the skin when fulfils certain conditions like adequate lipophilicity (log $P_{o/w} \cong$ 1-3) and a molecular weight <500 Da. Therefore, products aimed for dermal and transdermal delivery have been limited for those complying with these requirements. To overcome the obstacle, several new delivery systems have been developed for dermal (topical - minimal systematic absorption) and transdermal (systematic circulation) delivery. (14)

3.3.1. Passive and active permeation methods

Passive and active methods have been introduced to amplify the permeation *via* the skin. The passive technologies entail the use of formulation excipients, optimization of the drug-carrying vesicle, chemical penetration enhancers, and diverse types of micro and nano-delivery systems. The main goal of this strategy is the disruption of the rigid lipid layer of skin lipids. (15) On the other hand, the active approach uses an external driving force such as thermal (laser and radiofrequency thermal ablation), electrical (iontophoresis and electroporation), mechanical (microneedles), ultrasound, and velocity (jet injector) based approaches to enhance skin delivery (Figure 3). (13)

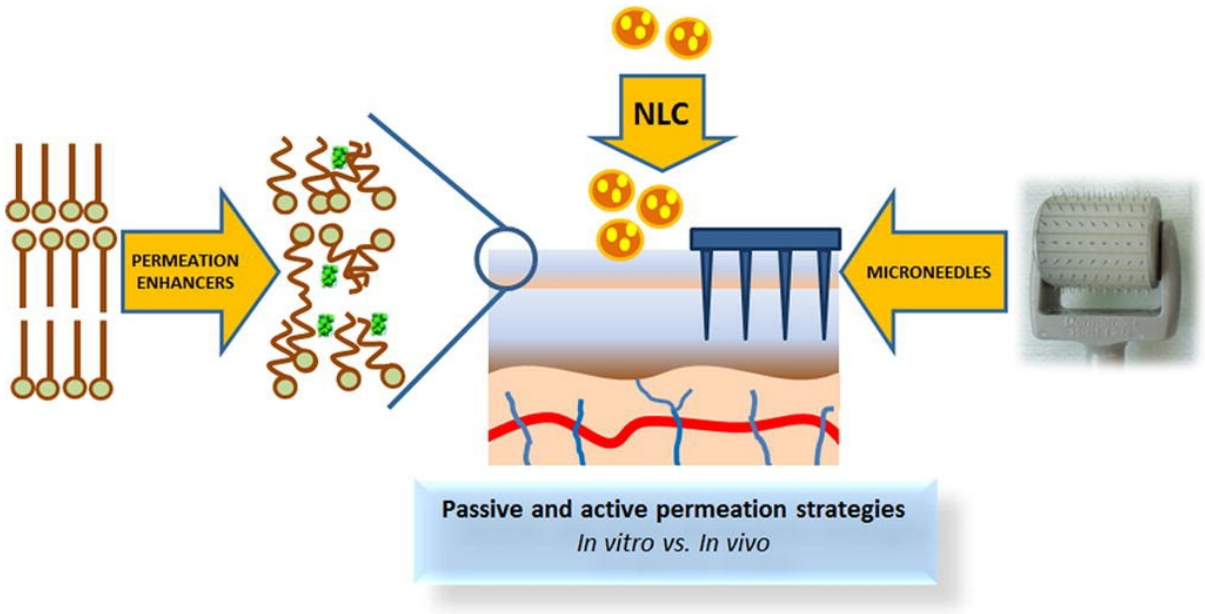

Figure 3- Passive (on the left) and active (on the right) permeation strategies (NLC-nanostructured lipid carrier) (16)

Classical examples of passive delivery systems are polymeric NPs, quantum dots, metal NPs and dendrimers. Lipid based colloid systems can also be used like liposomes and their different variations (transferosomes, ethosomes, niosomes) (Figure 4). (17) Nanoemulsions, nanostructured lipid carriers, and solid lipid NPs also present submicron-sized formulations acting as enhancements in transdermal drug delivery (TDD). These structures have the potential to target delivery to certain skin regions, enhance bioavailability and efficacy and promote the formulation of lipophilic poorly water-soluble compounds. (13)

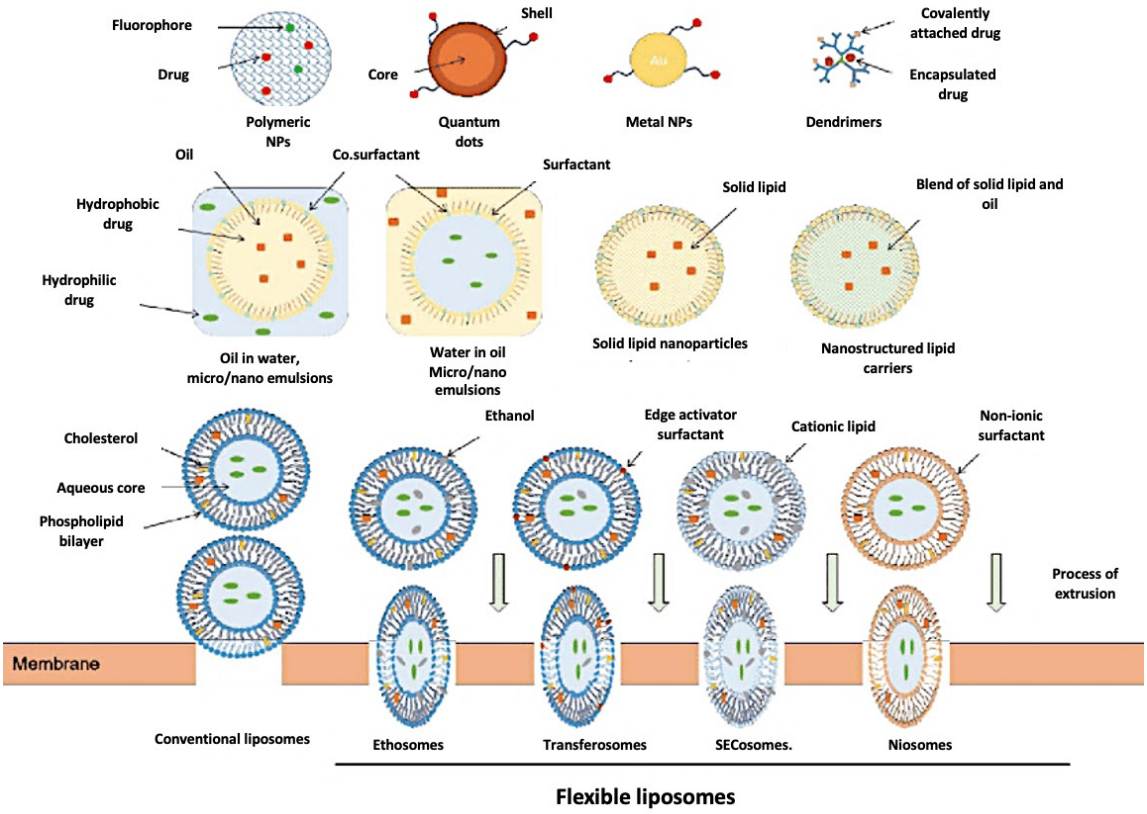

Figure 4- Nanodelivery systems used for topical and transdermal delivery (18)

Physical enhancement technologies (PET) can be carried out directly or indirectly. Indirect PET includes the application of acoustic (sonophoresis), electrical (electroporation and iontophoresis), magnetic and laser energy to increase the permeability of an applied drug in the SC. (19) Direct PET are outlined as marginally invasive methods that share one common trait and that is creating a hole or pore in the SC barrier through which can subsequently pass the drug to the epidermis. The methods in direct PET have the same outcome but they differ in using various methods. These may include mechanical, pressure-based, or thermal technologies. (13) (16)

3.3.2. Liposomes

Liposomes (Figure 5B) have their origin anchored in Cambridge where they were first discovered in the mid-1960s by British scientist Alec Bangham and his colleagues. (20) Natural lipids were basic components of firstly emerged liposomes. Nowadays they can contain natural and/or synthetic lipids and surfactants. Concentric phospholipids that form liposomes are amphiphilic molecules that have a hydrophilic head and two non-polar hydrophobic chains. The same molecules can be organized in micelles (Figure 5A) or lipid bilayers (Figure 5C). The structural differences between micelles, liposomes and lipid bilayers are schematically represented in Figure 5. (20) (21)

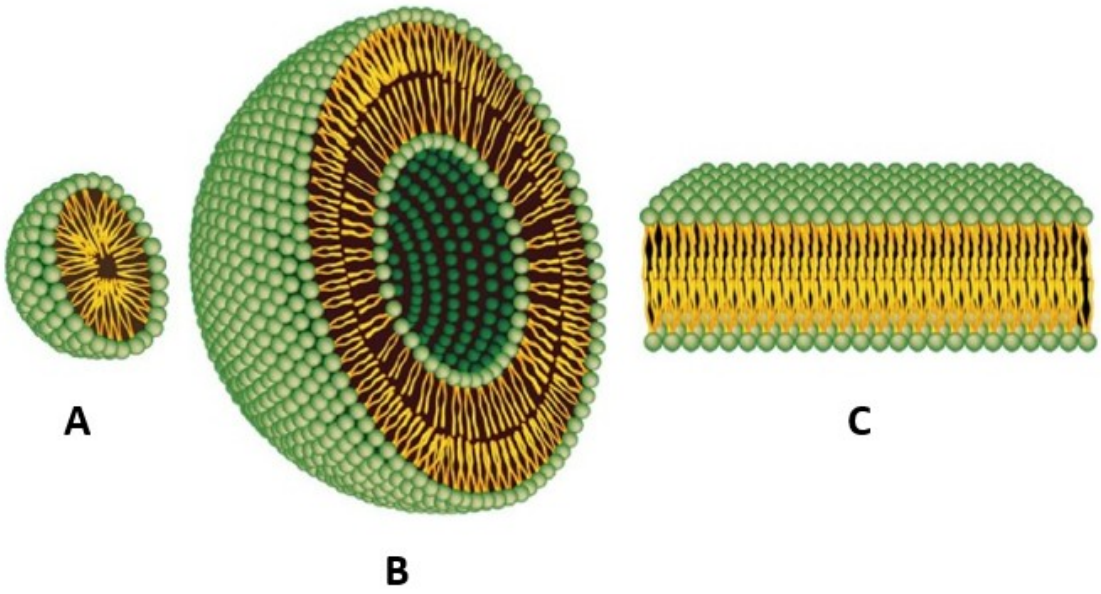

Figure 5- Structure of phospholipid-based structures (21)

3.3.2.1. Structure of liposomes

Since lipids are amphipathic (possessing both hydrophobic and hydrophilic part), they assert intrinsic propensity to self-assemble in aqueous media, and their thermodynamic phase characteristics affect entropically intended confiscation of their hydrophobic sections into spherical bilayers referred to as lamellae. They can respectively capture both lipophilic and hydrophilic agents whereas aqueous core captures hydrophilic molecules and lipid membrane on the other hand has a great affinity to lipophilic ones (Figure 5B). (22)

3.3.2.2. Stability of liposomes

In vitro and *in vivo* stability of liposomal systems with encapsulated drugs determine their lifetime and distribution within the organism. The circulation time of liposomes which are administered intravenously is shorter due to their direct interaction with lipoproteins and subsequent destabilization. Regional lymph nodes "swallow" liposomes after subcutaneous or intraperitoneal administration. Moreover, conventional liposomes are prone to degradation by deleterious effects of bile salts, gastric acids, and pancreatic lipases in the gastrointestinal tract (GIT) when administrated orally. Lipolytic enzymes such as lipases, phospholipase A2, and cholesterol esterases contained in pancreatic fluid disrupt liposomal structure by hydrolysis. Constant lysis caused by the factors mentioned previously leads to leakage of payloads. (23)

Cholesterol, as an intrinsic component of our skin and as an additive to the lipid bilayer, plays an important role in increasing liposomes' *in vivo* and *in vitro* stability and reducing their permeability. The presence of cholesterol is allowing phospholipids to densely stick together and inhibits their transfer to low- and high-density lipoprotein. Hydrophobic cholesterol interacts with the core of the membrane and consequently stabilizes it. (23) (24)

