Institute of Veterinary Pathology, Department of Veterinary Medicine

Freie Universität Berlin

Polymeric Core-Multishell-Nanocarriers for Topical Drug Delivery to the Skin: Nanocarrier Distribution, Cargo Delivery and Efficacy for the Treatment of Inflammatory Skin Conditions

Dissertation

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submitted by

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from Bonn, Bad Godesberg

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Dedicated to

a group of mice who never smelled spring air but should have

and all those trying to reduce experiments on – or otherwise trying to be less shitty to – non-human animals

Contents

List of Abbreviations

AD	Atopic dermatitis
ACD	Allergic contact dermatitis
CMS	Core-multishell nanocarriers
$_{C18}$CMS	hPG-amid-C18-mPEG CMS
bCMS	biodegradable hPG-PCL-mPEG CMS
C18	$C_{18}H_{36}$ saturated carbohydrate chain
ELISA	Enzyme-linked immunosorbent assay
EPR	Electron paramagnetic resonance spectroscopy
EtOH	Ethanol
FLIM	Fluorescence lifetime imaging microscopy
DFG	German Research Foundation (*Deutsche Forschungsgemeinschaft*)
DXM	Dexamethasone
HEC	Hydroxyethyl cellulose
hPG	Hyperbranched polyglycerol
HPLC-MS	High-pressure liquid chromatography-mass spectrometry
ICC	Indocarbocyanine
LC-MS	Liquid chromatography-mass spectrometry
mPEG	Monomethyl polyethylene glycol
LMWP	Low molecular weight protamine
MTT	3-(4,5-diMethylThiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate-buffered saline
PCA	3-(carboxy)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy
ROS	Reactive oxygen species
SC	Stratum corneum
TAC	Tacrolimus
TEWL	Transepidermal water loss
mTHPP	Meso-tetra (m-hydroxyphenyl) porphyrin

1. Introduction and literature

1.1. Introduction and context of this work

This project was part of the Collaborate Research Center (CRC) 1112 – **"Nanocarriers: Architecture, Transport, and Topical Application of Drugs for Therapeutic Use"** of the German Research Foundation (*Deutsche Forschungsgemeinschaft* [DFG]). The CRC was a multidisciplinary research effort with over 14 participating workgroups from the Departments/Institutes of Dermatology, Venerology and Allergy of the Charité Universitätsmedizin Berlin, Nutrition Science of the Universität Potsdam, Polymer Research of the Helmholtz-Zentrum Geesthacht, as well as Chemistry and Biochemistry, Pharmacy, Physics, and finally the Institute of Veterinary Pathology at Freie Universität Berlin, in which this work was conducted.

The **overall goal of the CRC** was both to explore and improve a set of promising novel nanomaterials, or "nanocarriers" for potential application in topical dermal drug delivery. The primary use cases envisioned were common, low- to moderately debilitating inflammatory skin diseases. The skin was chosen as a target particularly accessible to exploration. However, it was envisioned that this would also be a step-stone on the way towards more life-threatening, severe skin conditions, traumata, etc., as well as further development of systemic applications. A second important goal was to explore and improve the analytical methods that are used to investigate such nanoparticular delivery.

The **CRC comprised** 1.) work groups concerned with the design, synthesis, loading, and labeling of these nanocarriers, as well as their "simple" physicochemical characterization (e.g., size distribution after synthesis); 2.) groups concerned with their *in silico* modeling ("numerical models"); 3.) groups concerned with improving methods to detect and image the nanoparticles in tissue and explore their interaction with the latter; 4.) groups exploring their behavior from the toxicologic, general pharmacologic, and dermatologic side. The results from all groups were envisioned to help iteratively improve both nanocarriers and methods used.

A particular **knowledge gap** existed around whether results from *ex vivo, in vitro*, and *in silico* studies would translate to the situation *in vivo*, particularly to situations of complex inflammatory changes in the skin barrier.

This specific project was set up to address this gap. Its task was to explore the behavior of the CRC's most advanced nanocarriers (read: most well characterized and most promising) under *in vivo* conditions. For this, dendritic core-multishell nanocarriers (CMS) were chosen.

Specifically, we used the **hPG-amid-C18-mPEG CMS** (referred to as **$_{C18}$CMS** in this work) and the **biodegradable hPG-PCL$_{1.1K}$-mPEG$_{2k}$ CMS** (here referred to as **bCMS**). In addition, a thermoresponsive nanogel material was explored in a particular sub-task, albeit to a much smaller extent. These classes of nanocarriers had shown favorable properties *in vitro* and *ex vivo*: they seemed to be biocompatible and able to deliver and even increase penetration of cargo substances (Frombach et al. 2019; Du 2018; Hönzke 2018; Unbehauen et al. 2017; Du et al. 2016; Hönzke et al. 2016b; Yamamoto et al. 2016; Alnasif et al. 2014; Boreham et al. 2014; Do et al. 2014; Haag et al. 2011; Küchler et al. 2009a; Küchler et al. 2009b; Wolf et al. 2009). The current project aimed to explore a.) the **skin penetration of the nanocarriers** themselves, b.) the **effects of inflammatory skin alterations** on this penetration, c.) the potential **effects of the carriers on tissue**, d.) the effects of the carriers on the **skin penetration of cargo** substances loaded into the carriers, and e.) the **efficacy of drugs** when delivered by these carriers.

As **skin models,** healthy murine skin and an **oxazolone**-induced, murine **SKH1** model of inflammatory skin alterations were chosen, the latter as a substitute for skin alterations seen in **atopic dermatitis (AD)** and related conditions (based on Man et al. 2007). The **cargo substance loaded to the carriers** was **tacrolimus (TAC),** a drug that is important in the topical treatment of inflammatory skin diseases (Wollenberg et al. 2018a; Wollenberg et al. 2018b), but has a molecular weight slightly above the commonly assumed 500 Da ”cutoff” for skin penetration (Bos and Meinardi 2000). The primary **readouts** were the location of the carriers, the location of the cargo substances, clinical parameters, and histological changes in the tissue.

The direct results of this project were published in two peer-reviewed papers that comprise the core components of this cumulative dissertation (Radbruch et al. 2022; Radbruch et al. 2017), see section 2 and hereafter referred to as paper 2 and paper 1, respectively. In addition, a parallel project was performed by the author’s colleague Dr. Pischon, the author, and their working group, which examined similar questions, focusing on a skin model of psoriasis and CMS-facilitated delivery of Nile red into healthy skin. Nile red is a typical model cargo that is easy to visualize in tissue. These results are published in a further set of papers and are part of the dissertation of Dr. Pischon (Pischon 2018; Pischon et al. 2018; Pischon et al. 2017). In addition to this work, the author, Dr. Pischon, and their working group cooperated on several tasks with other working groups of the CRC 1112. Some of the results of these cooperations are published as part of a further set of papers, which are only referenced throughout the text (Wanjiku et al. 2019; Yamamoto et al. 2019; Balke et al. 2018; Hönzke et al. 2016a). The following sections will provide a review of the background and literature relevant to this project.

1.2. Basics of topical dermal delivery and the skin

1.2.1. Definitions – topical dermal vs. transdermal

Topical application is the application of drugs directly to, or close to, the target region of the drug. **Topical dermal delivery** means topical application to the skin, with the target being the skin itself (Brunaugh et al. 2019). Note that the objective of the research project outlined in this dissertation was not to explore **transdermal delivery**, in which the drug is also directly applied to the skin, but the goal is to reach target organs other than the skin itself. Further note that the term skin "penetration" is often used for a substance's penetration into the different layers of the skin, and the term "permeation" for a substance's movement through the skin into the bloodstream, or respectively a receptor medium in case of *in vitro*/*ex vivo* experiments (e.g., Selzer et al. 2013). While somewhat arbitrary and not consistently used in the literature (e.g., permeation is often used for movement between skin layers; Flaten et al. 2015), these definitions will be used throughout this dissertation.

1.2.2. General advantages of topical dermal delivery

Topical application has multiple potential advantages. Maybe the most important is that effective local concentrations can often be reached directly in, or close to, the target region of the drug, while **undesirable effects on other sites are reduced**. Such undesirable effects include gastrointestinal and systemic effects, which would be expected with oral and parenteral systemic application (Whalen et al. 2023). They also include effects on other regions of the skin, for example, the classical "thinning" of skin with chronic application of local corticosteroids (Wollenberg et al. 2018a). A further general advantage compared to systemic, particularly enteral, application is that it **decreases first-pass effects**, i.e., less drug is metabolized by the liver, enterocytes, and other organs and thus lost before reaching the target. In contrast to parenteral application, it of course has the obvious advantage that it is **less invasive** and can be more easily administered by the patients themselves (Whalen et al. 2023). Overall, all this makes topical dermal delivery desirable, particularly for many types of inflammatory skin diseases.

1.2.3. Basic microanatomy of the skin

The mammalian skin generally consists of 3 major layers (inside to outside; Sundberg et al. 2018; Zachary 2017; Kumar et al. 2015; Welsch and Sobotta 2009):

I. The **subcutis** (aka hypodermis) is a layer of loose connective tissue, adipose tissue, vessels, and, depending on the species in question, a skin muscle. It varies greatly in

thickness and connects the dermis to the underlying tissue (Sundberg et al. 2018; Zachary 2017; Welsch and Sobotta 2009). For the purpose of this work, the subcutis is mostly treated as part of the systemic circulation and not the skin proper.

II. The **dermis** (aka corium) is a layer of dense connective tissue. A primary function of the dermis is to provide mechanical strength. The principal cells managing this task are fibroblasts, which produce – and are surrounded by – an abundant extracellular matrix. The matrix mainly consists of collagens, elastin, proteoglycans, and glycoproteins. Furthermore, the dermis contains blood and lymphatic vessels, major populations of immune cells (incl. mast cells, lymphocytes, plasma cells, and various components of the mononuclear phagocyte system), and components of the peripheral nervous system (incl. Meissner's and Pacini's corpuscles as well as free sensory nerve endings). It also contains various specialized appendages, including hair follicles, arrector muscles, sebaceous glands, and apocrine and/or eccrine sweat glands, depending on region and species. The upper region of the dermis can, depending on the species and skin region, form extensive intercalations with the epidermis in the form of rete pegs, or more specialized structures, like the lamellae in the equine hoof (Sundberg et al. 2018; Zachary 2017; Kumar et al. 2015; Welsch and Sobotta 2009). For the context of this work, the dermis's main importance is its function as a substance's entry point into the systemic circulation (Moss et al. 2015), as a potential drug reservoir (see discussion of repeated dosing), and as a site of inflammatory changes and thus a main drug target (Radbruch et al. 2022; Undre et al. 2009; Man et al. 2007).

III. The **epidermis** is the skin's epithelial layer. The cells of the epidermis continuously divide in its innermost layer, then gradually differentiate toward the outside, where they cornify and eventually are shed and lost. Histomorphologically and functionally, it can generally be divided into:

   i. a **basement membrane**, that acts as a scaffold for epidermal cell attachment.
   ii. the **stratum basale**, which is the dividing stem cell population (basal keratinocytes).
   iii. multiple layers of **keratinocytes in varying stages of differentiation.** Depending on the species and skin region, these are further divided into a **stratum spinosum**, a **stratum granulosum**, and sometimes on hairless skin a **stratum lucidum**, e.g., on human palms/soles. Note that in specialized appendixes, further, specialized layers are differentiated.
   iv. the **stratum corneum (SC**; Sundberg et al. 2018; Zachary 2017; Kumar et al. 2015; Welsch and Sobotta 2009).

   The inner layers represent the **viable epidermis**, and its cells are called **keratinocytes**. The SC, on the other hand, can be called "non-viable" and its "cells" are called **corneocytes** or SC squames. The thickness of the layers of the epidermis varies greatly

between species and anatomical regions. Smaller species and areas with denser fur tend to have a thinner epidermis, while it tends to be much thicker in larger and sparsely-haired species, e.g., humans, and areas of particular mechanical stress, e.g., the feet (Zachary 2017). In addition, the epidermis produces complex keratin structures such as hair/feathers, nails/hooves/claws, horns, etc., and various glands. These are beyond the scope of this work, but it must be kept in mind that hair follicles and glands have been assumed to be routes of increased entry or reservoirs for some substances (Gorzelanny et al. 2020; Lademann et al. 2015; Knorr et al. 2009; Teichmann et al. 2006).

The epidermis does not contain blood vessels, it receives its supply via diffusion from the adjacent dermis. However, it does contain other non-keratinocyte cell populations. Most notably, these are Langerhans cells and epidermal dendritic cells, which constantly sample antigens, melanocytes, which produce melanin, and Merkel cells, which act as sensory organs (Sundberg et al. 2018; Welsch and Sobotta 2009).

For the context of this work, the epidermis's main importance is its role as the assumed primary barrier against TAC (Undre et al. 2009) and CMS (Pischon et al. 2017) penetration, as a potential drug reservoir (see discussion of repeated dosing), as well as a site of inflammation and thus drug target (Man et al. 2007).

1.2.4. Ultrastructure of the stratum corneum (SC)

The SC is often assumed to be the main rate-limiting barrier to substance penetration (see section 1.2.7). Its ultrastructure thus warrants more detailed consideration.

The SC's **corneocytes** are fully differentiated keratinocytes, which have lost their nucleus and ceased almost all metabolic activity. They have replaced their cytoplasm with a dense, dehydrated matrix dominated by keratins, a type of mechanically strong intermediate filament proteins (Sundberg et al. 2018; Welsch and Sobotta 2009), surrounded by a rigid "cornified envelope" (Évora et al. 2021). The cornified envelope is made up of several proteins, including loricrin, involucrin, filaggrin, and many others (Gorzelanny et al. 2020). Embedded in their extracellular lipid matrix, corneocytes are often depicted as the "brick" in a "brick and mortar"-like structure. This is important, as it is often assumed that many to most drugs may not actually diffuse through corneocytes, but more or less exclusively take the route through the extracellular matrix (e.g., Brunaugh et al. 2019). However, for making assumptions about the resulting diffusion path, it seems important to remember that these cells are flattened to an extreme extent. In humans, their thickness seems to only be around 0.2–0.4 µm thick, while their diameter is around 40 µm (Prausnitz et al. 2004). These values differ by body region, species/strain, and age (Évora et al. 2021; Allen and Potten 1976a). They also differ depending on the layers in the SC (Évora et al. 2021; Corcuff et al. 2001), which can roughly be divided

into a stratum compactum (freshly keratinized cells adjacent to the viable epidermis) and stratum disjunctum (superficial squames shortly before being shed). In mice, for dosal haired skin corneocyte thickness is reported to be around 0.07 µm in the stratum disjunctum to 0.21 µm in the stratum compactum and over 0.62 µm on hairless dorsal skin or over 0.75 µm on the tail and foot, while their diameters range between 17 and 51 µm (Allen and Potten 1976a). Their shape, when viewed from the side is thus not brick-like, as often depicted in schematic figures, but extremely flat*. The corneocyte shape, when viewed from the "top," appears to be roughly hexagonal (Évora et al. 2021). To be precise, their true 3D shape on average seems to be similar to a Kelvin tetrakaidecahedron†, albeit a very flattened one (Feuchter et al. 2006; Allen and Potten 1976b). Depending on the species and skin region, these squames can a.) be stacked one on top of each other in neat columns with little overlap but various degrees of "hooking into each other" in their interdigitating edge regions; b.) be layered with up to complete overlap; or c.) be arranged more or less haphazardly (Allen and Potten 1976a). In addition, their large "upper and lower" surfaces can either be comparatively flat or contain additional ridges that interlace with the squames above and below (Allen and Potten 1976a).

The **extracellular matrix** surrounding the corneocytes is a thin lamellar, lipid layer. Importantly, however, its structure is not to be confused with the lipid bilayer structure of the cellular membrane, from which it differs drastically in composition and sterical arrangement:

- **Composition**: While the cell membrane predominantly consists of phospho- and glycolipids and cholesterol, the SC matrix mostly consists of ceramides, free fatty acids (FFAs), and cholesterol (Boncheva 2014). In contrast to the cell membrane's phospholipids, the SC's ceramides seem to have smaller and less polar head regions. As ceramides, they not only contain a fatty acid but also a sphingosine tail. The chain lengths of ceramides and FFAs are longer, more varied, and predominantly unsaturated, i.e., sterically "straighter" (Boncheva 2014). Importantly, the SC's lipid matrix is almost completely dehydrated under normal conditions, in contrast to the water-rich cell membrane environment (approx. 1–2 H_2O/head region vs. 7–16; Boncheva 2014).
- **Sterical arrangement**: Laterally, lipids in cell membranes predominantly exist in a liquid-crystalline arrangement, allowing for considerable lateral diffusion. In the SC, the lipid arrangement seems to be dominated by a solid configuration without lateral diffusion, although liquid-crystalline regions also exist (Boncheva 2014). Vertically, there is not only one "simple" bilayer. Instead, there is usually a small number of repeating units, which

*Note: sometimes corneocytes are artificially swollen to make their structure visible under light-microscopy, e.g., 5x normal by applying alkali solutions. This can make them appear "brick and mortar"-like on light-microscopic images as well (e.g., see Évora et al. 2021).
† A shape generally suited to fill a space with approximately spherical cells with near 100% density and near minimal surface area (Feuchter et al. 2006).

can be electron-microscopically visualized as lamellarity (e.g., see Corcuff et al. 2001; Swartzendruber et al. 1989), although there are regions in which only a single unit can be seen. The molecular architecture of these lamellae may not be as simple as symmetrically repeating distinct lipid tail and polar head regions. It is only incompletely understood and different models exist. Different sterical arrangements are considered for the ceramides themselves (from completely folded and thus somewhat phospholipid-like to "stretched out," with ceramide and fatty acid tails on opposing sides of the polar region), different degrees of intercalation of tails, cholesterol, and other substances resulting in different degrees of asymmetry of short and long phases, and other options (Boncheva 2014). This is out of the scope of the current work, but it demonstrates the complexity of trying to gauge the potential interactions of nanoparticles with this environment.

The **intercellular adhesion** between corneocytes is assured by **corneosomes**, which are protein "spot welds" derived from the desmosomes of the predecessor keratinocytes. Depending on the layer in the SC and the skin region, they can be concentrated on the lateral "edges" of the corneocytes only (mature corneocytes of most regions) or distributed along their flat sides as well (immature corneocytes and regions needing higher stiffness, e.g., the feet; Évora et al. 2021; Corcuff et al. 2001). Their concentration can be altered by multiple factors, and their density seems to be somewhat correlated with the skin's permeability to water (Évora et al. 2021). Furthermore, the extracellular lipid matrix also seems to be anchored to the corneocytes: certain ceramides, fatty acids, and proteins like loricrin and involucrin are covalently connected to their cornified envelope. This structure is called the **cornified lipid envelope**, and it partially intercalates with the lipid extracellular matrix (Évora et al. 2021).

1.2.5. Human vs. murine skin anatomy

Rodents may be more closely related to primates than almost all mammalian clades (Doronina et al. 2022; Murphy et al. 2001) and murine skin is extensively used in research, with the assumption of *some* transferability to humans (Ngo et al. 2009; WHO 2006; Vecchia and Bunge 2005; OECD 2004). See section 3.1.1. of the discussion for major caveats about transferability. However, murine and human skin differ anatomically in several relevant aspects, most evidently:

- **Thickness:** Human skin layers are generally thicker, although the thickness varies immensely by region (see table 1). It can also vary by mouse strain, mechanical/chemical stress, and potentially hydration state (see discussion below). In addition, measurements can be derived from different microscopy techniques, which rely on various fixation methods that in turn may have different effects on the measured post-fixation thicknesses.

Histologically, the most visible difference is that the human stratum spinosum is usually multiple cells thick, while mice only have a single cell layer in the skin of the trunk (Sundberg et al. 2018). Overall, the general difference in thickness does seem to correlate with the observation that murine skin generally seems to be more penetrable (Godin and Touitou 2007).

Table 1. Examples of skin layer thicknesses as reported in the literature

	Mouse	**Hairless mouse**	**Human**
Stratum corneum thickness	9 µm [1] 5 µm (dorsum) [2] 0.7 µm (dorsum) [3] 6.27 µm (ear) [3] 19.5 µm (tail) [3] 340 µm (footpad) [3]	8.9 µm [2] 3.78 µm (dorsum) [3]	ca.17 µm (e.g., forearm) [1,2] 10–30 µm (most regions) [4] 185 µm (hand) [4]
Epidermal (overall) thickness	29 µm [1] 13 µm (dorsum) [2] 10–15 µm (thin skin body) [5] 70–80 µm (tail) [5] 150–400 µm (footpad) [5]	28.6 µm [2]	47 µm [1] 36 µm [2] 50–100 µm (thin skin) [5] 300–400 µm (palms, soles) [5]
Whole skin thickness	700 µm [1] 800 µm (dorsum) [2]	700 µm [2]	2970 µm [1] 1500 µm [2]
References:	[1] (Godin and Touitou 2007) [2] (Jung and Maibach 2015) [3] (Allen and Potten 1976a) [4] (Böhling et al. 2014) [5] (Sundberg et al. 2018)		

- **Hair**: Murine non-glabrous, particularly truncal skin of most strains is generally covered by thick fur and thus contains large numbers of hair follicles. Human skin on the other hand has comparatively sparce fur, except for the scalp. Furthermore, hair types and hair growth cycles differ (e.g., hair mostly in telogen and cycling in waves in mice, hair mostly in anagen and no waves in humans), although hair follicle structures are generally similar (Sundberg et al. 2018).
- **Glands**: Mice only possess eccrine sweat glands on their palms/soles, while these are ubiquitous in humans (Sundberg et al. 2018). Mice also lack apocrine sweat glands found in some regions of human skin, but both have similar sebaceous glands (Sundberg et al. 2018).
- **Vascularization**: The murine dermis is described as less well vascularized compared to the human dermis (Sundberg et al. 2018).

1.2.6. Special anatomy of the SKH1 mouse

The SKH1 mouse strain is a hairless, outbred, albino strain that carries the ***Hr^{hr}* allele of the *hairless (Hr) gene***. Several alleles of the *hairless (Hr) gene* are known that result in hairlessness in mice (and have been associated with it in humans; Benavides et al. 2009). The SKH1 (*Hr^{hr}/Hr^{hr}*) strain is particularly well characterized and often used as a skin model. Although *Hr^{hr}/Hr^{hr}* mice develop a normal first hair coat during their development, they almost completely lose their hair by 3 weeks of age and subsequently only develop a few, abnormal tylotrich (guard hair) follicles (Benavides et al. 2009). Histologically, their skin exhibits a few typical structures that differ from normal mouse skin:

- "**Utriculi**" are structures reminiscent of a hair follicle's infundibulum that are open to the skin surface and lined by hyperkeratotic epithelium.
- **Dermal cysts** are cystic structures in the deeper dermis – without connection to the skin surface – which are lined by keratinized epithelium and sometimes sebocytes.
- **Enlargement of sebaceous glands, dermal granulomas,** and relatively **rugose skin** can be present (Benavides et al. 2009).

Note that SKH1 mice are not to be confused with nu/nu nude mice. In contrast to the latter, SKH1 mice are neither athymic nor immunosuppressed. SKH1 mice are frequently used for studies on wound healing, carcinogenesis, toxicity, inflammatory skin conditions, and skin penetration. In contrast to other mouse strains, they do not require shaving, chemical depilation, or similar methods which can injure the skin to model sparsely-haired, human skin. However, it must be kept in mind that a.) they are an outbred strain, and thus there is a comparatively wide variation between individuals, and b.) their immune system is still less characterized than other mouse strains and there is some evidence that part of their immune responses may be altered compared to other mouse strains (Benavides et al. 2009).

1.2.7. Basics of skin penetration

The skin seems to have evolved to separate the inside from the outside, and it is not particularly surprising that it turns out to be a formidable barrier to the ingress of all sorts of substances. The main structures that must be considered for the barrier function of the mammalian skin are – from the outside to the inside – the SC (corneocytes or matrix), the viable epidermis (tight junctions and keratinocytes), epidermal basement membrane (although potentially of little relevance for small molecules; Gorzelanny et al. 2020), the dermis (extracellular matrix interspersed with various cell types; potentially of less relevance than its thickness may suggest due to the close proximity of dermal vessels to the epidermis), and the endothelium

of the dermal vessels (Gorzelanny et al. 2020; Sundberg et al. 2018; Zachary 2017; Kumar et al. 2015; Moss et al. 2015; Welsch and Sobotta 2009). The main rate-limiting step of penetration for many substances into and through the skin is assumed to be the SC (Gorzelanny et al. 2020; Brunaugh et al. 2019; EFSA et al. 2017; Selzer et al. 2013; Prausnitz et al. 2004), including for TAC (Hazama et al. 2017; Undre 2004). However, this cannot be assumed for all substances and may differ between exposure scenarios (see also discussion on TAC penetration). In addition to the diffusivity of a drug in a particular skin layer ("barrier against penetration"), the free energy profile/position-dependent partition coefficient of a substance must be considered (i.e., how much it "wants to be in a specific place in the skin"; Selzer et al. 2017). For example, the transition from the SC to the upper viable epidermis may also be an important barrier region (Yamamoto et al. 2016), whether the free energy profile of the drug makes the drug "want to stay" in this environment (e.g., modeled by Schulz et al. 2017), or whether tight junctions/cell membranes cause a diffusivity barrier (Gorzelanny et al. 2020).

Furthermore, a conceptual differentiation is often made between a.) an "extracellular route," i.e., a penetration of substances through the extracellular lipid SC matrix which is often assumed to be the predominant route for lipophilic but also many hydrophilic substances; b.) an "intracellular route" through the corneocytes; and c.) a "follicular route" through the follicles (Gorzelanny et al. 2020; Moss et al. 2015; Selzer et al. 2013). The latter may also constitute a reservoir for nanoparticles (Lademann et al. 2015). However, the exact route and type of penetration is probably very much dependent on the specific substance in question and is not well understood in many cases. Moreover, even if a substance preferentially penetrates through one route, it may also take the other routes to some degree. For example, substances that have a very low relative penetration through the corneocytes may still end up in there under steady-state conditions/after long penetration times (e.g., Feuchter et al. 2006; Wang et al. 2006). We found evidence that this is the case for TAC (Yamamoto et al. 2019).

There seems to be evidence for the following rules of thumb:

- **Size**: Generally, larger substances penetrate less effectively. In fact, there is a much-quoted "500 Dalton rule" which states that "the molecular weight (MW) of a compound must be under 500 Dalton to allow skin absorption" (Bos and Meinardi 2000). However, it is important to remember that this is an oversimplification. It cannot necessarily be assumed to be true for all substances, and it is important to define the rates of penetration in question. The rule was derived from the observation, that out of the substances used in the ICDRG patch test, i.e., the substances causing the most contact allergy, as well as a range of common topical drugs and drugs available for transdermal delivery, almost none

were above 500 Da (Bos and Meinardi 2000). Notable exceptions are, for example, the large calcitonin inhibitors TAC (>800 Da; Bos and Meinardi 2000) and even cyclosporine (>1000 Da; Lauerma et al. 1997)*. Furthermore, there is some evidence that certain nanoparticles may penetrate the skin barrier under certain conditions (European Commission, Directorate-General for Health and Food Safety 2021; WHO 2006), despite being *much* larger than 500 Da. In fact, the assumption that certain nanoparticles may penetrate the skin barrier was one of the basic assumptions of the CRC 1112 (see discussion of CMS penetration). The EFSA guidance on dermal absorption simply states that there is not sufficient evidence for any generalizations on this subject and thus the potential must be considered for each nanoparticle individually (EFSA et al. 2017).