3.3.2.3. Modifications of liposomes

Liposomes' technology has drastically developed by either moderating their lipid composition or by decorating their surface with a variety of ligands. The attachment of polyethylene glycol to the periphery of liposomes (PEGylation) is a modification which allows them to become therapeutically potent and mainly prolong their circulation time. This way, liposomes can defeat pharmacological challenges such as uptake by the reticuloendothelial system (RES), destabilization by blood protein, and rapid clearance from blood circulation (Figure 6). Clearance is also performed by a system of monocytes and macrophages in the liver, lymph nodes, spleen and mononuclear phagocyte system (MPS). (23) (25)

Several carbohydrates such as glucose, sucrose, lactose etc., as ligands on liposomes, have been found to become a promising opportunity for clinical use. They represent low intrinsic immunogenicity and very low toxicity. Liposomes decorated with these ligands have been used for various research purposes including tumor studies, pulmonary drug delivery and oral administration of insulin. (26)

Additional ligands such as small peptides, antibody fragments or whole antibodies are considered to be effective in tumor treatments, targeting mainly cancer cells in bladder, ovaries, lung or breast expressing EGFR as a target. (26) (27)

Immunoliposomes, carrying antibodies or their fragments, have emerged as solution for cells presenting antigen in diseases such as HER-2 and EGFR positive malignancies, infectious, autoimmune and neurodegenerative diseases etc. (26) (28)

As a novel drug transportation and delivery system, liposomes modified with enzymes (enzymosomes) have been introduced. (29) They are suitable for log duration treatments and have proven to be promising substitutes of conventional management of anti-platelet activities, gout etc. The most common enzymes that are used to enhance anti-tumor drugs include carboxypeptidase, alkaline phosphatase, β glucosidase and β lactamase. (30)

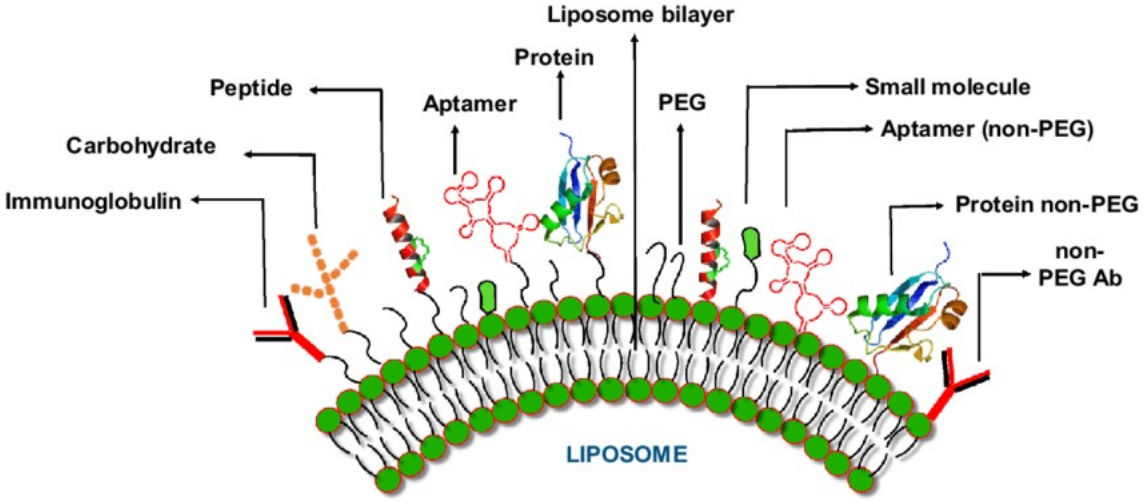

Figure 6- Modification of liposome surface with different ligands (targeted liposomes) (26)

3.3.2.4. Size of liposomes

The size of liposomes as well as their lamellarity can vary (Figure 7). These two characteristics determine in an appreciable extend their stability and encapsulation efficiency (EE). Multilamellar vesicles (MLVs), belonging to group of the largest liposomes, are inappropriate drug carriers due to their small core volume and their relatively low stability. They are used as precursors to form small unilamellar vesicles (SUVs) or large unilamellar vesicles (LUVs) pressed through polycarbonate filters with defined pore size. (31) There is an indirect proportion of size and clearance of liposomes. Small unilamellar liposomes/vesicles (SUVs) retain in the bloodstream for a prolonged time. The most appropriate candidates are

the liposomes with small diameter and with a tight distribution of sizes. PEGylated liposomes are sometimes commercially available, but there are some limitations for their use such as deficiency of specificity that hinder them from their clinical success. To increase the released amount of therapeutic agent and target-specificity of liposomes, multifunctional carriers and stimuli-sensitive liposomes for theranostics have been introduced. (23) (32)

Liposomes intended for internal use, an optimal size of liposomes is around 100nm or less. These range offers extended blood circulation achieved by lower hepatic and splenic uptake. Liposomes ranging from 120-810 nm have been used for skin delivery while the most favourable outcomes have been reported for a diameter around 120 nm. These liposomes have proven to have enhanced penetration in the skin compared to larger ones. (33) This observation makes them potential candidates for dermal delivery of active substances against melanoma since it is known that melanocytes, the cells responsible for melanoma, are also located at the lower layer of epidermis (basal layer). (34) The only research concerning dermal application of liposomes against melanoma was the effective siRNA delivery of edge-activated liposomes at basal epidermis for melanoma therapy. (35)

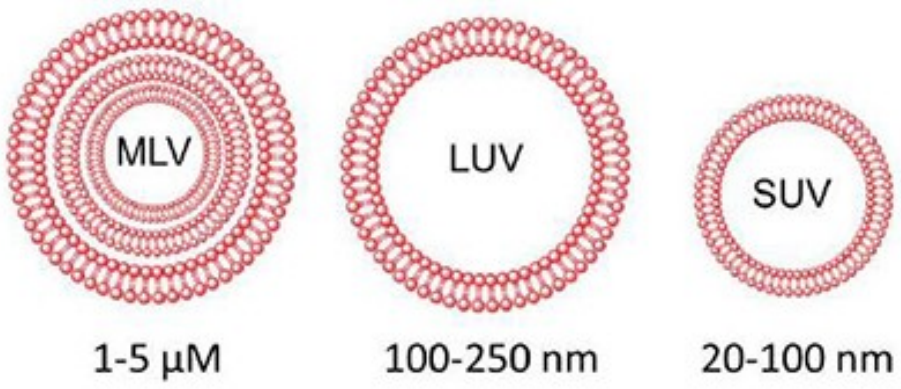

Figure 7- Classification of liposomes based on the lamellarity (36)

3.3.2.5. Preparation of liposomes

Preparation of liposomes can be carried out using a wide range of methods which influence the liposome characteristics such as lamellarity, size and EE. (37)

Liposome preparation generally consists of 4 stages:

a) drying down of a mixture of lipids from an organic solvent (thin film formation),

b) dispersion of lipids in aqueous media (formation of lipid bilayer),

c) separation, and purification of resultant liposomes, and eventually

d) analysis of the final product.

The schematic representation of liposomes' preparation is shown in Figure 8. (37)

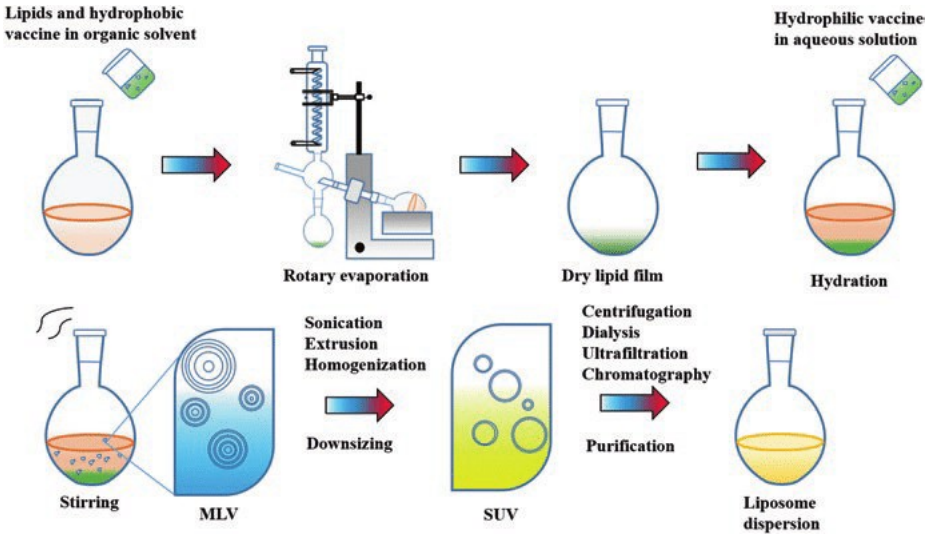

Figure 8- General scheme of liposomes preparation (38)

EE is a major characteristic of liposomes that expresses the percentage of the drug which was finally entrapped inside the lipidic vehicle. It is calculated by the determination of the difference: *total drug added during preparation – free non-entrapped drug* divided by the *total drug added during preparation*. The concentration of incorporated drugs can be determined using different analytical methods (HPLC, GC, GC-MS, UV-vis detection) depending on the incorporated drug. (37)

The main methods for the preparation of liposomes, together with their advantages and disadvantages, as well as the type of vesicles produced, are summarized in Table 1.

Table 1- Conventional methods of liposome preparation (37)

Method	*Advantages*	*Disadvantages*	*Types of vesicles*
Injection techniques	Simple	Difficult organic solvent removal, time consuming, needed sterilisation,	SMVs, LUVs
Electroformation methods	Homogenous size distribution	Expensive electrodes	GUVs
Microfluidic method	Good particle size control (PSC)	No appropriate for bulk production, difficult organic solvent removal	SUVs, LUVs
Thin-film hydration (Bangham method)	Simple	Low EE, difficult organic solvent removal, large vesicles without PSC, needed sterilisation	MLVs
Membrane extrusion	Simple, fast, good PSC, no contamination	Large-scale processing, can induce pores' clogging	MLVs
Detergent depletion	Homogenous product with good PSC, simple	Difficult organic solvent removal, time consuming, sterilisation needed	MLVs, LUVs
Heating method	No sterilisation needed, simple and fast process	High temperature	MLVs, SUVs
Reverse-phase evaporation technique	Simple process	Large amount of organic solvent, needed sterilisation, time consuming	MLVs, LUVs

Nowadays an innovative method called, “supercritical technology” has attracted the attention of many scientists using supercritical fluids as dispersing agents (e.g. CO_2), that are inexpensive and nontoxic in comparison to OS used in conventional methods. (39)

3.3.2.6. Methods for size reduction and uniform size distribution

After liposomes are formed by several methods, their size that ranges from tens of nanometers to several micrometers needs to be uniformed. (40) For the application of liposomes in the medical sphere and other *in vivo* implementations, the particle size of liposomes plays a crucial role since it affects EE, drug release, stability, mucoadhesion, and the cellular uptake of liposomes. Several different methods are used to reduce the size and the size distribution of hetero-dispersed suspensions of liposomes (Table 2) (Figure 9). (37)

Table 2- Different size-processing methods for uniform particle size liposome. (37) (40)

	Advantages	*Disadvantages*
Homogenisation	Suitable for large scale production	Quite broad liposome size distribution, possible metal and oil contamination
Sonication or ultrasonic irradiation	Suitable for SUVs preparation	Processing capacity is limited, overheating during the process can cause peroxidative damage to lipids, possible contamination with titanium
Extrusion	High sample reproducibility, quite narrow distribution of the liposome, different membrane/filter pore sizes	Large scale production is limited due to slow process, membrane/filter clogging and fragility, slow flow rate through the membrane

Figure 9- Hand extruder with gas-tight syringes (41)

3.3.3. Dendrimers

Dendrimers are spherical synthetic macromolecular polymers forming tree-like structures. The term *dendrimer* is derived from a combination of two Greek words; "*dendron*" and "*meros*" - in translation *tree* and *part*, emphasizing their branched structure. The history of these molecules dates back to the 1940s but the first synthesis and written evidence regarding their structure is from a research contacted by Vögtle and his co-workers in 1978. (42) These globular mono-dispersed carriers potentiate bioavailability for drugs that are either toxic, poorly soluble, or substances for efflux transporters. Furthermore, to enhance the release of drugs various stimuli can be applied externally such as magnetic fields, photons, pH, temperature, and X-rays. (43)

3.3.3.1. Structure of dendrimers

Dendrimers are composed of 3 basic components starting with initiation core, following repetitive generations (units) called dendrons stretching from the core, and functional units forming their periphery. (44) To determine the structure of the dendrimer, its cavities, and branches, it is crucial whether nitrogen, phosphorus, or carbon is anchored inside the initiation core as a central atom. On the other hand, possession of anionic, neutral, or cationic terminal functional groups on the surface can lead to hydrophilic or hydrophobic compounds.