- **Lipophilicity**: Generally, relatively lipophilic substances seem to penetrate better than hydrophilic ones (Selzer et al. 2013). Substances with comparatively high penetration have been said to have an octanol/water partition coefficient ($\log K_{o/w}$) in the range of 2–3 (Selzer et al. 2013). It is important to note, however, that this is also an oversimplification and probably largely depends on the substance as well as potential routes taken through the skin (Moss et al. 2015).

1.3. Atopic dermatitis (AD) – a common, complex condition of the skin

1.3.1. Introduction to AD and its epidemiology

Atopic dermatitis (AD), also called “atopic eczema” or “neurodermatitis,” is an inflammatory skin condition in humans (Ständer 2021; Wollenberg et al. 2018a), horses, dogs, cats (Marsella and De Benedetto 2017; Zachary 2017), and likely other species. In humans, it is one of the most **common** skin conditions. It has worldwide prevalence rates of up to 20% reported in children (Ständer 2021), although these rates show large regional variations (Odhiambo et al. 2009) and must of course be considered with some care without additional information on severity. Nevertheless, it has been reported to be the 15th most common nonfatal disease, and, more importantly, the skin disorder with the highest effect on disability-adjusted life years (Laughter et al. 2021).

1.3.2. Diagnosis, clinic, and histology of AD

The condition is diagnosed clinically by a catalog of features. The most characteristic features are **pruritus**, as well as sequelae of scratching (excoriation, erythema, edema/papulation, lichenification, hemorrhage). Further features include dry skin (xerosis), atypical vascular

* The original publication claimed that cyclosporine would not penetrate, including in atopic dermatitis.

responses, predisposition to bacterial, viral, and fungal skin diseases, and even systemic infections, as well as others (Ständer 2021). Clinical severity can be scored with 2 typical scoring systems: the “eczema area and severity index” (**EASI**), a modified version of which was used in this work, and the “scoring atopic dermatitis scale” (SCORAD; Ständer 2021). Histologically, lesional AD skin typically shows infiltrating T cells and dendritic cells, as well as mast cells and eosinophils (Martel et al. 2017).

1.3.3. Pathogenesis of AD

The pathogenesis of AD in humans is only incompletely understood and seems to be **complex, multifactorial, and heterogeneous**. In fact, it is so heterogeneous that it may be a common phenotype of different underlying causes/endotypes. Traditionally, the disease mechanisms were somewhat divided into two boundary cases: 1. an “inside to outside” hypothesis, which assumed a (hypersensitive) immune dysregulation as the root, which would then cause the alterations of the skin barrier, and 2. an “outside to inside” hypothesis, according to which the skin’s barrier alteration is the root, which then causes an unregulated influx of haptens, upon which hypersensitization ensues (Elias et al. 2008). However, it seems that the true situation may often lie in between these two poles, with mechanisms associated with both boundary cases present to varying degrees in individual patients. Generally, at least the following factors are thought to play a role in varying amounts and causal chains:

- Pathologically **Th2 leaning T-lymphocyte activation** (incl. release of IL 4, 5, 13, and 31; Sroka-Tomaszewska and Trzeciak 2021; Ständer 2021).
  In addition to Th2, activations of Th1, Th17, and Th22 pathways have been reported, but with differences between patient populations. Especially **Th17 involvement** seems to be a factor, but potentially only in patients of Asian but not of African or European ancestry (Sroka-Tomaszewska and Trzeciak 2021; Ständer 2021). In addition, as with other immunologic reactions, it is important to differentiate between more acute parts of the inflammation and more chronic phases. With the shift to the latter, in AD an *additional* significant contribution of **Th1-biased cytokines** starts to occur, including IFNγ (Martel et al. 2017; Bieber 2010), Il11, IL12, IL18, and TGFβ1 (Bieber 2010). There is also activation of ILCs (innate lymphoid cells; Sroka-Tomaszewska and Trzeciak 2021).
- Increased **IgE** levels, and **eosinophilia** (Sroka-Tomaszewska and Trzeciak 2021; Ständer 2021). Note that endotypes with and without exist (Martel et al. 2017).
- **Hypersensitivity** against *dermatophagoides farinae* and *pteronyssinus* house dust mites and other allergens (Martel et al. 2017).
- Decreased expression of **antimicrobial peptides** (Sroka-Tomaszewska and Trzeciak 2021; Ständer 2021).

- **Dysbiosis** of the skin's microbiome, including a shift towards *Staphylococcus spp.,* specifically pathogenic *S. aureus* (Patrick et al. 2021; Sroka-Tomaszewska and Trzeciak 2021; Ständer 2021).
- Genetic mutations of the **filaggrin gene (FLG)**. This is the prototypical genetic component. FLG loss-of-function mutations seem to be found in around 3–50% of human AD patients (Patrick et al. 2021), potentially varying relevantly between populations (e.g., Zhu et al. 2021; Margolis et al. 2019; On et al. 2017; Margolis et al. 2012). FLG mutations seem to play an important role, although their presence neither guarantees AD nor is it necessary for the development of AD (Kim et al. 2019; Marsella and De Benedetto 2017). FLG mutations seem to alter the SC's extracellular matrix lipid composition and function (Patrick et al. 2021; Sroka-Tomaszewska and Trzeciak 2021; Ständer 2021). This leads to loss of water from the skin and thus dryness, microbial dysbiosis, and potentially ingress of allergens (Ständer 2021).
- **Other genetic factors**: Overall, at least 31 different loci with AD susceptibility genes are reported, affecting the skin barrier, e.g., tight junctions between keratinocytes (Sroka-Tomaszewska and Trzeciak 2021) or loricrin (Martel et al. 2017), as well as the immune system, e.g., genes involved in mast cell and histamine metabolism, NOD-receptor genes, and specific Toll-like receptor polymorphisms (Sroka-Tomaszewska and Trzeciak 2021).
- **Environmental factors**: Extreme temperatures, UV radiation, epidermal aryl hydrocarbon receptor activation by air pollution, water hardness, and potentially the decrease of allergens in many individuals' environments in the sense of the hygiene hypothesis of hypersensitivity development (Ständer 2021).
- Environmentally-induced and then **epigenetically procreated** changes have recently been identified to potentially play a significant role as well. These seem to, again, affect both the skin barrier and the immune system. For example, it has been reported that exposure of a mother to tobacco smoke can cause DNA methylation changes which lead to lower Treg numbers and resulting atopy in her offspring (Sroka-Tomaszewska and Trzeciak 2021).
- Increased **sensitivity to itching** (Sroka-Tomaszewska and Trzeciak 2021).

Patients also often develop other **concurrent immune-mediated conditions**, including asthma, rhinitis/rhinoconjunctivitis, food allergies, inflammatory bowel disease, and rheumatoid arthritis. It has been assumed that such conditions can develop in the wake of the skin condition, in the form of an "atopic march," when the skin barrier is diminished by AD and the immune system is exposed to different types of haptens (Ständer 2021).

1.3.4. Treatment of AD

The recommended treatment regimens for humans with AD depend on the severity of the disease, as well as age and further patient factors (Wollenberg et al. 2018a; Wollenberg et al. 2018b) and can be approximately summarized as:

- **Always (including during pruritus-free periods):** A non-drug-based baseline therapy is considered important, particularly the use of proper emollients and avoidance of trigger factors (Ständer 2021; Wollenberg et al. 2018a; Wollenberg et al. 2018b).
- **Mild to moderate disease:** Topical medium strength to strong glucocorticoids or the topical calcineurin inhibitor TAC, either as reactive therapy ("when needed") or proactive therapy (i.e., chronic application even in symptom-free periods). Potential other options: wet wrap therapy with corticosteroids or TAC (Wollenberg et al. 2018a; Wollenberg et al. 2018b), topical pimecrolimus (a calcineurin inhibitor similar to TAC and cyclosporin; Ständer 2021), UV therapy (Wollenberg et al. 2018a; Wollenberg et al. 2018b), except in children (Ständer 2021), the topical phosphodiesterase-4 inhibitor crisaborole (Ständer 2021), and potentially antiseptics (Wollenberg et al. 2018a; Wollenberg et al. 2018b)
- **Severe disease:** Hospitalization, systemic immunosuppression with oral glucocorticoids or cyclosporine A (Wollenberg et al. 2018a; Wollenberg et al. 2018b). Other options include the systemic biologic Interleukin-4Rα antagonist dupilumab, as well as methotrexate, azathioprine (Ständer 2021; Wollenberg et al. 2018a; Wollenberg et al. 2018b), mycophenolate mofetil, alitretinoin, PUVA (psoralen and UV-A light) therapy (Wollenberg et al. 2018a; Wollenberg et al. 2018b), or the systemic oral jak inhibitor Baricitinib (Ständer 2021). In addition, various ongoing trials examine multiple biologics targeting the Th2 axis (Ständer 2021; Wollenberg et al. 2018a; Wollenberg et al. 2018b).

The two main drug classes for the topical treatment of AD are **glucocorticoids** and the calcineurin inhibitor **TAC**.

Topical glucocorticoids' main side effect is skin atrophy (which can be accompanied by others, such as telangiectasias, spontaneous scars and stretch marks, ecchymosis, hypertrichoses, and in infants diaper granulomas, and potentially iatrogenic Cushing). They are very effective, but because of their side effects, they are neither approved nor recommended to be used for longer periods at a time (mostly < 20 weeks) and their use should be limited to sensitive areas such as the face (Wollenberg et al. 2018a; Wollenberg et al. 2018b). Further risks are systemic uptake during pregnancy (only thin skin and strong glucocorticoids), which may induce iatrogenic Cushing effects, and the rosacea-like "red face" or "corticosteroid addiction

syndrome" (Wollenberg et al. 2018a; Wollenberg et al. 2018b). TAC will be further described in the next section.

1.3.5. Tacrolimus (TAC) in the treatment of AD

TAC is a calcineurin inhibitor, which generally prevents the activation of NFAT (nuclear factor of activated T cells; Murphy and Weaver 2016), although it acts via various pathways, including NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), JNK (c-Jun N-terminal kinase), p38 (p38 mitogen-activated protein kinases), and TGF-ß1(transforming growth factor-ß; Barbarino et al. 2013). It is potent in the **suppression of T cells.** Compared to the prototypical substance of the class, cyclosporine, TAC's ability to inhibit T cell activation seems to be 10–100 times as strong (Ruzicka et al. 1997). However, it may also inhibit natural killer cells (Barbarino et al. 2013) and others.

In AD, TAC 0.1% ointment is reported to have an anti-inflammatory effect approximately equivalent to medium strength (Wollenberg et al. 2018a; Wollenberg et al. 2018b; Cury Martins et al. 2015), or potentially even higher strength (Cury Martins et al. 2015), glucocorticoids. TAC 0.03% ointment, the lower of the two concentrations commercially available, also seems to be approximately equivalent to medium-strength glucocorticoids, or marginally less potent, depending on the study (Cury Martins et al. 2015). In contrast to glucocorticoids, TAC has been found to be safe for proactive application for at least up to 1 year, and there is safety data for 4-year treatment. It is approved for and used in children, except for children < 2 years of age, because of a lack of safety data for this age group. Compared to glucocorticoids, it seems similarly effective on the trunk and extremities, and even more effective in the face during chronic treatment. The most common adverse effect is a topical, usually short-lived burning sensation. Skin atrophy, a main adverse effect of glucocorticoids, is not observed (Wollenberg et al. 2018a; Wollenberg et al. 2018b; Cury Martins et al. 2015).

TAC is a relatively large macrocytic lactone, with a molecular mass of 804 Da, or rather **822 Da** in its commonly found hydrated form (Wallemacq and Reding 1993). It is **lipophilic**, and seems to have a solubility in water of only around 2.5 µg/ml (Yamamoto et al. 2019).

As discussed above, it is considered "on the size threshold" for skin penetration. However, it is very effective in the topical therapy of AD, which already suggests that it penetrates quite sufficiently. It has been reported that in human adult patients, 24 h after the first topical application, skin concentrations of 94 ± 20 ng/cm^{-3} were reached. During twice-daily treatment for 14 days, this seemed to increase to a steady state concentration of 595 ± 98 ng/cm^{-3} 24 h after the last treatment (Undre et al. 2009). The resulting blood concentrations in the same study were reported to be < 1 ng/ml in 94% of patients.

1.3.6. Animal models of AD

Multiple animal models of AD have been described. These include:

1. **Hapten-induced models**: A number of haptens, i.e., small molecules that can act as antigens when they become bound to larger molecules in the body, can penetrate into the skin when topically applied. There, they can induce a particularly reproducible and predictive immune response in mice. They usually require one application for sensitization, followed by further challenges. During the first challenges, many of them seem to elicit an acute, Th1-biased immune response, which is seen as a form of/model for "allergic contact dermatitis (ACD)." With continuing challenges, the reaction tends to shift towards the Th2 axis and is used as a model for AD (Martel et al. 2017). Common, strong haptens investigated utilized in AD models are oxazolone, dinitrofluorobenzene, dinitrochlorobenzene, trinitrochlorobenzene, and toluene diisocyanate. It is important to note that the types of infiltrating immune cells, cytokine biases, etc., do not only differ somewhat between the haptens but also between mouse strains. For example, if oxazolone is used in BALB/c mice instead of hairless Hr^{Hr}/Hr^{Hr} mice, it seems the response is more Th1 instead of Th2-biased. Overall, their advantage over most other models is quick and reproducible induction (Martel et al. 2017). For details on the oxazolone-induced model, see section 1.3.7 below.
2. **Allergen-induced models**: Similar to hapten-based induction, mixtures of larger molecular antigens can be used to topically induce inflammation. Typical mixtures are house dust mite antigens (HDM) or ovalbumin (OVA), which contain at least 48 and 10 different allergens, respectively. One potential disadvantage is that allergen compositions may vary between commercial suppliers. Furthermore, they usually do not elicit an immune response when applied to healthy skin (Martel et al. 2017) (most likely because these antigens are very large). This means that the skin barrier needs to be additionally compromised, e.g., by occlusion or use of Flaky Tail or NC/Nga mice (Martel et al. 2017).
3. **"Spontaneous" models**: In mice, there are the NC/NGA and Flaky Tail (ft/ft) strains. (The latter has filaggrin and matted (ma) mutations). Both strains show pruritic lesions and Th2 activation similar to humans, plus some of the other phenotypical features seen in human AD (Martel et al. 2017). Furthermore, spontaneous canine (Marsella and De Benedetto 2017; Martel et al. 2017), and equine AD (Marsella and De Benedetto 2017) resembles human AD. Spontaneous models are considered relatively unstandardized in onset and severity (Martel et al. 2017).

4. **Transgenic/knockout mice**: A long list of genetically engineered mouse models exists. They are usually created to model alterations in a single pathway, e.g., the expression of IL-4 in the skin. Because of this, however, they are often considered somewhat non-physiological for the overall disease process (Martel et al. 2017).
5. **Vitamin D analog-induced model**: Interestingly, topical application of Vitamin D or certain analogs also causes inflammation with some features of AD in mice, however, these are rather limited (Martel et al. 2017).

Most of these models elicit pruritic forms of dermatitis, with acute and chronic lesions and a Th2 bias similar to human AD. They also exhibit varying numbers of the other phenotypical features/immunologic endotypes seen in human AD and various further advantages and disadvantages that are out of the scope of this work. For a more detailed comparison, see (Martel et al. 2017; Man et al. 2007).

1.3.7. The oxazolone-induced models of allergic contact dermatitis (ACD) and AD

For the current project, the oxazolone-induced model of dermal inflammation was chosen for its purported simplicity and reproducibility (Martel et al. 2017; Man et al. 2007), and, more importantly, because it was assumed that by choosing a relatively small area of oxazolone application, a relatively low stress for the mouse can be achieved compared to models with systemic skin changes.

As described above, oxazolone is a hapten that elicits a strong immune response. If it is applied topically to the naive skin of hairless (Hr^{hr}/Hr^{hr}) mice (= sensitization), and then applied again once after a period in which the immune system can mount a specific response, i.e. approximately 7 d (= challenge), it causes an acute, mild to moderate dermatitis with features of ACD (Man et al. 2007). If this is followed by repeated challenges, i.e., 9–10 challenges over 22–25 d, the immune response shifts towards a Th2-biased, chronic dermatitis similar to AD (Man et al. 2007). The phenotypical features of this AD model are reported as [values in square brackets: phenotype after 1 challenge, i.e., in the ACD model]:

- **Clinical signs/macroscopic changes:**
  - moderate to severe **pruritus** with some scaling, scratching, and excoriation (Man et al. 2007)
- **Functional barrier alteration:**
  - transepidermal water loss (TEWL): increased (approx.10x) [unchanged] (Man et al. 2007)
  - SC hydration: decreased [unchanged] (Man et al. 2007)

  - skin surface pH: increased [unchanged] (Man et al. 2007)
- **Histology:**
  - prominent epidermal hyperplasia [milder hyperplasia] (Man et al. 2007)
  - lymphocyte-dominated inflammatory infiltrate in the dermis, with some invasion into the epidermis [modest infiltrates] (Man et al. 2007)
  - increased numbers of dermal mast cells [also increased] (Man et al. 2007)
  - increased numbers of eosinophils [only a few eosinophils present] (Man et al. 2007)
- **Further biomarkers of immune phenotype:**
  - the dermal inflammatory lymphocyte infiltrates are dominated by Th2 lymphocytes positive for the "chemoattractant receptor-homologous molecule expressed on Th2 cells" (CRTH2, also known as Prostaglandin D receptor), i.e., a marker for the Th2 cells [modest lymphocyte infiltrates which first are $CRTH2^-$ and then switch to $CRTH2^+$ and increase with the additional challenges] (Man et al. 2007)
  - increased IL4 immunostaining in the dermis [not markedly increased] (Man et al. 2007)
  - increased IgE serum levels (Man et al. 2007)
- **Further biomarkers of barrier alteration:**
  - proliferating cell nuclear antigen (PCNA) positive cells in the basal layers of the epidermis increase, corresponding to the histologic hypertrophy [already prominently increased, but slightly less] (Man et al. 2007)
  - the architecture of the SC extracellular matrix:
    - ceramides: total amount unchanged, but one of the 5 ceramide types in mice, Cer3, decreased [unchanged] (Man et al. 2007)
    - fatty acids: total amount decreased (Man et al. 2007)
    - cholesterol: increased (Man et al. 2007)
    - impaired lamellar body secretion (seen in electron microscopy; Man et al. 2007)
    - decreased number of lipid bilayers (seen in electron microscopy; Man et al. 2007)
    - premature separation of corneocytes from each other and viable epidermis (seen in electron microscopy; Man et al. 2007)
  - abnormal expression of filaggrin, loricrin, and involucrin in the epidermal layers (Man et al. 2007)
  - decreased expression of lamellar body antimicrobial peptide (CRAMP, mBD3) [already observable] (Man et al. 2007)
  - increase in epidermal serine protease activity (Man et al. 2007)
- **Response to glucocorticoid treatment:** Yes (Martel et al. 2017)

Note that this project used the full AD model to address the first question of whether CMS penetration is affected by barrier alterations in AD. For the second half of the project, however,

the primary focus lay on the CMS's effect on the delivery of TAC and the resulting efficacy, and an abbreviated protocol was used. This was deemed necessary for two main reasons: 1.) a shorter model was preferable to achieve 3 R goals; and 2.) the results from the first study showed that the primary, measurable endpoints for the efficacy (TEWL, skin hydration, and erythema) normalized significantly towards the end of the AD model (see paper 2, suppl. figure 2). Together with the interindividual variation in these values, which were much higher than expected, and the small group sizes aimed for, this meant that a measurable difference would not have been expected compared to the control groups. Thus, the model was abbreviated to end at the point of highest increase/decrease in these values, where measurable differences could reasonably be expected. However, this means that for the second part of the project, the inflammatory model may have retained some phenotypical and functional features of ACD-like inflammation. A response to TAC treatment could be expected, based on published results from a similarly abbreviated model (Ishii et al. 2013), and the short ACD-like protocols (Jung et al. 2013; Homey et al. 1998)[*]. For more details on the model induction, see the methodology sections of (papers 1 and 2).

[*]While the model is widely used in dermatologic research, reports on effects of TAC on this or other AD models seemed to be less readily available. To determine, whether oxazolone-induced murine models of inflammation could be expected to respond to TAC, a PubMed search was performed (state: 2019.01), with the search parameters ((“oxazolone”[MeSH Terms] OR “oxazolone”[All Fields]) AND (“tacrolimus”[MeSH Terms] OR “tacrolimus”[All Fields] OR “fk506”[All Fields])). Of the 14 results, 6 were excluded because they did not use oxazolone mouse models or treat with TAC. 3 used the short ACD protocol and reported an effect of topical TAC (Artym et al. 2018; Mączyński et al. 2016; Jung et al. 2013), 2 reported an effect of of oral TAC (Bavandi et al. 2006; Meingassner et al. 2003), 2 reported an effect on oxazolone-induced inflammation, but omitted a sensitization period (Homey et al. 1998; Homey et al. 1997), and one reported an effect in an AD protocol, which was however abbreviated (Ishii et al. 2013), closely resembling the AD protocol in the second half of the current project (see paper 2).

1.4. Nanocarriers

1.4.1. Nanoparticles

"**Nanoparticle**" is a somewhat vague term for, not surprisingly, particles in the nanometer size range. The WHO environmental health criteria for dermal absorption defined it as having a size of **< 100 nm in at least one dimension** (WHO 2006; European Commission, Directorate-General for Health and Food Safety 2021), but it is also frequently used for particles up to 1000 nm (e.g., Lademann et al. 2015) and often a bottom threshold of > 1 nm or other criteria are added (European Commission, Directorate-General for Health and Consumers 2010; Sayes and Warheit 2009; Buzea et al. 2007). The term is often used for substances that are otherwise normally found in larger (or smaller) sizes, and it makes a certain sense to use it in such cases. For such substances, varying the size of a particle in this range can create surprising effects. Typically quoted examples are the physical effects of extreme surface-to-volume ratios and the emerging influence of quantum effects, e.g., observed for certain metal particles. Naturally, when talking about biological systems it is immediately clear that such size matters, as this is simply the scale at which most biology happens anyway. In fact, pretty much any interesting molecule (including proteins or nuclear acids) and many complex structures (e.g., viruses) could be included under the definition[*]. Therefore, it must be remembered that for some specific purposes, it is useful to lump substances under "nanoparticle" purely by their size, but often it probably is not (e.g., see European Commission, Directorate-General for Health and Consumers 2010).

1.4.2. Nanocarriers for drug delivery

"**Nanocarrier**" is a term for nanoparticles used for delivering other molecules (cargo). The concept has recently become familiar to many people in the form of the widely employed and important lipid nanoparticle-delivered mRNA vaccines during the SarsCoV2 pandemic (Guerrini et al. 2022). The underlying problem that is spurring the development of nanocarriers is various typical challenges in drug delivery. For example, many drugs have suboptimal solubility (in vehicles, blood, or other biological liquids), suboptimal penetration through different biological barriers, suboptimal uptake by cells, low retention times in circulation or elsewhere, or the need to be protected from metabolization and excretion, and a need for more

[*] For scale: A typical IgG Antibody is roughly 10 nm (Reth 2013) at 15 000 Da (Murphy and Weaver 2016). In the skin, a typical repeating lamellar unit of the extracellular SC matrix is roughly 13 nm (Boncheva 2014) and there seem to be only one to a few of these between neighboring corneocytes.

specific targeting to the required region of action (Lukowiak et al. 2015). Various nanocarriers are explored to solve these problems through very different properties.

1.4.3. Dendritic core-multishell-nanocarriers (CMS)

One particularly promising type of nanocarrier is dendritic, polymeric core-multishell nanocarriers (CMS)[*]. They are a family of related molecules with a compelling design: they mimic the structure of micelles or liposomes, i.e., structures that are widely involved in the transportation of a wide variety of substances in all cellular life forms. They are created to act as **universal carriers**, to transport both hydrophobic as well as hydrophilic molecules, both for hydrophilic and hydrophobic biological environments and drug formulations. Like their natural counterparts, they contain relatively long amphiphilic chains with non-polar and polar regions. These regions create different domains which are approximately arranged in concentric "shells," hence the name. However, in contrast to their natural counterpart, these chains are covalently linked to a central core. They thus form **"unimolecular micelles or liposomes"** (Kurniasih et al. 2015; Lukowiak et al. 2015). A main advantage aimed for with this is standardization and stability: They do not fall apart due to dilution below their critical micellar concentration or shear forces, a risk their non-covalently assembled natural counterparts are subject to (Lukowiak et al. 2015).

By modifying the central core, the components of the shells/amphiphilic side chains, and the type of bonds between them, a wide variety of CMS can be created (Lukowiak et al. 2015). In the most common configuration, the core is hydrophilic, followed by a lipophilic inner and a hydrophilic outer shell. Cargo molecules can be taken up by the different shells, depending on their polarity. However, the overall steric arrangement may altogether be somewhat more complicated in at least two ways:

a. The polar and non-polar regions may not be strictly distributed in concentric shells. Instead, they may form smaller, more randomly distributed domains due to the flexibility of the structure. It has been suggested that they may be more accurately termed **"multi-domain" nanocarriers** (Rabe et al. 2014). In non-polar solvents, their outer polar regions have actually been reported to partially fold inward, exposing the polar domains (Rabe et al. 2014). This is somewhat similar to "regular" micelles reversing their polarity and may explain their solvability in non-polar environments (Rabe et al. 2014).
b. The carriers can form **supramolecular aggregates** depending on the cargo and environment. Individual carriers (unimers) are roughly in the size range of approximately

[*] Not to be confused with a wide variety of totally unrelated, non-polymeric and often metal based "core-multishell nanocarrier" structures, e.g., see (Singh and Bhateria 2021).

16 nm (and a mass of 70 000 u; Alnasif et al. 2014), depending on the exact architecture and method of measurement. The supramolecular aggregates can be comparatively small (< 30 nm; Radowski et al. 2007) or large (at least 240 nm; Alnasif et al. 2014). In addition to their larger size, these aggregates create additional environments in between the individual carrier molecules, which may participate in cargo encapsulation.

1.4.4. Dendritic core-multishell nanocarriers for topical dermal delivery

Interestingly, CMS were reported to exhibit two additional properties that make them interesting for topical dermal delivery:

1. Somewhat surprisingly, they not only seem to function well as vehicles for topical delivery, they were actually always reported to ***enhance* penetration of cargo substances into the skin** in *ex vivo* studies (see table 2; Frombach et al. 2019; Du 2018; Saeidpour et al. 2017; Du et al. 2016; Hönzke et al. 2016b; Yamamoto et al. 2016; Do et al. 2014; Haag et al. 2011; Küchler et al. 2009b; Küchler et al. 2009a).
2. The CMS themselves seem to penetrate into the SC. However, as expected due to their size, they **generally do not seem to penetrate the viable layers of the skin** in large amounts. This means that they may not interact much with living cells, neither in the skin nor with other organs after potentially entering the systemic circulation. This means they are expected to potentially cause fewer unwanted effects than other penetration enhancers, like classical, small molecular solvents. However, there is conflicting evidence that shows that they may always penetrate – at least in small quantities – or show increased penetration under some conditions (see sections 1.4.5 and 3.2.2, and table 3 for details; Graff et al. 2022; Dommisch et al. 2021; Rajes et al. 2021; Brodwolf et al. 2020; Rajes et al. 2020; Frombach et al. 2019; Du 2018; Löwenau et al. 2017; Du et al. 2016; Alnasif et al. 2014).

Of course, it is important to remember that CMS may differ widely in their architecture, and thus these and other properties cannot be assumed to generally be transferable to the whole class. The two particularly promising architectures that were explored in this project are further described in the next two sections.