The structure of dendrimers is also defined by the generation (G) which is expressing the repetitive synthesis process for their isolation. A number standing behind the letter G represents the branch layer as depicted in Figure 10. (42) Generally lower generations remain in an open structure. Although the higher generation gets, the more globular, compact, and dense it becomes. (45)

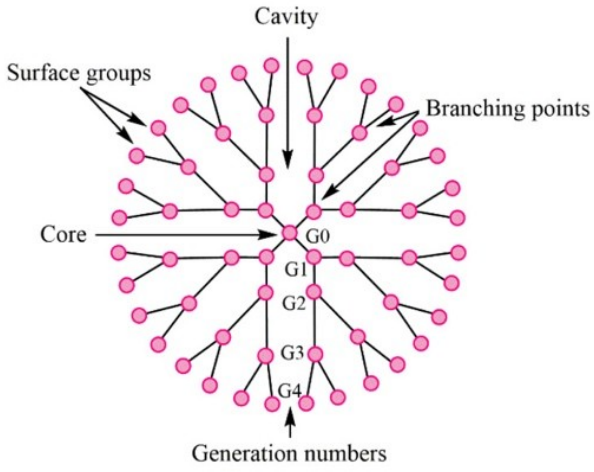

Figure 10- Structure of dendrimer (42)

3.3.3.2. Types of dendrimers

Many different types of dendrimers have been developed and used since the 1980s. (46) Based on the core type, the monomer type, the dendrimer shape, the internal cavities, and the functional-ending groups they are classified into many different categories (Figure 11). (47) Poly(amidoamine) (PAMAM) dendrimers are the most well-characterized class of dendrimers. Their core is composed of linear chain molecules containing primary amines. The most used molecules forming the core are ethylenediamine, ammonia, or cysteine. The branching units are based on alkyl acrylate (creating half-generations i.e. G0.5) and ethylenediamine (creating full dendrimer generations i.e. G1). They can be used to improve solubility, residence time, bioavailability, and skin permeation of the drug molecule. As a result, PAMAM dendrimers play a crucial role in various delivery systems, especially transdermal, pulmonary and ocular. Their extensive use in different applications made them commercially available. (46) (48) (49).

Several dendrimer structures have been found to be analogous to the shape and dimensional size of different biological structures like cytochrome C, haemoglobin, insulin, hemerythrin, and prealbumin. (45)

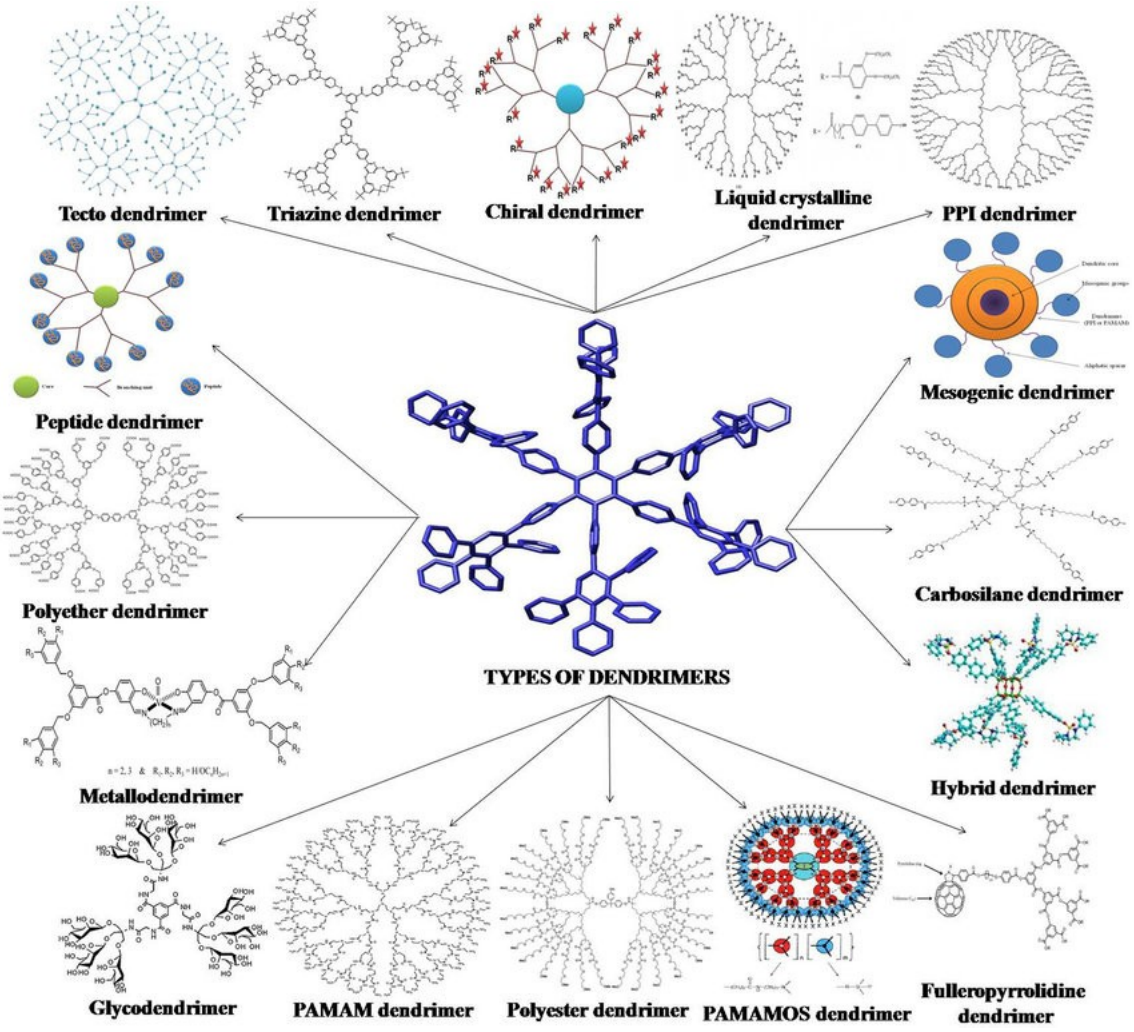

Figure 11- Different types of dendrimers (50)

3.3.3.3. Properties of dendrimers

One considerably important property that makes dendrimers good potential pharmaceutical excipients is multivalency. The term multivalency or polyvalency means that the molecules possess several reactive places or functional groups on their periphery. These places can interact with many biological molecules such as cells, viruses, polymers, and proteins. Monovalent receptor-ligand binding is very weak in contrast to the multivalent that may amplify the signal transduction. The higher the generation of the dendrimer, the more functional groups it has, thus favouring the interaction with biological targets. In comparison with linear polymers the advantages of using hyper-branched polymers (dendrimers) as delivery systems are included in Table 3. (46)

Table 3- Comparison of properties of linear polymer and dendrimer (51)

Property	*Dendrimer*	*Linear Polymer*
Structure	Compact and Globular	Not Compact
Shape	Spherical	Random Coil
Architecture	Regular	Irregular
Synthesis	Stepwise growth	Single step poly condensation
Crystallinity	Non-crystalline and amorphous	Semi crystalline/crystalline
Aqueous solubility	High	Low
Non-polar solubility	High	Low
Compressibility	Low	High

3.3.3.4. Synthesis of dendrimers

The synthesis of dendrimers is quite closer to molecular chemistry than to polymer synthesis but has characteristics from both. Step-by-step controlled synthesis is well known for molecular chemistry while, on the other hand, repetitive sequences. i.e. monomers are used in the creation of polymers. Versatile dendrimers are synthesized in a mode of reactive steps that lead to a creation of the first generation (G1) followed by the second (G1+1) and so on. (52).

Two major approaches have been applied in the synthesis of dendrimers: the divergent approach (Figure 12) and the convergent approach (Figure 13).

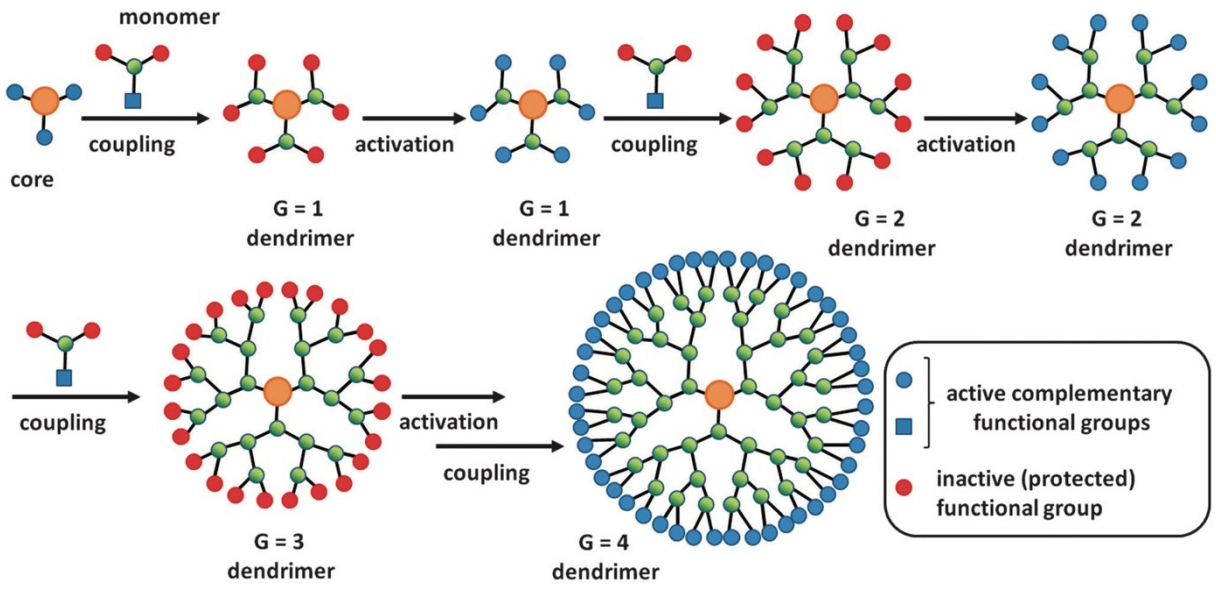

Figure 12- Schematic representation of the divergent approach

The divergent approach is starting from the core and continues outwards with subsequent attachment of building blocks forming the "arms" of the dendrimer in an exhaustive manner.

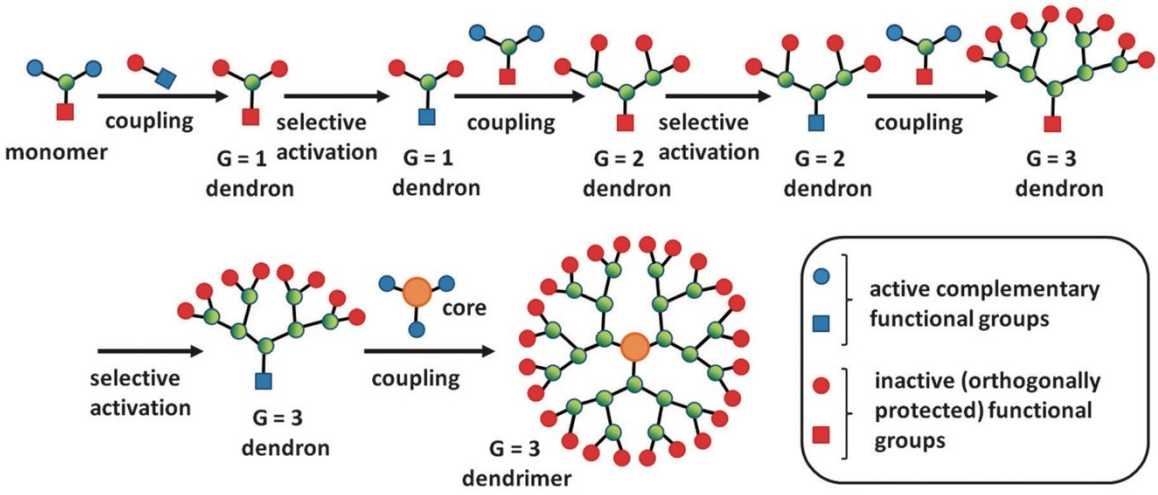

Figure 13- Schematic representation of the convergent approach

In the convergent approach, the synthesis starts with the formation of the "arms" (or "dendrons" in this case) that become the outermost parts of the dendrimer after their attachment to the appropriate core. (53) Since the final generation number is pre-determined, the synthesis necessitates the branches of various sizes in advance for each generation. (46)

3.3.3.5. Drug - dendrimer interactions

In terms of possible interactions between the drug molecule and dendrimers, three strategies may occur. The first strategy is its incorporation realised through the formation of hydrogen bonds, electrostatic and hydrophobic interactions, and noncovalent associations known as *physical interaction*. These interactions can take place in the central core, between the branches of the dendrimers or even at their periphery. (54) Contrarily to the first strategy, a more stable covalent conjugation can take place between the periphery functional end groups of dendrimers with the drug molecule, otherwise known as *chemical interaction*. The additional third option is the combination of the previously described methods where active substances can be noncovalently and covalently incorporated in the dendrimer. The schematic representation of the three cases is shown in Figure 14. (42)

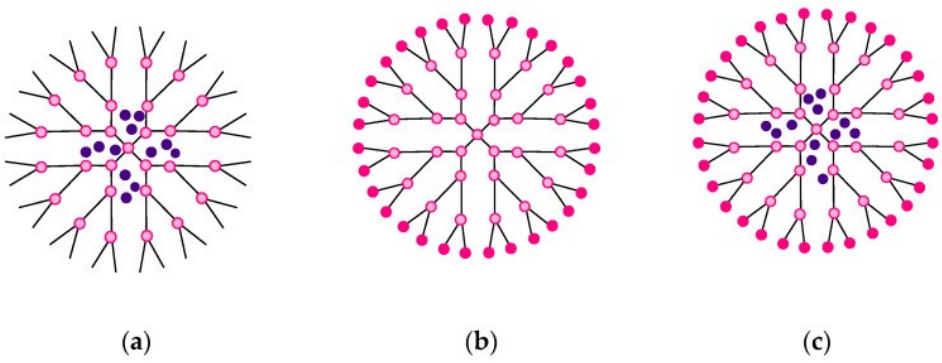

Figure 14- 3 ways of conjugation or complexation of the drug molecule with a dendrimer molecule: (A) physical interaction, (B) chemical interaction, (C) both interactions simultaneously (42)

3.3.3.6. Dendrimers for dermal and transdermal delivery

Even though dendrimers are relatively large in their molecular size, they still can be used as candidates for skin penetration and transdermal permeation due to their unique physicochemical properties. (55) They can enhance drug delivery into the skin despite their limited capability to penetrate the SC by two possible mechanisms. The first is to act as a drug release modifier. The skin permeation rate of an active agent is given by its thermodynamic activity that may have drug particle very low (lipophilic drug) but in combination with dendrimer the thermodynamic activity of the drug is elevated with its rapid release into the vehicle (water) with subsequent skin permeation. (56)

The second possible option for dendrimers to accelerate permeation of the skin, is to act as the vehicle-dependent penetration enhancement. Some vehicles e.g. *isopropyl myristate* can liquefy lipids of the lamellar-gel phase of SC or partially dissolve them to weaken the

barrier properties of the skin. When particularly low generation dendrimers together with such vehicles are combined, the permeation extent is considerably increased. (56) PAMAM dendrimers as firstly emerged dendrimers can enhance stability or water-solubility of hydrophobic drugs and make them one of the best potential enhancers invented for TDD. Many additional dendrimer-drug complexes have been investigated and subsequently evaluated in the transdermal route of drug administration (Table 4). (57)

Table 4- Examples of drugs with certain generations of dendrimers used for transdermal delivery (57)

Guest molecule	*Dendrimers*	*Interaction pattern*	*Goal*	*Administration route*
Tamsulosin hydrochlorid	G3 PAMAM	Simple encapsulation	Enhance transdermal delivery efficacy	Transdermal
CAT reporter transgene	PAMAM	Electrostatic interaction	Skin gene transfection	Transdermal
Ketoprofen and diflunisal	G5 PAMAM	Electrostatic interaction	Anti-inflammatory	Transdermal
Indomethacin	G4 and G4.5 PAMAM	Electrostatic interaction	Anti-inflammatory	Transdermal

Iontophoresis has been recently proposed as a new opportunity for dendrimers to be applied on the skin. This non-invasive effective local technique driven by a weak electric field provides the possibility to deliver ionic drugs into different tissues. This practice is limited mainly to dendrimers that possess a large number of charges on the outer layer. This technology is significant mainly in transdermal and ocular delivery systems. (57)

3.4. Advanced drug delivery nanosystems

The previously described drug delivery nanosystems (DDnSs), liposomes and dendrimers, are classical examples of *conventional* DDnSs (cDDnSs). These cDDnSs can be (and have been) used independently for the effective delivery of active substances. The possibility of size, structure and morphology control are their major advantages. In a further extend, the combination of cDDnSs is providing alternative structures which can be created either of different (*chimeric*) or the same (*hybridic*) nanosystems. These combinations are providing the so called *advanced* DDnSs (aDDnSs) which are encompassing bio-materials that carry active pharmaceutical ingredients. (58) aDDnSs can be used for high and low-molecular-weight polar and nonpolar therapeutics for systematic and non-invasive routes of administration whilst enabling epithelial or subepithelial absorption of the therapeutical agent. (59)

Classical example of chimeric aDDnSs is the case where a combination of liposomal and dendrimeric technologies was used for the encapsulation of the anticancer drug doxorubicin. (60) On the other hand, an example of hybridic aDDnSs is the combination of liposomes in liposomes for the encapsulation of Leuprolide. (61) Additional examples of aDDnSs are included in the following Table 5. (62)

Table 5- Examples of aDDnSs (62)

System	*Drug/Bioactive compound*	*Biomaterials and/or Inorganic materials*
Liposomes in liposomes (LiLs)	Leuprolide	Lipids
MLCRs/Chi-aDDnSs	Doxorubicin	Lipids (newly synthesized) Dendrimers
MLCRs/Chi-aDDnSs	Anti-cancer drug	Superparamagnetic iron oxide NPs/pH-sensitive amphiphilic polymer
Chi-aDDnSs (targeted nanospheres and pretargeted radioimmunotherapy)	Paclitaxel	Polymers (ABA-type copolymers, PEG)
Chi-aDDnSs	Indomethacin	Lipids/Gradient block copolymers

3.5. Imiquimod

Imiquimod (IMQ) is a synthetic immune response modifier that belongs to a class of drugs referred to as imidazoquinolones (Figure 15). The molecule has been shown to possess anti-viral and anti-tumor activity. (63) IMQ is commercially available in the form of cream in two different concentrations - 3.75% (brand name Zyclara®) and 5% (brand names Aldara® and Bascellex®). (64)

IMQ formulation in dosage forms is highly challenging owing to its low solubility in either hydrophilic or lipophilic vehicles. Moreover, despite the low molecular weight, IMQ penetrate the skin very poorly, probably due to very low solubility in SC and underlying tissues. (65)

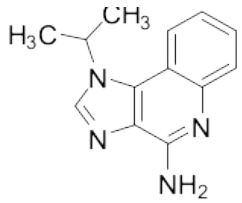

Figure 15- Chemical structure od imiquimod (66)

IMQ acts indirectly by enhancing the body's natural ability to heal through the induction of cell-mediated and innate pathways. (67) Innate pathway includes Langerhans cells, which begin to release immunomodulatory and proinflammatory cytokines, including interferon α, interleukin 1, and tumor necrosis factor as depicted in Figure 16. (68) Direct binding to toll-like receptor (TLR) 7 on monocytes/macrophages and dendritic cells of the epidermis and dermis on the other hand activates apoptosis. Activation of adaptive immune response is attributed to IMQ's effectiveness also on Langerhans cells that can present antigens on their surface and later on migrate to regional lymph nodes where they present the antigens to the T cells. (63)

IMQ is used to treat perigenital and perianal papillomavirus vegetations (*condylomata acuminata*), and in 2004 it was approved for treating superficial basal cell carcinoma and actinic keratosis. Especially in warts IMQ is becoming a very popular and effective method of choice in current clinical practice. So far, it has been most often treated with cryotherapy, local podophyllotoxin, or curettage. (63) (68)

Side effects of IMQ may be local or systemic. They are intensified when applying a larger amount of cream, by prolonging the duration of action, increasing the frequency of application or the total duration of therapy. Depending on the indication, the side effects may vary. The most common local side effects that people experience are erythema (around 60%), erosion, excoriation / desquamation, edema while the most common systematic ones are headache, myalgia and the least common are flu-like symptoms. (68)

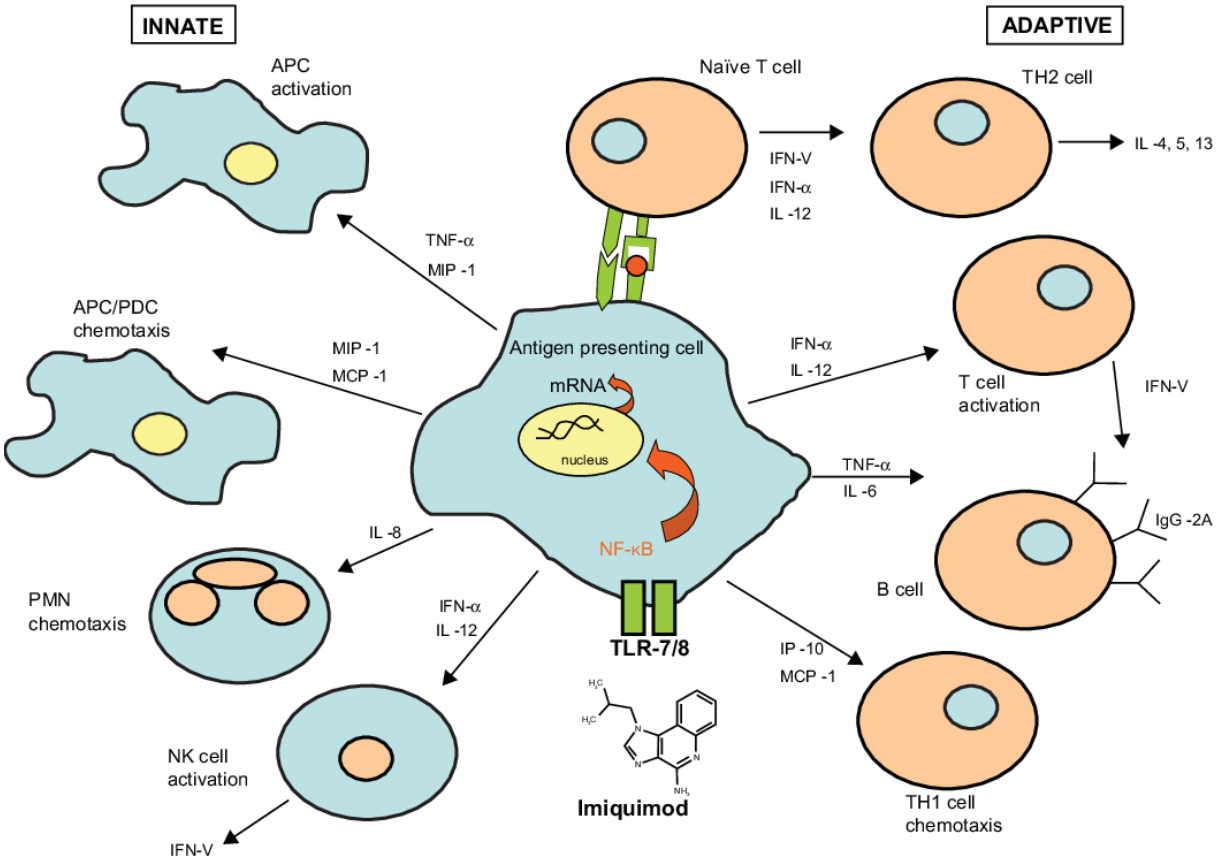

Figure 16- Activation of adaptive and innate immunity after binding to TLR-7/8 receptor (67)