1.4.5. hPG-amid-C18-mPEG CMS ($_{C18}$CMS)

$_{C18}$CMS are the “prototypical” CMS and were most extensively characterized at the beginning of this project (Pischon et al. 2017; Radbruch et al. 2017; Unbehauen et al. 2017; Hönzke et al. 2016b; Yamamoto et al. 2016; Alnasif et al. 2014; Boreham et al. 2014; Do et al. 2014). They possess a hydrophilic **hyperbranched polyglycerol (hPG) core**. To this, side chains

are attached via **amide bonds**. The side chains are di-block copolymers consisting of a **$C_{18}H_{36}$ alkane**, forming the inner, lipophilic shell, and **monomethyl polyethylene glycol (mPEG)**, forming the outer, hydrophilic chain (see paper 1). Their unimer size is reported as **approx. 12 nm** (7 to 25 nm, dependent on conditions of measurement; Radbruch et al. 2017; Pischon et al. 2017; Hönzke et al. 2016b; Do et al. 2014; Alnasif et al. 2014; Boreham et al. 2014; Unbehauen et al. 2017).

$_{C18}$CMS are reported to increase penetration of **Dexamethasone (DXM)** (Frombach et al. 2019; Hönzke et al. 2016b) and the model cargo **Nile red** (Hönzke et al. 2016b) compared to delivery by cream vehicles in ***ex vivo* models using human skin**. They are also reported to increase the penetration of the loaded model cargos Nile red (Küchler et al. 2009a), **Rhodamine B** (Küchler et al. 2009b), and **PCA** (Haag et al. 2011) in ***ex vivo* models using pig skin**. When comparing this effect to solid lipid nanoparticles, CMS were reported to have a larger penetration enhancement (Küchler et al. 2009a). It has even been concluded that they increase the penetration of **small cell-penetrating peptides** into human skin *ex vivo* (but **not larger peptides,** likely independent of loading, and conclusions were indirectly drawn, see Do et al. 2014).

However, when exploring the efficaciousness of **opioids** delivered by these CMS in wound *ex vivo* models, they were actually found to reduce the effects of the drugs (in contrast to solid lipid nanoparticles, which increased them; Wolf et al. 2009).

The carrier molecules themselves generally seem to penetrate into the SC. They did not penetrate into the deeper, viable skin in ***ex vivo* models of healthy human skin** and ***in vitro* models of reconstructed human skin** (Hönzke 2018; Alnasif et al. 2014) when incubation times of 6 h and 3 h respectively were used.

However, there is evidence that they may penetrate under certain conditions, including longer incubation times of 24 h and 6 h respectively (Alnasif et al. 2014), application on an ***in vitro* model of non-melanoma skin cancer** even for only 3 h (Alnasif et al. 2014), and after removing major parts of the SC by **tape stripping** (Alnasif et al. 2014). On the contrary, no increased penetration was observed in an ***in vitro* model of generalized peeling skin disease**, a keratinization disorder with orthokeratotic hyperkeratosis and increased corneocyte detachment from the granular layer (Alnasif et al. 2014). Some newer evidence was interpreted to show that they can be taken up by Langerhans cells in the epidermis (Frombach et al. 2019). Note that the latter study took place after the study described in this dissertation.

The carriers had been interpreted to display satisfactory biocompatibility in *ex vivo* tests (Wolf et al. 2009). However, see section 3.3 of the discussion on conflicting reports (Hönzke et al. 2016b) and questions about interaction with the immune system.

These $_{C18}$CMS were selected to be used as the model nanocarrier for the first study of this dissertation (see paper 1), as well as its sister project on psoriasis (Pischon 2018; Pischon et al. 2017). Concurrently to the studies in this dissertation, in the sister project, $_{C18}$CMS were similarly examined *in vivo,* in an **imiquimod-induced mouse model of psoriasis**. No penetration into viable layers of the skin in intact, as well as barrier- altered inflamed mouse skin, was shown. No systemic distribution was observed following topical application and no adverse effects of the NC were observed locally in the skin or systemically. When examining their effect on the penetration of loaded **Nile red** in **healthy murine skin** *in vivo*, they did seem to enhance Nile red penetration into the viable epidermis, but not into the dermis (Pischon 2018; Pischon et al. 2017).

1.4.6. Biodegradable hPG-PCL$_{1.1K}$-mPEG$_{2k}$ CMS (bCMS)

A more recent development is the "biodegradable" hPG-PCL$_{1.1K}$-mPEG$_{2k}$-CMS (hereafter referred to as bCMS). Like their predecessors, bCMS utilize hyperbranched polyglycerol (hPG) as their core and mPEG as their outer shell. However, Du et al. succeeded in replacing the inner alkane-based shell with **polycaprolactone (PCL**; Du 2018; Du et al. 2016). PCL is FDA-approved for various biodegradable medical implants, drug-delivery devices, and sutures (Malikmammadov et al. 2018). The PCL backbone consists of ester bonds, which can be enzymatically **cleaved by lipases and other esterases**, effectively disassembling the particle. There is esterase activity in the epidermis (Pyo and Maibach 2019; Beisson et al. 2001; Menon et al. 1986) as well as throughout the mammalian body (Satoh and Hosokawa 1998). This was assumed to decrease the risk that bCMS particles could accumulate and cause adverse reactions, even if they should enter into the viable skin or bloodstream. At the same time, PCL provides a lipophilic nano-environment similar to octanol suitable for carrying a variety of lipophilic cargo (Schwarzl et al. 2017). Of note, the enzymatic degradation reaction is too slow to likely have a relevant influence on drug release during topical application (Du et al. 2016).

bCMS were reported to increase the penetration of the model cargo **Nile red** into **human skin *ex vivo*** (Du 2018; Du et al. 2016). More importantly, it also was shown to increase the active drug **TAC** in the viable epidermis and dermis compared to the commercially available gold standard ointment (Du 2018). The carriers themselves were not observed in layers deeper than the SC (Du 2018; Du et al. 2016), even after removing parts of the SC by tape stripping (Du 2018).

bCMS were not available at the beginning of the project, but were chosen for the second study of the current project once they became available (see paper 2).

1.5. Scientific questions

First part of the project:

- If applied topically to the skin *in vivo*, do indocarbocyanine (ICC)-tagged $_{C18}$CMS penetrate into the stratum corneum (SC), the viable epidermis, the dermis, or even permeate into the subcutis and systemic circulation?
- Is this penetration behavior affected by the altered skin barrier of an oxazolone-induced murine model of human AD?
- In case $_{C18}$CMS *would* fully permeate through the skin barrier (as it could be caused by extreme barrier alteration or cuts; mimicked by subcutaneous injection), in which organs would $_{C18}$CMS be deposited?
- Do $_{C18}$CMS cause adverse effects *in vivo*, locally, or systemically, under the conditions of these studies?

Second part of the project:

- Do bCMS exhibit the same penetration behavior into oxazolone-altered skin, as found for $_{C18}$CMS?
- Does loading of the active drug TAC to bCMS increase its penetration into the viable skin, compared to delivery via the gold standard ointment formulation, when applied topically to oxazolone-altered skin?
- Does such delivery of TAC in bCMS alter the drug's penetration into the systemic circulation?
- Does such delivery of TAC in bCMS increase the efficacy of topical TAC treatment of the oxazolone-induced inflammation?
- Do bCMS cause any adverse effects under the conditions of this study?

2. Own research publications in scientific journals

2.1. Dendritic Core-Multishell Nanocarriers in Murine Models of Healthy and Atopic Skin

Authors: Radbruch M, Pischon H, Ostrowski A, Volz P, Brodwolf R, Neumann F, Unbehauen M, Kleuser B, Haag R, Ma N, Alexiev U, Mundhenk L, Gruber AD.

Year: 2017

Journal: Nanoscale Research Letters

DOI: 10.1186/s11671-017-1835-0.

Bibliographic Source: *Radbruch M, Pischon H, Ostrowski A, Volz P, Brodwolf R, Neumann F, Unbehauen M, Kleuser B, Haag R, Ma N, Alexiev U, Mundhenk L, Gruber AD. Dendritic Core-Multishell Nanocarriers in Murine Models of Healthy and Atopic Skin. Nanoscale Res Lett., 2017, 12: article 64, doi: 10.1186/s11671-017-1835-0, PMID: 28116609, PMCID: PMC5256633.*

Declaration of own portion of work in this research publication:

Contributions of M Radbruch:

1. Preparation, performance, and evaluation of investigations.
2. Subsequent setup of the entire manuscript (with the exception of help with methods for particle synthesis, characterization, and *in vitro* toxicological investigations as well as fluorescence lifetime imaging microscopy).

Contributions of the co-authors: All co-authors participated in the development of the study design, evaluation of the experimental results, and the setup and review of the manuscript. H Pischon and L Mundhenk, furthermore, assisted with the performance of *in vivo* experiments. M Unbehauen prepared, conducted, and evaluated NC preparation and characterization. F Neumann prepared, conducted, and evaluated the *in vitro* toxicological analysis. P Volz prepared, conducted, and evaluated the fluorescence lifetime imaging microscopy. A D Gruber and L Mundhenk conceived the overall project and decided on the use of *in vivo* studies to investigate the scientific questions.

Declaration on ethics:

All animal procedures were approved by the Ethics Committee of the local governmental authorities (Landesamt für Gesundheit und Soziales Berlin, approval ID G 0126/13) and were conducted in strict accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations for the care and use of laboratory animals (Guillen 2012).

2.2. Biodegradable core-multishell nanocarrier: Topical tacrolimus delivery for treatment of dermatitis

Authors: Radbruch M, Pischon H, Du F, Haag R, Schumacher F, Kleuser B, Mundhenk L, Gruber AD.

Year: 2022

Journal: Journal of Controlled Release

DOI: 10.1016/j.jconrel.2022.07.025

Bibliographic Source: *Radbruch M, Pischon H, Du F, Haag R, Schumacher F, Kleuser B, Mundhenk L, Gruber AD. Biodegradable core-multishell nanocarrier: Topical tacrolimus delivery for treatment of dermatitis. J Control Release., 2022, 349:917-928, doi: 10.1016/j.jconrel.2022.07.025, PMID: 35905785.*

Declaration of own portion of work in this research publication:

Contributions of M Radbruch:

1. Preparation, performance, and evaluation of investigations, histology, morphometry as well as TAC extraction from skin layers for the quantification of TAC.

2. Subsequent preparation of the entire manuscript (with the exception of the information on TAC quantification by liquid chromatography tandem-mass spectrometry).

Contributions of the other authors: All co-authors participated in the development of the study design, evaluation of the experimental results, and the setup and review of the manuscript. H Pischon and L Mundhenk, furthermore, assisted with the performance of *in vivo* experiments. F Schumacher prepared, conducted, and evaluated the quantification of TAC by liquid chromatography tandem-mass spectrometry (LC-MS/MS). A D Gruber and L Mundhenk conceived the overall project and decided on the use of *in vivo* studies to investigate the scientific questions.

Declaration on ethics:

All animal procedures were approved by the Ethics Committee of the local governmental authorities (Landesamt für Gesundheit und Soziales Berlin, approval ID G 0126/13 and G 0038/15) and were conducted in strict accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations for the care and use of laboratory animals (Guillen 2012).

3. Concluding discussion and outlook

3.1. Cargo delivery by CMS – do CMS enhance drug penetration and efficacy?

3.1.1. Brief summary of results

Does loading of the active drug TAC to bCMS increase its penetration into the viable skin, compared to delivery via the gold standard ointment formulation, when applied topically to oxazolone-altered skin?

In contrast to the project's hypothesis, no evidence was found that bCMS increase the penetration of TAC into the skin. (There even seemed to be a trend for lower penetration compared to ointment, although this was non-significant when applying statistical evaluation compensating for multiple comparisons, and thus cannot be directly interpreted at the power of the study; paper 2).

Does such delivery of TAC in bCMS alter the drug's penetration into the systemic circulation?

Delivery in bCMS did lead to significantly lower TAC concentrations measured in the systemic circulation, which could suggest a lower flux of TAC into the viable skin when delivered by bCMS (paper 2).

Does such delivery of TAC in bCMS increase the efficacy of topical TAC treatment of the oxazolone-induced inflammation?

In contrast to the project's initial hypothesis – but in accordance with the observed lack of penetration enhancement – bCMS delivery did not result in increased efficacy of TAC. (Efficacy was not significantly different than the gold standard cream formulation; paper 2).

3.1.2. Comparison to additional work and the literature

3.1.2.1. Comparison to bCMS delivery of TAC and Nile red under ex vivo conditions

The most important observation of this project was that the penetration enhancement that was reported in *in vitro* and *ex vivo* models could not be confirmed under the *in vivo* conditions studied here (paper 2, figures 3 and 4).

Du et al. reported penetration enhancement of bCMS for TAC (Du 2018) and Nile red (Du et al. 2016). They used excised healthy human skin. For TAC, they compared bCMS delivery to the commercially available, gold standard ointment formulation. To do so, they performed

horizontal sectioning of frozen skin samples to approximately distinguish skin layers, extracted TAC from tissue homogenates, and measured TAC by mass spectrometry (MS). They found an approximately 6-fold increase of TAC in the SC and epidermis, as well as a trend toward an increase in the dermis after an incubation time of 24 h. The effect was further increased after 48 h (Du 2018). For Nile red, they compared bCMS delivery to an in-house cream formulation and reported a significant, 3- to 4-fold increase of Nile red fluorescence signal in the SC, a 4- to 7-fold increase in the viable epidermis, and a trend of increased signal in the dermis (Du et al. 2016).

The difference to the current results (paper 2) is particularly important, as this is only the second study to measure topical penetration enhancement for any type of polymeric CMS under *in vivo* conditions (the first is described in section 3.1.2.2 below), and the first to measure the resulting treatment efficacy and blood concentrations. This means that our findings caution that there could be a systematic difference in penetration enhancement *in vivo* and *in vitro*. However, the *in vitro*/*ex vivo* systems used by our colleagues are generally considered suitable to investigate skin penetration, have been widely studied, and may actually have a number of advantages compared to the models used here (e.g., see WHO 2006; OECD 2004). Thus, it seems likely that the difference can primarily be explained by differences in the protocols used. For future studies, it will be important to scrutinize these differences, so that either our results can be dismissed, or, if they cannot be dismissed, the *ex vivo*/*in vitro* protocols can be refined. There are multiple potential explanations for the difference to the current results, the most important of which are discussed below.

3.1.2.2. Comparison to $_{C18}$CMS delivery of Nile red in vivo (parallel study)

Supporting the hypothesis that lack of TAC penetration enhancement is not an absolute consequence of the differences between the *in vivo* and *in vitro*/*ex vivo* situation is the following: The current penetration results for TAC in bCMS are also somewhat in conflict with the only other *in vivo* penetration study on topical CMS delivery, which was performed by the same team during the first part of the project, with an almost identical treatment protocol (Pischon et al. 2017). That study used the prototypical $_{C18}$CMS architecture instead of bCMS, the cargo was the model substance Nile red instead of TAC, the skin model was a depilated skin area of healthy Balb/c mice instead of an SKH1-based inflammatory model, and the cargo was detected by semiquantitative fluorescence microscopy on cryosectioned tissue slides instead of mass spectrometry. Doing so, a significantly higher cargo signal could be observed with Nile red delivered in the aqueous $_{C18}$CMS formulation, compared to Nile red delivered in the control cream formulation. The difference was only observable in the epidermis, and not the SC or the dermis. However, the method was semi-quantitative and did not allow a

meaningful determination of how large and how relevant the difference in apparent penetration was. Subjectively, the effect seemed less pronounced than what was observed in the literature (see below). In addition, there was a relatively large interindividual- and inter-sample variation. The difference in results is not completely clear, however, the simplest explanation may be the difference in cargo. Nile red and TAC both have high lipophilicity, which is considered a prime determinant for skin penetration. Their respective logP values for octanol/water are approximately 5 for Nile red (Küchler et al. 2009b), although much lower values like 3.8 (NCBI PubChem 2020) are also frequently reported in the literature, and approximately 4.63 for TAC (Undre 2004), although interestingly here, too, values from at least 2.74 (Lauerma et al. 1997) to 5.28 (Du 2018) are reported. However, the Nile red molecule is substantially smaller than TAC at 318.4 Da (NCBI PubChem 2020) compared to 804 Da, or rather 822 Da in the common hydrated form (Wallemacq and Reding 1993). The size of TAC is considered on the upper threshold for regular skin penetration (Bos and Meinardi 2000), and this may have had an influence on penetration enhancement by CMS.

3.1.2.3. Comparison to TAC delivery by thermo-responsive nanogels (parallel study)

The current results are, however, in accordance with another small study run in parallel to the studies reported here, which used the same treatment conditions, the same model, and the same detection method, but a completely different nanocarrier architecture: thermoresponsive nanogels (unpublished data). Like CMS, they had been observed to enhance the penetration of model cargo under *in vitro* conditions. As for CMS, we were not able to confirm this penetration enhancement under the *in vivo* conditions used (n = 3 only). To some degree, this supports an assumption that the treatment conditions *in vivo* diminished the observable penetration enhancement effect of CMS.

3.1.2.4. Comparison to penetration enhancement in other in vitro/ex vivo studies

Including the studies of this project, there are at least 22 published studies examining cargo delivery by various CMS architectures and under various conditions. Table 2 gives a high-level overview. Studies that examined the penetration enhancement effect almost unanimously found one. There are a number of plausible explanations for the difference from the current results. The ones we deemed particularly important were discussed in paper 2. They broadly fall into three categories, 1.) potential shortcomings of our study, which are discussed in the limitations section below, 2.) differences intrinsic to the model, i.e., the inflammatory model, species differences in the skin barrier, and the physiologic state of the skin (see paper 2), and 3.) potential “limitations” of the *in vitro*/*ex vivo* studies, summarized here:

- **Long incubation times:** *In vitro*/*ex vivo* studies generally used longer incubation times (3 to 48 h, see table 2), compared to 1h (with repeated application) in the current study (paper 2) and its parallel study (Pischon et al. 2017). It seems plausible that this could unrealistically increase penetration by itself. However, such incubation times seem to be well established in these models and are taken into consideration when interpreting the results. The significance of the long incubation times may lie in combination with the next point.
- **Occlusion while comparing aqueous to lipophilic formulations:** In the current project, relatively realistic treatment conditions were used, which included unoccluded application (paper 2, Pischon et al 2017). This meant that the water-based, and in the case of bCMS hydroxyethyl cellulose (HEC) thickened, CMS formulations dried rapidly. After less than 10 min, the water content had generally evaporated, leaving a small, dry remnant. It seems plausible that under such conditions, CMS may have become less mobile in the collapsing hydrogel formulation, CMS may have altered their steric configuration to be less favorable for TAC release, and/or the hydrophobic TAC may have crystalized due to its increasing concentration in the solution, all of which could have decreased the transition from the formulation to the skin once dry[*].

  However, we suspect that in most or even all previous *in vitro*/*ex vivo* studies on CMS, occluded conditions were used, which may have completely prevented the drying of CMS formulations, or at least relevantly delayed it. One study using the Franz cell setup reported covering the donor chamber (Küchler et al. 2009a). Unfortunately, the status of occluded vs. non-occluded is not reported in the remaining studies. However, in personal communication, we confirmed at least for some studies that the Franz cell donor chambers were also covered as part of the standard protocol (e.g., in Hönzke et al. 2016b). For non-Franz cell setups, it seems that incubators with a humidity of up to 100% were used, which we speculate could have a similar effect. It seems plausible that this may have dramatically prolonged the period in which CMS and/or cargo could have transitioned from the liquid formulation into the SC. This, in turn , may have artificially increased the effectivity of CMS delivery *ex vivo*.

  It must be noted that the situation could be more complex than “just” a potential decrease in transition after drying. This is because: a.) occlusion can generally increase skin penetration of substances, b.) occlusion can cause overhydration, c.) leaving aqueous

[*]Note that drying of a topical drug formulation does not necessarily have to *de*crease penetration. On the contrary, for some substances drying of very volatile vehicle formulations like ethanol has been described as a potential mechanism for penetration enhancement. The assumption is that drying rapidly increases the concentration of the substance in the formulation, supersaturates it, and thus “pushes” the substance from the formulation into the skin (Williams and Barry 2012). However, such an effect would not explain our observations.

substances on the skin for a prolonged time can also cause overhydration, d.) overhydration can cause penetration enhancement, and e.) ointment formulations can be occlusive and can thus also cause some amount of overhydration (Sheshala et al. 2019; Mojumdar et al. 2017; Hafeez and Maibach 2014; Selzer et al. 2013; Samaras et al. 2012; Williams and Barry 2012; Björklund et al. 2010; WHO 2006; OECD 2004; Warner et al. 2003; Zhai and Maibach 2002; Zhai and Maibach 2001; Spears et al. 1999).

Three different aspects must be considered here: I. The direct effect of occluding the donor chamber or high environmental humidity in *ex vivo/in vitro* setups (in combination with potential diffusion of water from the acceptor compartment in Franz Cell setups into the skin) may have been more or less the same for the aqueous CMS group and ointment control groups. II. The additional occlusive effect of the ointment should in fact have had a larger relative influence in the current study and could have created a bias towards the cream group (see limitations, and figure 1). III. However, a potential additional direct effect of leaving a water-based formulation on the skin for a prolonged time only exists in the aqueous CMS group, and only if the water does not evaporate. This could have induced a bias towards CMS *ex vivo/in vitro*. Note that previous *ex vivo* studies on the penetration enhancement effect of CMS aimed to use a "finite dose approach." This means that only a relatively small film of the water-based formulation was available on the skin. This raises the question of whether such a small amount of water could relevantly overhydrate the SC. The author was not able to find direct data on the amount of water necessary to cause overhydration. However, as a very rough Fermi estimate, severe soaking seems to increase the SC's thickness up to 4-fold (Warner et al. 2003). Assuming a thickness of 30 µm, and that the complete additional volume is made up of water, this would mean that taking up roughly 0.012 µl water/cm^2 would constitute a relevant overhydration of the SC. Previous *ex vivo* studies used approx. 14 µl and 20 µl of aqueous CMS formulation/cm^2 (Du 2018; Du et al. 2016). This would seem enough to fully overhydrate the SC, even if the guesstimate of the required 0.012 µl/cm^2 is too low by a factor of 100. Thus, it seems at least plausible that this mechanism could have contributed. In fact, we seem to have found ultrastructural evidence of overhydration of the SC under similar *ex vivo* conditions, using x-ray microscopy (Yamamoto et al. 2019).

Next, it is difficult to predict the effect that ultrastructural SC changes induced by overhydration may have on the interaction of CMS with the SC. There is preliminary evidence that CMS may alter/interact with the SC lipid confirmation (Du 2018). This is particularly plausible, as the "arms" of the carriers approximately resemble amphiphilic lipid structures found in lipid membranes (Hönzke et al. 2016b). As we have speculated in paper 1, it seems plausible that the particles thus intercalate with the SC extracellular lipid lamellae. Consequently, it also seems theoretically possible that the penetration effect of

CMS may be synergistic with, or co-dependent on overhydration, as described for other penetration enhancers (Pham et al. 2016).

Overall, this means that occlusion could have been a factor causing bias towards CMS penetration enhancement *in vitro/ex vivo*. It also means that multiple simultaneous effects must be weighed and that the treatment regime used in the current study may have given the ointment formulation an unfair advantage (see limitations in section 3.1.3). Future experiments that compare aqueous, or more broadly volatile formulations with less volatile and potentially occluding formulations, may have to pay more attention to whether a treatment protocol could give unequal weight to any of these factors. If so, the protocol may have to be adjusted to what is expected under real-life conditions.

- **Single vs. repeated dosing and potential accumulation:** Reviewing the literature, it seems that if TAC is applied repeatedly, twice daily, its skin concentration rises until it quickly reaches a steady-state concentration. This concentration is still present 24 hours after the last application and then decreases (Undre et al. 2009; EMA Committee for Medicinal Products for Human Use 2006). Blood concentrations seem relevantly increased for longer than 24 hours as well, even though these publications point out that this is not an accumulation. The profile along the skin seems to indicate that during these “steady state” conditions, TAC is not found in the SC only, but in the SC, the epidermis, and the superficial dermis (Undre et al. 2009). Under such conditions, it is possible to speculate that TAC concentrations in the skin predominantly start to depend on TAC’s free energy profile in the viable epidermis, dermis, dermal capillary network, and circulatory sink, instead of its penetration through the SC. In other words, the “desire of TAC to leave the viable skin again, once it is there.” The importance of not underestimating the free energy and diffusivity profiles of these non-SC structures for topical treatment (Selzer et al. 2017) has been demonstrated, among others, by our colleagues with the example of dexamethasone (Schulz et al. 2017). However, CMS generally seem to penetrate into the SC, but not into the viable skin, at least not in very large amounts, and in healthy skin (see discussion on CMS penetration in section 3.2). In consequence, CMS may have little effect on these factors. This in turn means that under conditions of repeated dosing, they may still affect the overall flux of TAC through the SC but have less of an effect on the skin concentrations. Assuming that bCMS actually *decreased,* the flux of TAC through the SC compared to ointment in the current study (paper 2), e.g., because of the rapidly drying formulation described above, this could be an elegant explanation for why we found comparatively little difference in skin concentrations, but significantly decreased systemic concentrations.

  At least under conditions of single-dosing experiments, on the other hand, it seems to be understood that TAC penetration is predominantly rate-limited by the SC (Undre 2004).

This means that if CMS increase the flux through the SC, increased penetration would be measured under such conditions. It seems plausible that this is what was predominantly measured during *in vitro*/*ex vivo* studies.

It is important to reiterate that this possibility is purely the author's speculation at this stage. The current study (paper 2) did not allow observation of pharmacokinetic profiles over time, and so unfortunately no direct conclusions can be drawn from the data. It is merely listed here as a potential option to be excluded in future studies. Importantly, it is furthermore not an argument for the use of *in vivo* setups. Quite the contrary, the author believes that *in vitro*/*ex vivo* setups can more accurately depict the real-life situation (see suggestions for further research in section 3.4).

In addition, a slight effect of a publication bias towards penetration enhancement cannot be fully excluded, particularly since some of the methods and group sizes in the published studies only allowed for evaluations of trends. However, the conclusions drawn in the papers listed in table 2 generally seem robust given the overall data presented. Nevertheless, the author himself did notice a certain amount of pressure toward the publication of positive results/resistance against the publication of negative results, so continuing vigilance is, as always, necessary.