4. Experimental part

4.1. Used chemicals and equipment

The water used during this study was always of ultra-pure (Mili-Q) quality. MeOH and Acetonitrile were of HPLC quality (Sigma-Aldrich, Germany) and Chloroform was of pro analysis quality (Penta, Czech Republic). IMQ was purchased from Tokyo Chemical Industry CO., Japan and L-α-Phosphatidylcholine was purchased from Sigma-Aldrich, Germany. Four different generation of dendrimers (G0, G1, G2 and G3) were used during this study and were synthesized from members of our group. (69) (70)

Analytical balances, Ohaus Pioneer and Ohaus Discovery, were used to weigh the individual raw materials. Liquids were dispensed using Transferpette S, Brand adjustable volume pipettes. A Vortex MS 3 Digital, IKA instrument was used for solutions' homogenization and an ultrasonic bath K-12 LE, Kraintek 12 was used for their sonication. Evaporation of the organic solvent was performed at a rotary vacuum evaporator IKA RV 10, Basic. Extrusion of the prepared liposomal mixtures was performed using a Mini Extruder, Avanti Polar Lipids. The extruder was heated with a temperature-adjustable MR Hei-Standard magnetic stirrer, Heidolph. The liposomes were extruded using 1000 μL gas-tight syringes, Avanti Polar Lipids, through 400 nm and 100 nm Nuclepore membranes, Whatman. The membrane was always anchored between two 10 mm support filters, Avanti Polar Lipids. The liposomes's size and Polydispersity Index (PDI) values were measured with a Zetasizer Nano-ZS, Malvern, and the samples were placed in DTS 1060 cuvettes, Malvern for the measurements. Centrifuges MPW 260 R were used to centrifuge the prepared liposomes during purification and an LT3 mechanical shaker from Sigma Aldrich was used for long sample shaking. pH values of the prepared buffers were measured with a micro-pH meter: Hanna precision pH meter Model pH 210 from Sigma Aldrich (Schnelldorf, Germany). Prolonged drying of samples was performed with a Vacuubrand RC 6 hybrid pump (Wertheim, Germany).

The human skin which was used for the permeation experiments was from Caucasian female individuals who underwent abdominal plastic surgery and was used with the approval of the Ethics Committee of the Sanus Surgical Centre in Hradec Králové (No. 5/4/2018), according to the principles of the Declaration of Helsinki. Written informed consent has been obtained from all individuals. The subcutaneous fat tissue was carefully removed, and the remaining full-thickness skin was washed with saline, blotted dry, and stored at −20 °C.

Phosphate-buffered saline (PBS), filled in the acceptor compartment of Franz diffusion cells for skin experiment, was made of 0,312 g $NaH_2PO_4.2H_2O$ (156 g/mol; 2 mM), 2,865 g $Na_2HPO_4.12H_2O$ (358 g/mol; 8 mM), 8 g NaCl (58,4 g/mol; 137 mM), 0,201 g KCl (74,6 g/mol; 2,7 mM), 50 mg of gentamicin, all those substances were dissolved in 900 ml of H_20 and then refilled with a rest of H_20 to 1000 ml (final pH values was modified to 7.40 by addition of NaOH and H_3PO_4 solutions) before final filtration. WNB-Memmert GmbH water bath at a temperature of 32 °C was used during the permeation experiment.

Determination of the concentration of IMQ (before and after liposomes' purification for the calculation of the EE as well as after the permeation experiment) was enabled using an Agilent 1200 series instrument (Agilent Technologies, Germany) equipped with a G1379B degasser, G1310A isocratic pump, G1316A thermostatted column, G1329A autosampler, and G1321B fluorescence detector. IMQ was analysed on an HS Discovery C-18 150×4.6 mm column (5 μm particles with a 100 Å porosity) at 25°C. The mobile phase consisted of MeOH/acetonitrile/acetate buffer (100 mM, pH = 4) 185:275:540 v/v at a 1 mL/min flow rate with a 1 μL injection volume. Acetate buffer was prepared by mixing following substances: firstly 5,84 g of NaCl (58,4 g/mol) and 6 g of CH_3COOH (60,52 g/mol; 1,05 g/ml) were weighed and subsequently dissolved in 900 ml of H_2O, refilled with the remaining H_2O to the 1000 ml. The final pH value was modified to 4.00 by addition of NaOH and CH_3COOH solutions.

The HPLC run time was 5 minutes and IMQ was detected using λexc = 240 nm and λem = 360 nm after 3.2 minutes. The calibration curve was linear over a range of 0.02 – 10 μg/mL ($R^2 \geq 0.999$, $p < 0.001$)

4.2. Preparation of liposomes with encapsulated imiquimod and dendrimers

4.2.1. Structures of used generations of dendrimers

The structures of the dendrimers used during this study are represented in the Figures 17-20.

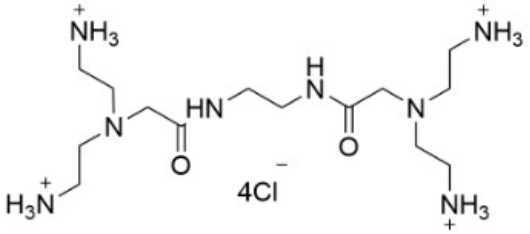

Figure 17- Structure of G0 dendrimer

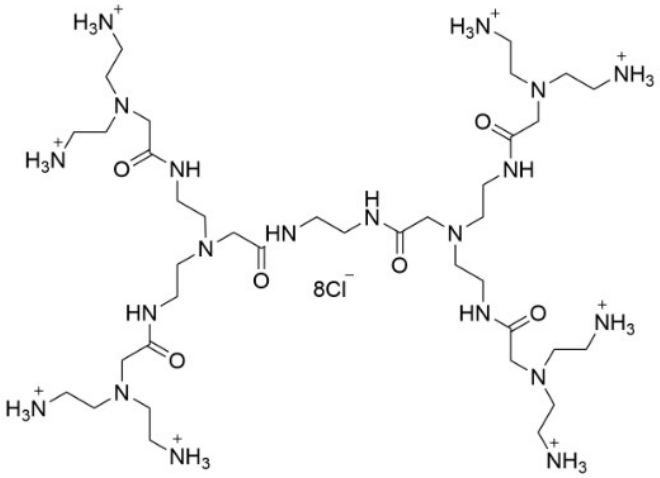

Figure 18- Structure of the G1-dendrimer

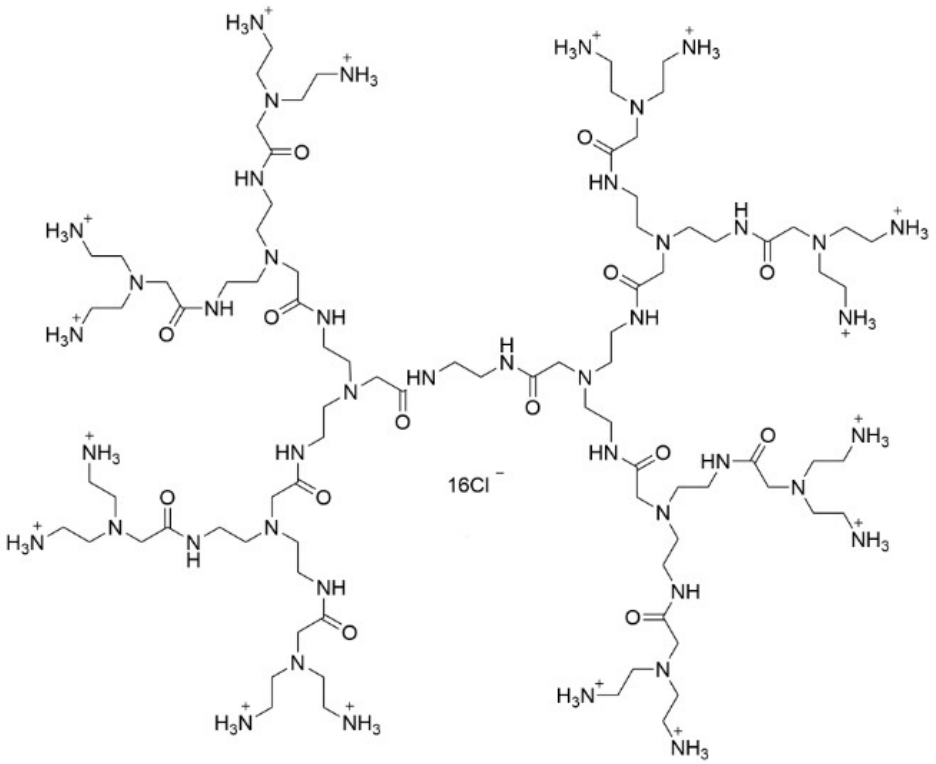

Figure 19- Structure of the G2-dendrimer

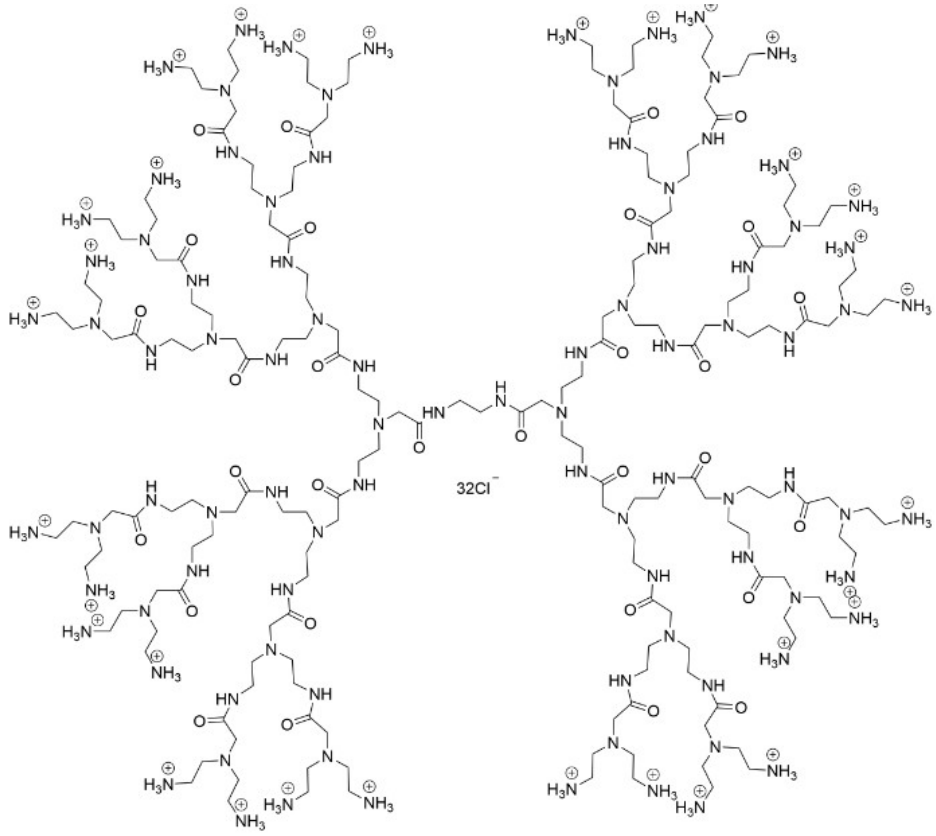

Figure 20- Structure of the G3-dendrimer

4.2.2. Imiquimod in dendrimer solution

Specific amounts of dendrimers (depending on generation) were completely dissolved in water (3 mL) to create aqueous dendrimer solutions of different concentrations for each generation. More specifically, the dendrimer concentrations were 20, 10, 5 and 2.6 mM for the G0, G1, G2 and G3 respectively. The amounts (mg) of dendrimer used for each generation are provided in Table 6 together with the molecular weights of each generation. An excess of IMQ (30 mg) was added to each aqueous dendrimer solution to create saturated solutions. After the IMQ addition, the mixtures were vortexed, sonicated for 20 minutes (extra IMQ was added if the initially added amount was dissolved), mechanically shaked overnight and then centrifuged at 10,000 rpm for 10 minutes at 20 °C. Subsequently the supernatant was carefully removed and transferred in a clean 4 ml vial. 100 μl from each supernatant was diluted (1000x) with methanol (MeOH) and the concentration of IMQ was measured on the HPLC.

Table 6- Dendrimer-IMQ solution specifications

Sample	*Dendrimer generation*	*Dendrimer Mw*	*Dendrimer concentration (mM)*	*Dendrimer amount (mg)*
G0-20	G0	492	20	32.09
G1-10	G1	1211	10	36.55
G2-5	G2	2648	5	39.90
G3-2.6	G3	5522	2.6	43.15

4.2.3. Preparation of liposomes and aDDnSs

4.2.3.1. Preparation of empty liposomes

Empty liposomes (with no drug or dendrimers) were prepared by weighing 200 mg of egg PC in a 25 mL round bottomed flask and dissolving them in 10 ml of Chloroform:MeOH (4:1). The flask with this mixture was placed in a sonicator bath for 10 minutes. The organic solvent was removed at rotary evaporator under reduced pressure until a thin layer of lipid film formed at the bottom of the flask. To ensure complete solvent evaporation, the lipid film was further evaporated using a high vacuum pump for 1 hour. This was followed by hydration of the lipid film with 2 ml of ultrapure water for 1 hour at transition temperature *(Tc*~65 ºC*)* under stirring (rotary evaporator without vacuum). The resulting mixture was sonicated for an additional period of 1 hour. Subsequently, the sample stored at 4 ºC (fridge) until further used.

4.2.4. Preparation of liposomes and aDDnSs

The same procedure as for the preparation of empty liposomes was followed with the difference that the dried lipids were hydrated with 2 ml IMQ-dendrimer solution (of each generation) instead of ultrapure water.

4.2.4.1. Extrusion of liposomes

The manual extrusion method was chosen to reduce the size of the prepared liposomes to the desired values. The extrusion took place above the lipid phase transition temperature (> 65 °C). Thus, a magnetic stirrer with a heating surface was set to 70ºC and the extruder stand was placed on the heating surface while a probe inserted in the well of the extruder.

The extruder was equipped with a polycarbonate membrane of pore size 400 nm between two support filters and placed on the support stand. Prior to each extrusion, the used membrane and the supporting filters were wetted with water. The membrane wetting was accomplished by filling one of the gas-tight syringes with water and inserting it into one end of the extruder, while inserting the other empty syringe with the plunger set to zero into the opposite end of the extruder. Subsequently, water was forced through the membrane from one syringe to another, for a total of 15 extrusions. The syringe was then emptied, immediately refilled with liposomal suspension and inserted into one end of the extruder, while the other syringe on the opposite side remained empty. The formulation was left in the extruder on a heating block for 15 minutes to equilibrate to the desired level of temperature *Tc*. The plunger of the filled syringe was gently pushed, avoiding the membrane rupture, until all liposomal suspension was transfer through the membrane into the opposite syringe. This procedure

repeated 15 times. After extrusion with a 400 nm membrane, the suspension was transferred to a test tube. The extruder was cleaned and equipped with a new polycarbonate membrane of pore size 100 nm between two new support filters. The new membrane was wetted similar like before, and the already extruded suspension from the vial introduced to the extruder. The suspension was extruded 15 times through the new membrane and after the extrusion was complete, the mixture was stored in the refrigerator.

After all samples were extruded the IMQ concentration was measured.

4.2.4.2. Measurement of size and PDI

Dynamic Light Scattering (DLS) characterization of the vesicles was performed by measurements on a ZetaSizer were used to determine the size obtained after extrusion. In parallel, the PDI values, which is a measure of the heterogeneity of the sample, were measured.

The measurement was performed in a specially designed cuvette by taking 0.1 ml of the extruded sample and mixing it with 0.5 ml of ultrapure water. Proper conditions were set, and each measurement was performed in triplicates.

4.2.4.3. Purification

Before liposomes' purification to remove the unencapsulated drug, the formulations were tested for the determination of total IMQ amount. Each sample was diluted 1000x with MeOH.

The liposomes' purification was performed using a centrifugation method. Tubes with syringes inside were placed in the centrifuge (Figure 21). Each syringe was filled with 3 ml of Sephadex gel, which was prepared by swelling Sephadex G-50 loose powder in water (for every 1 g of powder 20 ml of water were used).

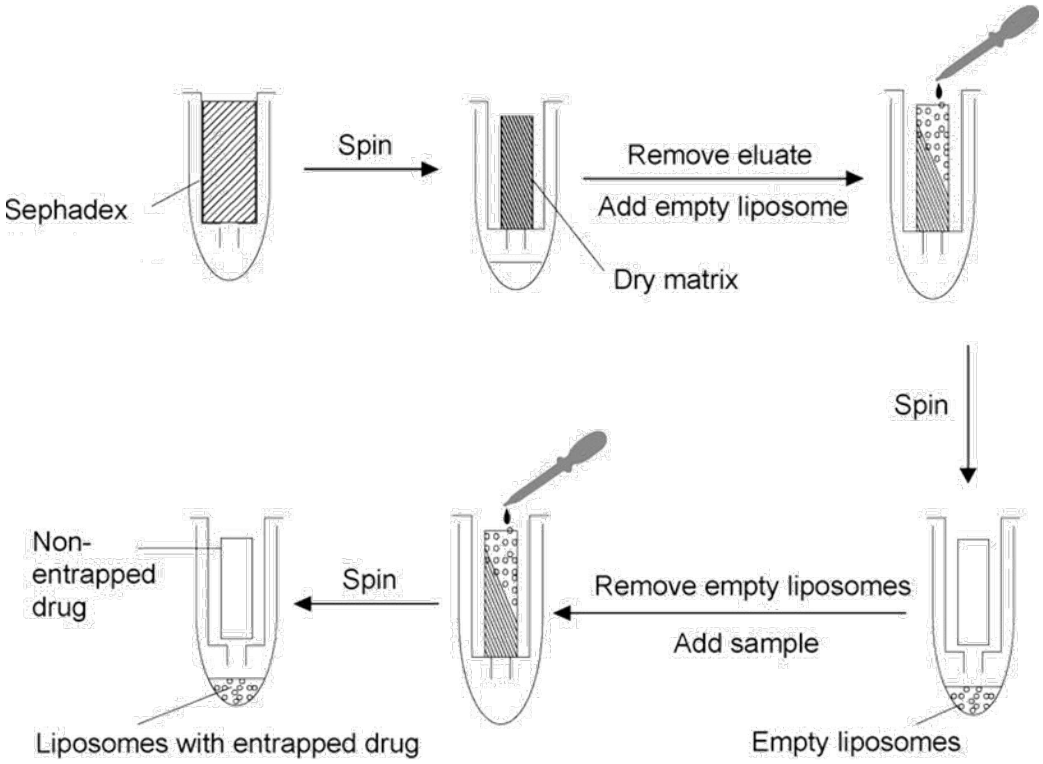

Figure 21- Scheme of centrifugation

The gel was prepared the day before purification, covered with aluminum foil and stored in a refrigerator overnight. A roll of cotton wool was inserted into the ends of the syringes so that only the liquid was filtered during centrifugation. After filling the syringes with the gel, the remaining amount of solution from the gel was centrifuged into the tubes at 1500 rpm for 3 minutes at 20 ^{o}C.

Each 1 ml of the gel was saturated with 0.044 ml of empty liposomes, i.e. 3 ml of the gel was saturated with 0.132 ml and centrifuged under the same conditions specified before. After saturating the gel with empty liposomes, the gel was washed with 0.2 ml of water and centrifuged under the same conditions specified before. This step was repeated together 3

times in a row. The next step was to remove the filtered solution tubes and insert the syringes into new tubes for later collection of purified liposomes. Then, 0.3 ml of the sample was applied to the centre of the gel and centrifuged twice under the same conditions specified before.

To measure the concentration of the encapsulated IMQ, 0.1 ml of purified sample was taken and diluted 1000 x with MeOH similarly like previously explained. 0.3 ml of the diluted sample was taken and filtered through cotton wool before the IMQ concentration evaluated by HPLC.

4.3. aDDnSs Skin experiment with Imiquimod

IMQ skin permeability was evaluated using modified Franz diffusion cells with an acceptor volume of 7.0 ± 0.2 mL (Figure 22). The frozen human skin was taken out of the fridge slowly thawed, cut into pieces (approximately size 2 x 2 cm), placed between Teflon holders with an available diffusion area of 1cm^2, and sealed with silicon grease. The holders were mounted to the diffusion cells with the skin dermal side facing the acceptor compartment. The acceptor compartment was filled with phosphate-buffered saline (PBS) at pH = 7.4 (containing 0.005% of gentamicin sulphate as a preservative) and stirred in a water bath at 32 °C throughout the experiment. 28 Franz cells were prepared and left to equilibrate overnight. The next day, the following formulations were applied: 25 mg of Aldara cream (IMQ applied=1.25 mg) in 5 cells and 25 µl of each purified liposomal formulation in 5 replicants. 3 cells remained untreated serving as control ones.

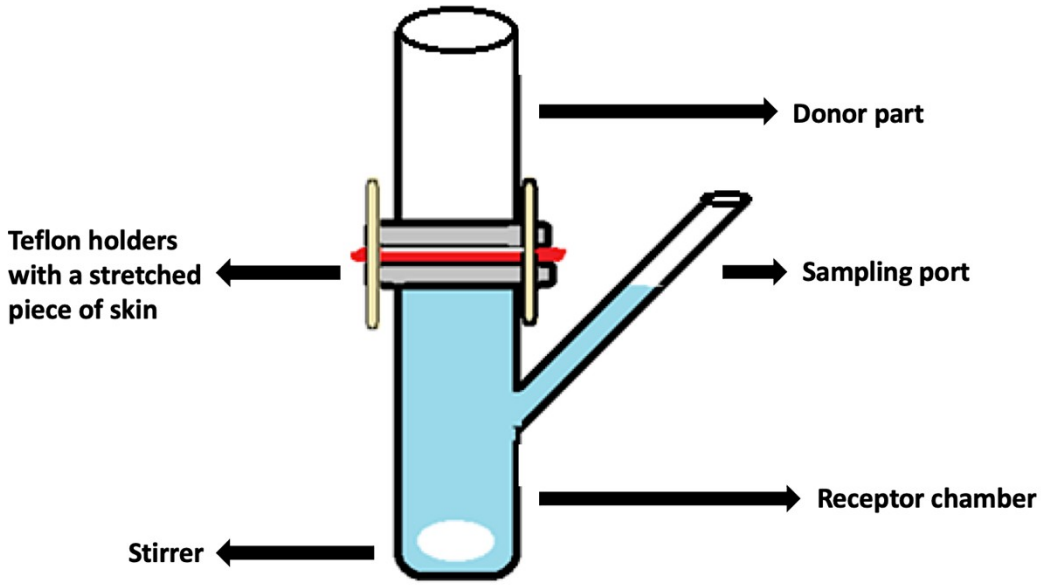

Figure 22- Diffusion Franz cell

8 hours after the application, the applied formulations were cleaned off from the skin surface by using ear cleaner and H_2O. The cells were left in the bath for an additional time of 16 hours. After 16 hours, the cells were dismounted, and the skin was washed with H_2O and ear cleaner. The tissue that had been exposed to the donor sample was punched out, wrapped in aluminium foil, and heated to 80 °C for 1 minute in the oven. The epidermis was then carefully peeled off from the dermis.