Table 2. Available literature on cargo delivery by CMS (Y: yes, N: no, n.e.: not examined)

CMS architecture	Study	Conditions			Conclusions that CMS enhanced … [Y/N/n.e.]		Comments, shortcomings, and potential reasons for differences from the results reported here
		Epithelial model Incl. species	**Cargo** (and control)	**Incubation** (application)	**Cargo penetration into viable skin**	**Efficacy of delivered drug**	
bCMS (hPG-PCL-mPEG)	Paper 2 (Radbruch et al. 2022)	Murine oxazolone-induced model for inflamed skin *in vivo*	**TAC** (control: standard TAC ointment)	1h (5d x 2x/d)	N	N	Suggests *decreased* penetration; unoccluded setup; measured by: LC-MS Differentiations of skin layers: tape stripping plus heat separation
	(Du 2018) (note: dissertation)	Excised human skin *ex vivo* (Franz cell)	**TAC** (control: standard TAC ointment)	24 h + 48 h	Y	n.e.	Long incubation times; differentiation of skin layers: horizontal cutting; TAC extracted by: EtOH Measured by: LC-MS
			Nile red (control: Nile red in cream)	6 h	Y	n.e.	Measured by: fluorescence microscopy
		Excised human skin *ex vivo* with severe SC alteration: 30 tape strips (Franz cell)	BSA-FITS (control: BSA-FITS PBS solution)	6 h	N	n.e.	bCMS *decreased* BSA penetration (assumption made: the complex formed increases the already large size of BSA. CMS-DEA, another architecture, increased penetration); measured by: fluorescence microscopy
	(Du et al. 2016)	Excised human skin *ex vivo* (Franz cell)	**Nile red** (control: Nile red in cream)	6h	Y	n.e.	Measured by: fluorescence microscopy
C_{18}CMS (hPG-A-C18-mPEG)	(Pischon et al. 2017)	Murine skin *in vivo*	**Nile red** (control: Nile red in cream)	1h (5d x 2x/d)	Y	n.e.	See section 3.1.2.2; unoccluded; measured by: fluorescence microscopy
	(Frombach et al. 2020)	Excised human skin *ex vivo* (incubator) Healthy vs. model for inflammatory barrier alteration, induced by serine protease	DXM (control: DXM cream)	16 h	Y	n.e.	Barrier alteration is potentially more severe than in the current project (pre-treatment includes chloroform and methanol); long incubation; 100% humidity → see discussion of occlusion above; (no statistical test reported, trend seems present)
	(Frombach et al. 2019)	Excised human skin *ex vivo* (incubator)	DXM (control: DXM cream; DXM in 10% ethanol)	4 h + 16h	Y	n.e.	Penetration already shown at 4 h; shows CMS co-localization with Langerhans cells by IHC + confocal microscopy; DXM measured by: HPLC-MS, fluorescence microscopy, ELISA; (significance only with fluorescence microscopy, but trend in all 3; there seems little difference to EtOH solution) 100% humidity → over-hydration not excluded; layer extraction for ELISA and HPLC may not have removed parts of SC
	(Saeidpour et al. 2017)	Excised porcine skin *ex vivo* (ear)	PCA-labeled DXM	4 h + 6 h	n.e. (Y)	n.e.	Measured by: EPR; differentiation of skin layers only by first 3 tape strips vs. rest of the skin → conclusion on penetration into the viable skin cannot really be derived
	(Hönzke et al. 2016b)	Excised human skin *ex vivo* (Franz cell)	DXM (control: DXM cream)	6 h + 12 h	Y	n.e.	Validated penetration study method; measured by: RP-HPLC; epidermis and SC seem not differentiated

	(In addition to $_{C18}$CMS, 3 novel ester-based ones are tested: hPG-E-12-mPEG hPG-E-15-mPEG hPG-E-18-mPEG)						(significance only after 12 h, but trend visible after 6 h);
			Nile Red (control: Nile red cream)	6 h	Y	n.e.	The effect is not significant for $_{C18}$CMS but the trend is relatively pronounced; measured by: fluorescence microscopy
		Reconstructed human skin *in vitro* with TNF-alpha-induced inflammatory barrier change	DXM (control: DXM cream)	6 h	n.e.	Y	Only performed for hPG-E-15-mPEG CMS, not $_{C18}$CMS; readout: IL8 and Il1beta; effect not significant in all doses for IL8 and none for Il1beta, but trend visible
	(Yamamoto et al. 2016)	Excised Human skin *ex vivo*	DXM (control: DXM in HEC gel)	16.66 h	Y	n.e.	Measured by: x-ray microscopy (label-free detection of DXM and CMS at resolutions near electron microscopy); note: only allows for measurement on a very small region of interest (conclusion based on trends)
	(Do et al. 2014)	Excised human skin *ex vivo*	Peptides: LMWP and its degradation products VSR (938 Da) VSRRRRRR (1502 Da) VSRRRRRR GGR, (1772 Da)	6 + 24 h	(Y)	n.e.	CMS seems to not increase penetration of the whole peptide (2457 Da), slightly decrease penetration after 6 h, and increase penetration of the following degradation fragments after 24 h: VSR (938 Da) VSRRRRRR (1502 Da) VSRRRRRRGGR (1772 Da) peptides were not loaded into CMS, but applied concomitantly, pre-incubation overnight with enzyme inhibitors in PBS → may have caused over-hydration; no statistical tests were performed it seems; not clear how skin layers were differentiated, and whether the effect is measured for whole skin or viable epidermis; (also note that LMWP does penetrate in small amounts into the skin without CMS)
	(Alnasif et al. 2014)	Excised human skin *ex vivo* (Franz cell)	**Nile red** (control: no control)	6 h + 24 h	n.e.	n.e.	Shows successful delivery of Nile red by CMS, but the focus is on the influence of different barrier alterations and skin models on the penetration of CMS and the loaded Nile red; penetration enhancement (i.e., compared to a cream or similar control) is not tested
		Excised human skin *ex vivo*, tape stripped (30 tapes)		6 h	n.e.	n.e.	
		Reconstructed human skin (commercial and validated EpiDermFt plus in-house constructs)		3h + 6 h	n.e.	n.e.	
		Non-melanoma skin cancer model		3 h	n.e.	n.e.	
		Generalized peeling skin disease model		3 h	n.e.	n.e.	
	(Haag et al. 2011)	Excised porcine skin *ex vivo* (Franz cell)	PCA (control: PCA in solution; PCA in invasomes)	0.5 h	(Y)	n.e.	Penetration seems increased by CMS in the full-thickness skin, but no enhancement is seen after removal of the first tape strip → if enhancement, then only uppermost SC. (Invasomes in contrast seemed to increase concentration in the following few superficial layers of SC); measured by: EPR

	(Küchler et al. 2009a)	Excised porcine skin *ex vivo* (Franz cell)	**Nile red** (control: Nile red in cream)	6h	Y	n.e.	Reports sealing the donor compartment with Nescofilm during incubation, i.e., occluded conditions → see discussion of occlusion above; measured by: fluorescence microscopy
	(Küchler et al. 2009b)	Excised porcine skin *ex vivo* (Franz cell)	rhodamine B (control: rhodamine B in cream; rhodamine B in solid lipid nanoparticle)	6 h	Y	n.e.	Rhodamine B is hydrophilic (logP < 1; Küchler et al. 2009b); significant penetration enhancement not only into SC, epidermis, and dermis but also significant vs. SLN in epidermis and dermis; measured by: fluorescence microscopy
hPG-E-15-mPEG	(Graff et al. 2022)	Novel reconstructed human skin *in vitro* model with AD-like barrier alteration created through Filaggrin knockout and IL4/IL13 activation	DXM (control: DXM cream)	"6 h" 2x (1x/d, approx. 48h apart)	n.e.	Y	Barrier alteration is potentially more severe than in the current project; Not clear whether the conclusion is supported by the statistical tests shown; readout: TSLP and involucrin expression; glucocorticoid receptor expression and translocation
	(Jager et al. 2018)	Excised buccal and masticatory mucosa *ex vivo* (Franz cell)	PCA labeled DXM (control: DXM in macrogol formulation)	6h	(Y)	n.e.	No differentiation into different layers is performed, i.e., SC is included; measured by: EPR
	(Hönzke et al. 2016b)	See above	See above	See above	Y	Y	See above
hPG-E-15-mPEG hPG-E-12-mPEG hPG-E-18-mPEG	(Hönzke et al. 2016b)	See above	See above	See above	Y	n.e.	See above
ccCMS osCMS1 osCMS2a osCMS2b osCMS2c (oxidation and redox-sensitive CMS; and non-sensitive CMS)	(Rajes et al. 2021)	Excised human skin *ex vivo*, healthy vs. novel model of inflammatory alteration by serine protease	mTHPP (control: mTHPP in vehicle)	24 h	n.e.	n.e.	Shows successful delivery of cargo by CMS, but only compares 5 CMS architectures and does not use a non-CMS vehicle; Barrier alteration is potentially more severe than in the current project (pre-treatment includes increasing skin penetrability for serine protease through short incubations with chloroform and methanol); long incubation; 100% humidity → see discussion of occlusion above
osCMS2b rsCMS0 (oxidation and redox-sensitive CMS)	(Rancan et al. 2021)	Excised human skin *ex vivo*, healthy vs. novel model of inflammatory alteration by serine protease co-cultured with human T cells	**rapamycin** (control: rapamycin in vehicle)	24 h	Y (osCMS2 in inflamed skin)/N (rsCMS0 in inflamed and both architectures in and healthy skin)	Y (healthy and inflamed)	**Rapamycin is similar to TAC, but even larger** (914 Da; logP = 4.3); barrier alteration is potentially more severe than in the current project (pre-treatment includes increasing skin penetrability for serine protease through short incubations with chloroform, methanol); long incubation; incubation with 95% humidity over the skin → see discussion of occlusion above; (conclusion for efficacy is based on trends only, but believable as e.g., for osCMS2, p is 0.051 and 0.059; there is a very large overlap between individuals of groups however)
	(Rajes et al. 2020)	Excised human skin *ex vivo* (cell culture medium)	mTHPP (control: mTHPP in vehicle)	24 h	n.e.	n.e.	Shows successful CMS delivery, but only compares 2 CMS architectures and does not use a non-CMS

							vehicle; long incubation; 100% humidity → see discussion of occlusion above
CMS Patent: WO2015172769A2, DendroSol®	(Böhm et al. 2021)	Cat, *in vivo*	Thiamazole (control: *oral* thiamazole)	repeated daily application for up to 120 d, without removal	n.e.	n.e.	Clinical study; shows treatment efficacy of thiamazole topically delivered with CMS, but the control is oral application, i.e., penetration enhancement was not examined (penetration enhancement is claimed in Böhm et al.2020, but there is no data supporting it)
	(Böhm et al. 2020)	Cat, *in vivo*	Thiamazole (no control)	repeated daily application, without removal	n.e.	n.e.	

Abbreviations:
n.e.: not examined, Y: yes, N: no
DXM: Dexamethasone, a prototypical corticosteroid
Rhodamine B: a hydrophilic, fluorescent model cargo
PCA: 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy, a spin label
mTHPP: meso-tetra (m-hydroxyphenyl) porphyrin (mTHPP), a fluorescent model cargo
LMWP: low molecular weight protamine, a cell-penetrating peptide
EPR: electron paramagnetic resonance spectroscopy
ELISA: enzyme-linked immunosorbent assay
LC-MS/HPLC-MS: (high pressure) liquid chromatography-mass spectrometry
RP-HPLC: reverse-phase high-pressure liquid chromatography
PBS: phosphate-buffered saline

3.1.3. Limitations of the current study for bCMS penetration enhancement

Scrutinizing the method and protocols used reveals a number of limitations of the current study (paper 1 and 2), the most important of which may explain the difference to *in vitro* results equally well or better than the points discussed above:

- **Species:** The greatest limitation is the use of murine skin (paper 1 and 2). While there is relatively rich data on skin penetration comparing rodent to human skin, data on differences in penetration enhancement effects seems scarce. Hairless mice are heavily used to investigate skin penetration, including that of TAC in AD (Jia-You et al. 2016), TAC delivered by nanocarriers (Gabriel et al. 2016; Lapteva et al. 2014; Pople and Singh 2013; Goebel et al. 2011), and also penetration enhancement effects (e.g., Fuh et al. 2019; Herman and Herman 2015; Mutalik and Udupa 2003; Sinha and Kaur 2000; Okamoto et al. 1990). However, the validity of this approach should not be taken for granted. Two good, high-level reminders for this are a.) that over 80% of potential therapeutics fail when tested in humans even after successful tests in other species (Perrin 2014), and b.) and that when comparing sets of genes between humans and mice which are highly expressed in the skin but have little expression elsewhere, only a 30% overlap was found (Gerber et al. 2014). Where penetration enhancement effects have been compared between species, it seems that the murine skin may usually *over-* rather than *under*estimate penetration enhancement effects (Chantasart and Li 2012; Ruland et al. 1994; Rigg and Barry 1990; Bond and Barry

1988). This increases our confidence, that since we found a *lack* of penetration enhancement (paper 2), this should translate to human skin *ex vivo*. However, the data interpretation may have been more difficult if a penetration enhancement effect had been found. More importantly, at least one study found that there was no consistent relationship between penetration enhancement effects on human abdominal skin and hairless mouse skin for the substance tested there, and recommended that the model should not be used (Bond and Barry 1988). Finally, it cannot be excluded that a potential penetration enhancement effect of the gold standard ointment formulation may have been increased in the murine skin to a relatively larger degree than a potential enhancement effect of CMS. In the author's opinion, this means that no further *in vivo* studies should be performed to assess the penetration enhancement effect of any type of CMS (or other nanocarrier for that matter), at least until all other potential explanations for the difference between the current results and the results obtained on human skin *ex vivo* have been excluded (via *in vitro*/*ex vivo* studies).

- **Method of application/incubation:** Potentially the second largest limitation of the current study is that there may have been some degree of systematic bias induced by the method of application/incubation, similar to what was discussed in the "occlusion" section for the *in vitro*/*ex vivo* studies, but in the opposite direction. To model realistic treatment conditions, formulations were topically applied, gently massaged into the skin, and left unoccluded for 1 h, after which the remnants were removed (paper 2). As described above (section 3.1.2.4), this resulted in the rapid drying of the aqueous, HEC gel-based bCMS solution. This was considered a good thing, as it is what would realistically be expected in real-life treatment conditions. In fact, fast drying is one of the reasons that makes aqueous solutions desirable for topical applications. However, the commercial ointment formulation of TAC is non-volatile, and after 1 h, small remnants of the control ointment formulation could still be discerned on the skin. This may not be what would realistically be expected during real-life treatment. Instead, formulations would probably either be spread to adjacent areas or otherwise removed, assuming that neither humans nor other animals generally tolerate greasy, xenobiotic substances on their skin. This means that the TAC in the ointment formulation may have had some degree of "unfair advantage" in the form of a prolonged time to transition into the SC. In addition, ointments can theoretically cause an occlusive effect, which may constitute a form of penetration enhancement of its own. While the quantities of formulations used were small and considered as a finite dose situation, it is nevertheless possible that this had some effect on the current results.
- **Lack of time-resolved pharmacokinetic profile:** A further limitation is that the TAC measurements were performed as a single snapshot in time, and no pharmacokinetic profile was available (paper 2). This is also true for the *in vitro*/*ex vivo* studies listed in table 2.