The epidermis and dermis were weighed and extracted with 1 and 2 ml of acetonitrile: ammonium acetate (98:2 v/v, 5 mM, pH = 2.7) respectively, for 24 hours (shaker). The extract was filtered through cotton and analysed by HPLC.

5. Results and discussion

When SC is intact and skin barrier is uncompromised, the permeation *via* the uppermost part is limited to the molecules that are relatively small, uncharged and lipophilic. (18) Liposomes, thanks to their technological and biological advantages, are considered to be the most rewarding drug-carrier system according to many studies so far. (21) (26) (27) Also dendrimers, belonging to the group of nanocarriers, can render drugs greater bioavailability, biocompatibility and water-solubility. (57)

Liposomes combined with dendrimers or liposomal locked dendrimers (LLDs) have already been mentioned in a study comparing the encapsulation efficacy of the cytostatic doxorubicin. It was encapsulated in one case in liposomes together with dendrimers, in the other case in pure liposomes. The results clearly showed that the encapsulation efficiency was significantly higher with doxorubicin encapsulated in the LLD. (60) aDDnSs have also shown promising potential in preclinical studies for NSAIDs and their administration *via* the transdermal route. (71)

The subject of this work was the combination of the above-mentioned formulations in order to achieve an increased concentration of the adjuvant chemotherapeutic agent IMQ at the site of targeted action (in the epidermis) while avoiding undesired transdermal delivery.

5.1. Liposomes' preparation

5.1.1. Thin-film method

For the preparation of the liposomes, the thin-film hydration method was chosen. Some studies point to the potential disadvantages of this process like low EE, large vesicles without PSC or difficult removal of OS. (37) The Bangham method was chosen for several reasons including the lipophilicity of the drug, the later need for extrusion to obtain particles of defined size and ease of use. Among other things, at the forefront of this thesis was the tendency to follow a similar preparation procedure as in the previous work, which main purpose was to determine encapsulation efficiency of IMQ in combination with dendrimers and liposomes. (72)

5.1.2. Lipids

Egg PC was used as lipid mixture for the thin-film formation. Egg PC is a neutral lipid belonging to the group of glycerophospholipids, which is one of the most used lipids for the formation of liposomes. However, the literature also mentions the possibility of applying other phospholipids to influence the properties of final liposomes. These are, for example the introduction of phosphatidylglycerol, which imparts a charge to the lipids to prevent particle fusion, or the introduction of cholesterol to reduce the permeability of the lipid bilayer. (73)

5.1.3. Hydration media

The hydration of the lipid mixture after thin-film formation is a crucial step for the liposomes' preparation. For this study, the lipid hydration was performed with dendrimers' aqueous solutions which were saturated with IMQ. Different concentrations of each generation (G0-G3) were chosen based on previous IMQ solubility experiments where the optimum dendrimer concentration was evaluated for each generation. As optimum dendrimer concentration was chosen the one which gave the highest IMQ solubility. These dendrimer concentrations for G0, G1, G2 and G3 were 20, 10, 5 and 2.6 mM respectively. (74)

After saturating the above dendrimer solutions with IMQ, the concentration of IMQ in each solution was measured with HPLC. Due to their high drug concentration, all solutions were diluted 1000x before the HPLC experiment to follow the range of the calibration curve. The following table (Table 7) is summarizing all the different dendrimers solutions together with the final IMQ concentration found. The values obtained from HPLC were in accordance with our initial experiments. (74) More specifically, the IMQ concentration in the saturated dendrimers' solutions was 7.34, 9.17, 9.24 and 8.45 mg/ml for the G0, G1, G2 and G3 respectively.

Table 7- Dendrimer-IMQ solution specifications with measured IMQ concentartion values

Sample	*Dendrimer generation*	*Dendrimer Mw*	*Dendrimer concentration (mM)*	*Dendrimer amount (mg)*	*IMQ concentration (mg/ml)*
G0-20	G0	492	20.0	32.09	7.34
G1-10	G1	1211	10.0	36.55	9.17
G2-5	G2	2648	5.0	39.90	9.24
G3-2.6	G3	5522	2.6	43.15	8.45

After the confirmation of IMQ concentration, the saturated solutions were used for the lipid hydration. The hydration was performed at the lipids' transition temperature (Tc~65 °C) under stirring to ensure increased mobility of the lipidic mixture during liposomes' formation.

5.1.4. Size reduction - extrusion

As the first step of particle size reduction, sonication in an ultrasonic bath was used to prepare the particles for subsequent extrusion. A study comparing the effects of homogenization, sonication and extrusion mentions this method as less effective in reducing particles' size. It states that prolonged exposure to ultrasound can lead to peroxidative lipid damage or degradation of the substance/ drug that is encapsulated inside the lipid. Therefore, prolonged application of ultrasound is not appropriate. (37) (40) During the experimental part of this diploma thesis, the samples were sonicated for 1 hour after their preparation.

The extrusion was performed above the lipid phase transition temperature throughout the whole process to ensure increased mobility and elasticity of lipids. In relation to the adequate number of extrusions of the sample through the membrane, the value of 15 × was chosen to create the most homogeneous samples. Usually, 5 to 10 extrusions through a membrane with pore dimensions of 100 nm should ensure the formation of unilamellar vesicles with an average size of 110-120 nm. (72) During our approach, we have gradually reduced the size of the liposomes to avoid blocking of small membranes. Initially the vesicles were extruded through 400 nm membranes and later through 100 nm membranes. 15 passages through each membrane ensured the homogeneity of the samples and the avoidance of contamination with large vehicles.

5.1.5. Liposomes' characterization

After the size reduction (extrusion) the liposomes needed to be purified for the removal of the unencapsulated drug and characterised before their use in the permeation experiment. In addition, the total drug present in the formulations before and after the purification had to be measured to get an insight of the method's EE.

For the total drug concentration (free + encapsulated) the non-purified as well as the purified samples were diluted with MeOH (1000x). MeOH was used as a dilution medium since it has the ability to disrupt the lipid bilayers of the liposomes and release the encapsulated drug. Together with Triton, MeOH is the most commonly used lipid bilayer disruption tools. Triton is a powerful non-ionic detergent which disrupting the liposomes by altering the phospholipid organization and forming highly nonsymmetrical structures. (75) When MeOH or other short chain alcohols are added to the liposomal mixture, the planar membrane structure is altered, and the activation energy required for fusion is increased since the membrane fluidity is also increased. (76) Triton is a preferred option when antibiotic-loaded lipidic vesicles are evaluated against bacteria due to its decreased toxicity in

comparison with MeOH. (77) On the other hand, MeOH is more efficiently disrupting the lipid bilayer in comparison to Triton and that's why it was used in this study. (78)

The total IMQ concentration found in the non-purified samples was 7.31, 7.69, 8.34 and 8.75 mg/ml for the formulations made from zero (Lipo G0), 1st (Lipo-G1), 2nd (Lipo-G2) and 3rd (Lipo-G3) generation dendrimers respectively. There is a slight difference between the IMQ concentrations measured for the saturated aqueous dendrimer solutions (Table 5) and the non-purified liposome samples which can be explained from the fact that MeOH is in fact disrupting the liposomal formulations to a great extend but not always quantitively. (78) After purification, the IMQ concentration found to be encapsulated in the liposomes was 1.53, 1.98, 1.73 and 1.37 mg/ml for the Lipo-G0, Lipo-G1, Lipo-G2 and Lipo-G3 respectively. The difference between the IMQ concentration before and after purification is shown in Figure 23.

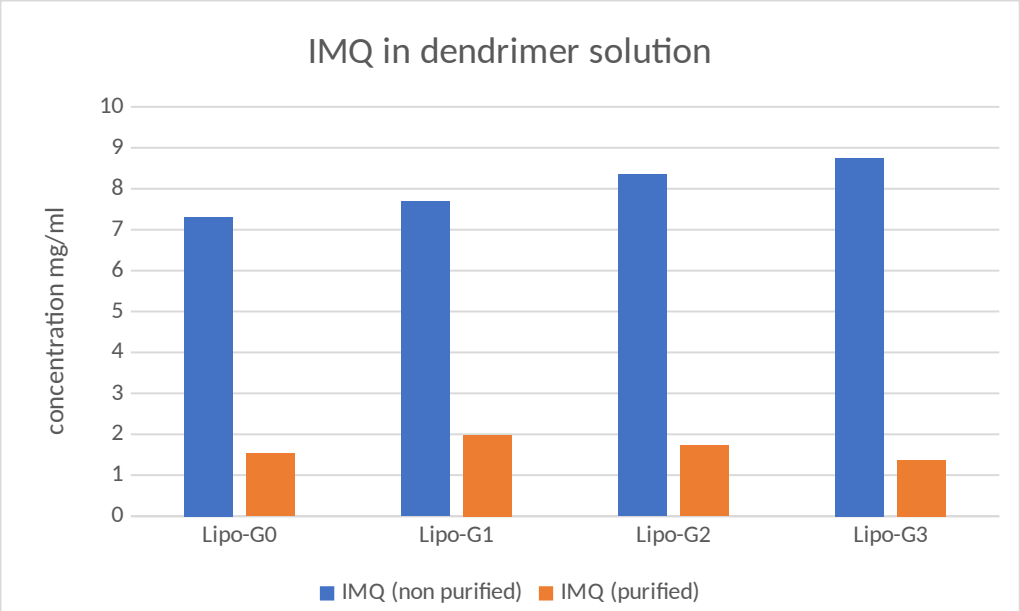

Figure 23- Comparison of total IMQ concentration (unencapsulated and encapsulated) and IMQ concentration after purification (encapsulated only)

Based on the results obtained, the EE for the different dendrimer generations was calculated to be 20.93, 25.74, 20.74 and 15.65 % for the Lipo-G0, Lipo-G1, Lipo-G2 and Lipo-G3 respectively (Table 8). The highest EE was observed for Lipo-G1 and there was no correlation between the EE and the dendrimer generation. In a previous study from our group where liposomes were prepared either by having IMQ incorporated in the lipidic mixture or dissolved in the hydration media, it was shown that the method where IMQ is dissolved in the hydration media is providing a higher EE with values ranging between 27-40 %. (72) In the

present study the EE values are lower, but this phenomenon can be explained from the fact that the hydration media in the present study were having different initial IMQ concentration.

Table 8- Characteristics of the prepared liposomes

Sample	*Z-average (nm ± SD)*	*PDI (± SD)*	$c_{non\ purified}$ *(mg/ml)*	$c_{purified}$ *(mg/ml)*	*%EE*
Lipo-GO	106.97 ± 0.67	0.218 ± 0.009	7.31	1.53	20.93%
Lipo-G1	125.57 ± 3.62	0.367 ± 0.046	7.69	1.98	25.74%
Lipo-G2	132.37 ± 0.84	0.256 ± 0.012	8.34	1.73	20.74%
Lipo-G3	111.53 ± 2.23	0.189 ± 0.007	8.75	1.37	15.65%

After the calculation of the EE, the purified loaded liposomes were evaluated by light scattering technique to get an insight for their size and PDI. All liposomal formulations were in a size range of 107-133 nm. More specifically, Lipo-G0, Lipo-G1, Lipo-G2 and Lipo-G3 were found to be 106.97 ± 0.67, 125.57 ± 3.62, 132.37 ± 0.84 and 111.53 ± 2.23 nm respectively. The results confirm the size reduction to the expected range after using membranes with minimum pore size of 100 nm for the samples' extrusion. In addition, these values are around the size of 120 nm which was proved to be appropriate for liposomes intended for skin delivery. (33)

Concerning the size polydispersity of the samples, all liposomal formulations were in a PDI range of 0.189-0.367. More specifically, Lipo-G0, Lipo-G1, Lipo-G2 and Lipo-G3 had a PDI of 0.218 ± 0.009, 0.367 ± 0.046, 0.256 ± 0.012 and 0.189 ± 0.007 respectively. PDI values can range from 0.0 (perfectly uniform samples) to 1.0 (highly polydisperse samples with multiple particle size populations). For liposomal formulations, a PDI of 0.3 and bellow is considered to be acceptable indicating a population of homogenous lipid carriers. (79) (80) (81) The results of the present study indicated that the majority of the formed liposomes were homogenous (PDI≤0.3) with the exception of Lipo-G1. This liposomal formulation was also found to have the greatest size standard deviation (± 3.62) among all formulations. Even if the PDI of this formulation was not ideal, the formulation was used in the following skin permeation experiment since it had the highest EE.

5.2. Skin permeation experiment

The prepared dendrimer containing liposomal formulations were tested for their ability to deliver the active substance (IMQ) to the difference layers of human skin. For this study, the commercially available cream Aldara was used as control. The high concentration of IMQ in Aldara (5 %) is a limitation factor which does not allow us to apply comparable amounts of active substance to all Franz cells since the concentration of IMQ to the liposomal formulations is much lower. Thus, it was decided to apply comparable amounts of formulations (25mg of Aldara and 25 µl of each liposomal formulation) having in mind that the applied amount of active substance is different. The exact amount of each formulation applied as well as the amount of active substance is given in the following Table 9.

Table 9- Amounts applied during skin experiment

	IMQ concentration (mg/ml)	*Application amount*	*IMQ amount applied (mg)*	*Ratio between IMQ amount in Aldara/IMQ in the given formulation*
Aldara	5% (50 mg/1g)	25 mg	1.25	1
Lipo-G0	1.53 mg/ml	25 µl	0.038	33
Lipo-G1	1.98 mg/ml	25 µl	0.050	25
Lipo-G2	1.73 mg/ml	25 µl	0.043	29
Lipo-G3	1.37 mg/ml	25 µl	0.034	37

The water bath temperature where the permeation experiment was performed was kept at 32 ºC to mimic the skin's temperature and the applied formulations were removed 8 hours after application to closely mimic the application suggested for the commercial formulation Aldara.

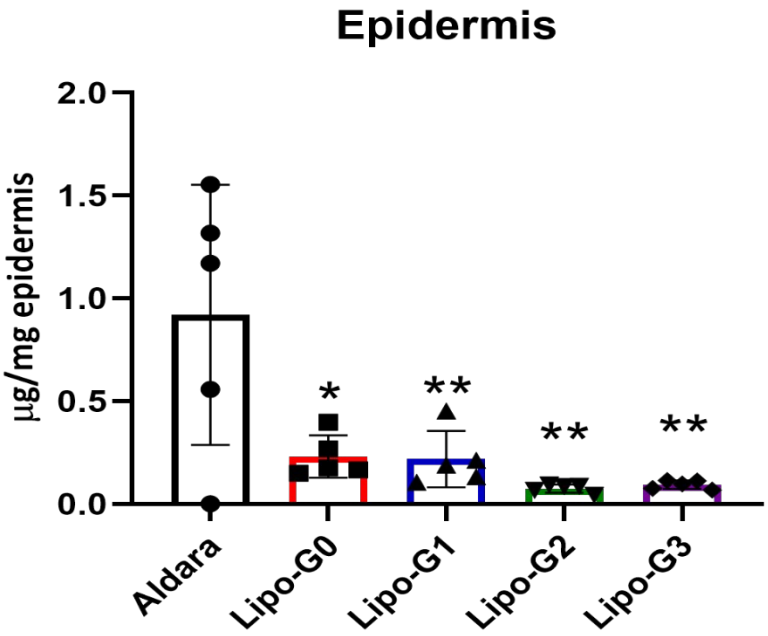

Figure 24- Deposition of IMQ in epidermis

The deposition of IMQ in epidermis is shown in Figure 24. More specifically, the amounts found were 0.920 ± 0.633, 0.231 ± 0.104, 0,218 ± 0.137, 0.074 ± 0.020, 0.094 ± 0.021 µg of IMQ per mg of epidermis for the formulations Aldara, Lipo-G0, Lipo-G1, Lipo-G2 and Lipo-G3 respectively. It is obvious that Aldara is depositing to epidermis almost four times higher amount of IMQ compared with the liposomal formulations. On the other hand, it should be noticed that all liposomal formulations containing at least 25 times less IMQ compared to Aldara (Table 9). Thus, it can be said that the liposomal formulations are more efficient than the commercially available formulation since they are delivering a higher portion of their IMQ content. All liposomal formulations deposited to epidermis comparable amounts of IMQ (no statistical significance observed) which is showing that the deposition is non depended on the dendrimer generation.

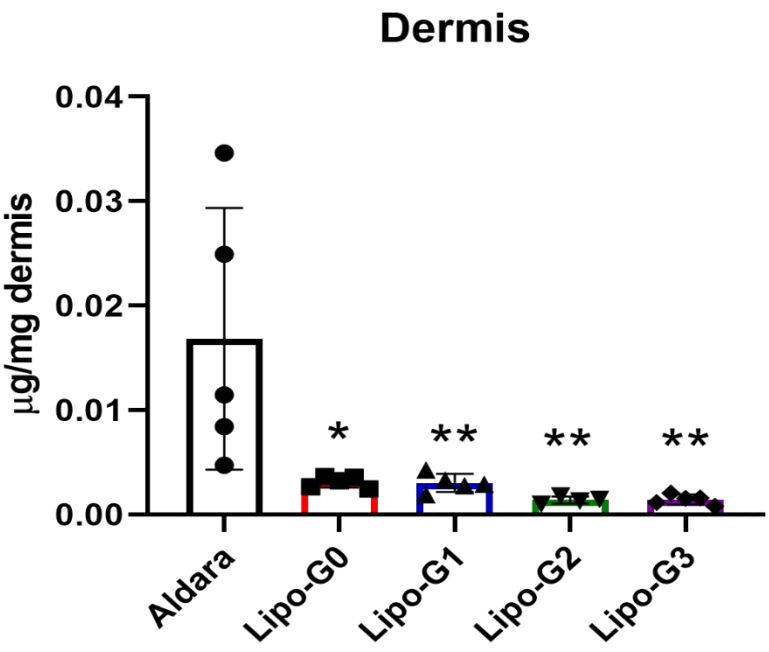

Figure 25- Deposition of IMQ in dermis

The deposition of IMQ in dermis is shown in Figure 25. More specifically, the amounts found were 0.017 ± 0.013, 0.003 ± 0.001, 0.003 ± 0.001, 0.002 ± 0.002, 0.001 ± 0 µg of IMQ per mg of dermis for the formulations Aldara, Lipo-G0, Lipo-G1, Lipo-G2 and Lipo-G3 respectively. It is again visible that the difference between Aldara and the liposomal formulations is statistically significant. Similarly like for epidermis, there are no major differences between the IMQ amount deposited to dermis from the different liposomal formulations which is showing that the different generations are not having some effect.

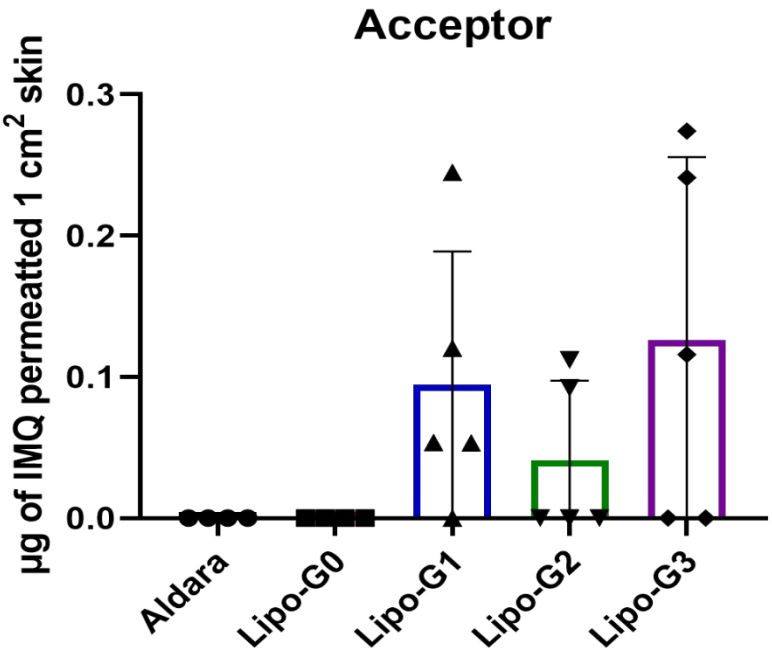

Figure 26- Delivery of IMQ in the acceptor phase

Figure 26 is showing the IMQ amount permeated to the acceptor phase per 1cm^2 of skin area after each formulation applied. Aldara and Lipo-G0 were found to deliver comparable amounts of IMQ very close to the detection limits (0.015 ± 0:013 µg per 1cm^2 of skin) when Lipo-G1, Lipo-G2 and Lipo-G3 were found to deliver 0.118 ± 0.094, 0.102 ± 0.056 and 0.210 ± 0.130 µg of IMQ per 1cm^2 of skin respectively. The drug amount found in acceptor represents the systemic deposition of active substance. Thus, it can be concluded that Aldara and Lipo-G0 cause the lower systemic side effects among the formulations tested.

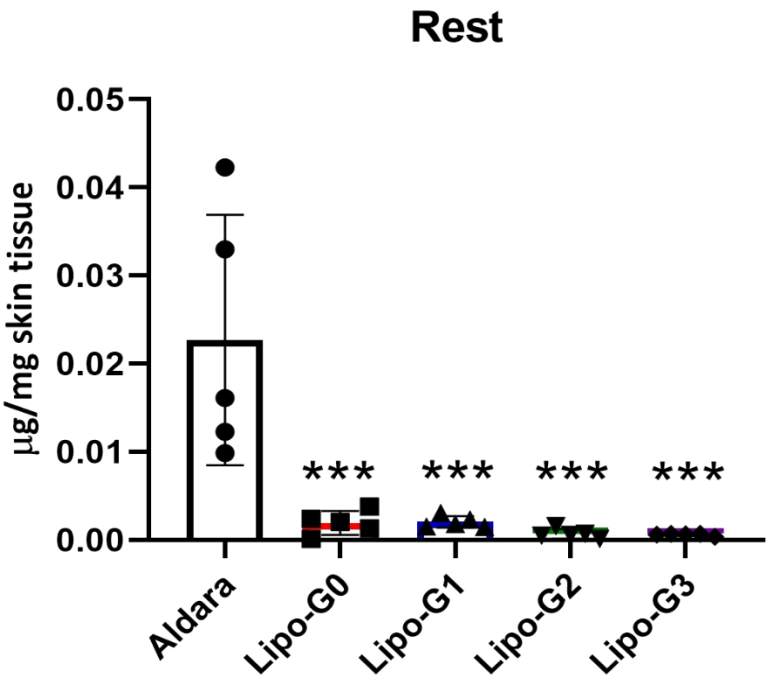

Figure 27- Delivery of IMQ in the surrounding tissue

Figure 27 is showing the IMQ amount deposited in the tissue around the application area. Aldara deposited the highest amount of IMQ 0.023 ± 0.014 µg per mg of skin surrounding tissue) while the liposomal formulations Lipo-G0, Lipo-G1, Lipo-G2 and Lipo-G3 deposited 0.001 ± 0.001 µg. The results confirm that the IMQ delivery from the liposomal formulations is effectively targeted to the application area and the active substance is not escaping to the surrounding tissue thus reducing potential side effects.

6. Conclusion

This diploma thesis combined the beneficial properties of nanocarriers, especially dendrimers and liposomes, to create a novel formulation called aDDnSs for skin drug delivery. Zero, first, second and third generation dendrimers at given concentrations were used to increase the solubility of the lipophilic drug IMQ and thus to achieve a higher concentration within the liposomal particles. Subsequently prepared formulations together with original product Aldara were applied on skin to observe drug's deposition and permeation in and *via* the skin.

The permeation experiment with IMQ showed that these nanocarriers are able to deliver the active substance to the different layers of skin in a generation non-depended manner. Notably, the formulation containing the zero-generation dendrimer did not deliver any detectable amount of IMQ in the acceptor phase, suggesting lower systemic deposition and decreased side effects and thus, it appears to be the most suitable of all the formulations tested.

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