However, here TAC measurements were performed 1 h after the last application in an attempt to simulate realistic real-life treatment conditions (paper 2), while TAC and other cargos were measured 3–48 h after application in *in vitro*/*ex vivo* studies (see table 2). In addition, functional systemic circulation and metabolism make the results more complex to interpret. For example, theoretically, it cannot be ruled out that CMS increased the penetration of TAC, but the flux from the skin into the circulation, as well as the metabolization in the liver, were fast enough to negate any measurable penetration enhancement. While the data on the comparatively long blood half-life of TAC in human patients (Undre et al. 2009) gives us reason not to assume this, it is a reminder that pharmacokinetics over time are important. Together with the argument on repeated dosing and dependency on free energy and diffusivity profiles in viable skin layers which was discussed above (section 3.1.2.4), this suggests that it may be crucial to gain a better, time-resolved understanding of CMS effects. (In the author's opinion, this should be investigated using *in vivo*/*ex vivo* setups, see suggestions for further research in section 3.4).

- **TAC extraction:** Another large limitation is the lack of knowledge of the TAC extraction efficiency from tissue in the current study (paper 2). While we used very precise mass spectrometry to quantify TAC with comparatively low limits of detection and quantification, the tissue extraction step could have played a relevant role. However, since the main observation of our results is relative (CMS compared to ointment), this may not alter the conclusions drawn. Furthermore, it must be noted that the *in vivo* experiment used ethanol as an extraction medium for TAC (Du 2018), while the current study used an extraction medium consisting of 10% ethanol and 0.005 w/w% Tween 20 in phosphate-buffered saline for the tape-stripped SC, and 10% ethanol with 100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 1% Triton-x-100 for extraction from the heat separated and homogenized epidermis and dermis (paper 2). While we are not aware of an immediate reason why this could have differentially decreased TAC extraction efficacy in the epidermis and dermis of CMS-treated compared to ointment-treated skin, it cannot be excluded. However, the latter extraction medium has been used in other *ex vivo* studies of the CRC, for example when demonstrating that CMS increased DXM penetration (Frombach et al. 2020).

Finally, an interesting observation we were not able to explain was that we found a much larger concentration of TAC in the blood plasma than in erythrocytes (paper 2), even though the literature reports that a larger fraction of TAC is usually found bound to erythrocytes (EMA Committee for Medicinal Products for Human Use 2006; Venkataramanan et al. 1995).

3.1.4. Clinical and histological efficacy and limitations of the current study for evaluation of efficacy

A primary goal of the current project, and one of the main reasons that the CRC required using an *in vivo* proof of concept, was to confirm that the enhanced drug delivery would result in enhanced efficacy. The current results confirmed efficacy, but the expected increase in efficacy could not be observed (paper 2, figures 6 and 7). This is not surprising due to the lack of penetration enhancement discussed above and has been discussed in the paper. Here, only important limitations will be further discussed:

- A noteworthy, non-primary finding of the current study was the extremely large interindividual variance in the severity of the induced inflammation and, subsequently, all clinical and histological parameters measured, even before treatment. This introduced a large amount of noise (see paper 2, figures 5, 6, S4, S5, and S7). It made it necessary to use a non-standard normalization method for TEWL values. TEWL increases during model induction, and with the beginning of treatment, it falls again. However, the peak TEWL values had extreme interindividual variability (paper 2, figure S4). Since the group sizes were kept small, there was also inter-group variability in the peak (paper 2, figure 6b). To infer meaningful differences, we in effect normalized to the largest difference between healthy skin and inflamed skin for each individual. This was interpreted as the degree of individual improvement towards normal. However, we only switched to this metric *after* observing the large variability in clinical values. Doing this, of course, carries a risk of choosing a metric that confirms already held beliefs or even p-harking. However, the conclusion that TAC delivered by bCMS was effective, but not more effective than when delivered by conventional ointment, does seem justified even when looking at the raw data. The confidence in this is, of course, increased by the fact that it matches what we would expect from the TAC penetration results and that it is confirmed by the histological parameters.

  Overall, the high variability of the model does not change our interpretation, but it will have decreased the sensitivity for differences between groups.
- Note that the factors that lead to the variance are not clear. SKH1 is an outbred strain, so the null hypothesis is genetic variability. However, other factors are plausible. Particularly interesting is the fact that oxazolone-induced dermatitis seems to be dependent on the intestinal microbiome. Sensitivity to oxazolone may even be transferable between mice with the microbiome transfer (Zachariassen et al. 2017; Lundberg et al. 2012). This was neither considered nor controlled for during the study. (The study was performed under SPF conditions; this however does not control for the general microbiome).

- A second, equally important limitation concerns the question of whether an increase in efficacy compared to the standard ointment formulation could actually have been detected at the concentrations used. An additional control group showed that standard ointment containing only 0.03% TAC instead of the 0.1% TAC used for the main study already seemed to be as efficacious as the latter (paper 2, figure S9). This does not change the conclusions drawn. However, it suggests that detecting a difference in efficacy brought about by a difference in drug delivery by any of the two formulations may have required a down-titration of the dose.
- Furthermore, note that non-inferiority statistics were not calculated for treatment efficacy metrics in the current project, due to lack of meaningful non-inferiority thresholds. While the means of all efficacy metrics were observed to be equal or better in the TAC-loaded CMS group compared to the standard TAC ointment (paper 2, figures 6 and 7), it is important to remember that the statistical tests performed can only show a.) evidence for an increased efficacy compared to vehicle controls, and b.) a lack of evidence for a difference between the TAC-loaded CMS group and the standard TAC ointment, and not a lack of difference (Kishore and Mahajan 2020).
- Also note that measured parameters on erythema, SC hydration, and ear thickness were excluded from the interpretation. The same considerations about potential bias/p-harking already discussed apply (see paper 2, suppl material section 3.3.).
- A further limitation concerns the question of to what degree the utilized models represent AD. During the TAC delivery study, the induction period of the oxazolone-induced model of AD was shortened, as described in the introduction and paper 2. It is important to remember that it thus may have retained characteristics of the more acute, more Th1-biased inflammatory response found in allergic contact dermatitis (Man et al. 2007).

3.2. Local and systemic distribution of CMS – do CMS penetrate into the viable skin in intact and barrier-disrupted skin?

3.2.1. Brief summary of results

Scientific question 2: If applied topically to the skin in vivo, do indocarbocyanine ICC-tagged $_{C18}$CMS penetrate into the stratum corneum (SC), the viable epidermis, the dermis, or even permeate into the subcutis and systemic circulation?

After topical application (2x/day for 5 days) $_{C18}$CMS were found throughout the SC, but no penetration to the viable epidermis or dermis was observed (paper 1).

Is this penetration behavior affected by the altered skin barrier of an oxazolone-induced murine model of human AD?

The penetration behavior was not altered by the AD model, i.e., $_{C18}$CMS were found in the SC, but not beyond (paper 1).

In case $_{C18}$CMS would fully permeate through the skin barrier (as it could be caused by extreme barrier alterations, e.g., open wounds; mimicked by subcutaneous injection), in which organs would $_{C18}$CMS be deposited?

$_{C18}$CMS signal was found in the draining lymph nodes and lower amounts in the liver, spleen, lung, and kidney. They were not found in any other organs (paper 1).

Do bCMS exhibit the same penetration behavior into oxazolone-altered skin, as found for $_{C18}$CMS?

The penetration behavior into barrier-altered skin observed for $_{C18}$CMS could be confirmed for bCMS, i.e., bCMS were only found in the SC, and no penetration into the viable epidermis or beyond was observed (paper 2).

3.2.2. Local distribution

The fact that CMS nanocarriers did not seem to penetrate into the skin in observable amounts (papers 1 and 2) is generally in accordance with the "500 Da rule," often cited in dermatopharmacology, which suggests that molecules above approximately 500 Da should usually not be assumed to penetrate past the SC in relevant amounts (Bos and Meinardi 2000)*. We measured $_{C18}$CMS to have an average molecular mass of approximately 42 000 Da (number average molecular weight, at a weight average molecular weight of 77 000 Da; unpublished results). bCMS were measured as approximately 163 000 Da (number average molecular weight, at a weight average molecular weight of 277 000Da; paper 2). Note that the molecular mass of these polymers is not uniform across all individual molecules but exists as a mass distribution. Nevertheless, this means that it is not completely surprising that the carriers did not seem to penetrate, at least into the healthy skin.

However, the results from *in vitro* experiments on $_{C18}$CMS showed that the carriers did seem to penetrate into the viable skin in large amounts under some conditions, and particularly that penetration was increased further in models of an altered skin barrier (Alnasif et al. 2014), as outlined below. In addition, CMS particles are flexible, and there is evidence that they can flatten down to < 1nm (Alnasif et al. 2014), which seems to increase the plausibility that they could "squeeze through" extracellular space or even between strata of lipid matrix lamellae. Plus their side chains show a similar architecture to components of cellular membranes, which led us and others (Hönzke et al. 2016b) to speculate that they may intercalate with lipid components of the lipid matrix. Whatever the potential mechanism or pathway of penetration, it has been suggested that CMS have an advantage because of a "high stratum corneum penetration ability" (Rancan et al. 2021). It has in fact been interpreted during the planning stages of the CRC as a potential mechanism for the penetration enhancement effect simultaneously observed for CMS. The tentative hypothesis was, that CMS may themselves penetrate through the SC (by some mechanism not yet understood), and in doing so, they would literally "carry" loaded drugs through the SC and release them in the viable skin. What is more, there was some hope that if the carriers would preferably penetrate into barrier-compromised, inflamed skin, this could be used as a form of targeting the inflamed regions. Since our data suggest that this may not be the case, at least not for AD without large areas

* Relevant amounts mostly meaning "drug development should probably focus on molecules smaller than 500 Da if topical or transdermal applications are aimed for." However, it is partially derived from the observation that all of the most common skin allergens seem to be below this threshold (Bos and Meinardi 2000), so there is no direct reason to believe that it should not be the baseline assumption for substances like CMS as well, until proven otherwise.

of SC laceration (papers 1 and 2), as well as psoriasis (via the parallel project; Pischon et al. 2017), this justifies some more detailed discussion.

3.2.2.1. Comparison of bCMS to $_{C18}$CMS

The observation that the skin penetration pattern of bCMS (paper 2) matched that of $_{C18}$CMS (paper 1) is not particularly surprising, as both carrier systems have a large mass (as described above), size (diameter of the unimers in water: approx. 12 and 29 nm respectively), and overall architecture (dendritic CMS with mPEG outer shell and hPG core). However, it must be stressed that the mechanics of the interaction between the SC and CMS (as well as other complex molecules in general) are incompletely understood. Thus, there currently seems to be no definite way of predicting whether an architectural difference in CMS like the replacement of the C18 carbohydrate chain by PCL will have an effect on SC penetration. In other words, the information that bCMS showed the same behavior is not surprising, but neither is it redundant.

3.2.2.2. Comparison to the parallel project on the Balb/c strain

Importantly, the skin penetration pattern of $_{C18}$CMS found here generally matched the penetration behavior observed for these carriers in the working group's parallel project, which examined their penetration when on the skin of healthy Balb/c mice and a Balb/c, Imiquimod-induced model of psoriasis-like dermatitis (Pischon et al. 2017). In this study, the same treatment regime was used and $_{C18}$CMS were also almost exclusively found in the SC, with no bulk penetration to deeper layers.

It must be mentioned, however, that a very small number of solitary spots of fluorescence signal could be detected in the viable epidermis, both in the healthy skin and the inflamed Balb/c skin (Pischon et al. 2017). Due to their co-localization with CD207 positive epidermal cells, these signals were assumed to be solitary carriers, taken up by Langerhans cells, potentially sampling them directly from the SC, i.e., without the particles first penetrating into the viable epidermis (Pischon et al. 2017). The two most likely explanations why this was observed in the Balb/c models but not in the current studies seem to be as follows:

1. The sensitivity of the methods used here may not have been high enough to detect all individual carrier molecules, and the observation of a few singular events in the Balb/c in contrast to the SKH1 models may have simply been due to chance. This potential of trace amount penetrations is further discussed below. On the other hand, no method was available to confirm that the signal was indeed tagged CMS, and it is possible that it either constituted accumulations of free ICC dye tags after degradation of the particles or, even

more likely, an artifact. The latter possibility is supported to some extent by the fact that the signal was not seen in the cluster-fluorescence lifetime imaging microscopy (FLIM) evaluation.

2. In contrast to SKH1 mice, Balb/c mice have fur. Correspondingly, the epidermis of Balb/c mice was, as expected, found to be slightly thinner (see paper 2 and Pischon et al. 2017). Note that no reliable method was available to measure the true SC thickness, and the TEWL of healthy skin was in the same baseline range of approx. 10g/h/m^{2*}. Furthermore, the fur made it necessary to depilate the area of treatment 2 days before induction of the psoriasis model. It seems plausible that a slightly thinner SC, and/or small, histologically unrecognizable alteration caused by the depilation could have allowed for the penetration of small numbers of CMS molecules.

Nevertheless, the observation that the penetration behavior was generally repeatable when examining $_{C18}$CMS on the healthy skin of two different mouse strains and two different types of inflammatory barrier alteration, with regular fluorescence microscopy and additional FLIM microscopy, plus for bCMS in the inflamed skin, under conditions of realistic and repeated application, increases the confidence that CMS should probably not be expected to show bulk penetration into the viable skin under conditions similar to the ones used in these 3 studies (paper 1, paper 2, Pischon et al. 2017), and when considering the limitations of the method discussed below.

3.2.2.3. Comparison to the in vitro results from other workgroups of the CRC and the literature

Polymeric CMS generally seem to penetrate into the SC when topically applied to the skin or oral mucosa under all conditions published (Graff et al. 2022; Dommisch et al. 2021; Rajes et al. 2021; Brodwolf et al. 2020; Rajes et al. 2020; Frombach et al. 2019; Du 2018; Löwenau et al. 2017; Du et al. 2016; Alnasif et al. 2014). Concerning permeation through the SC into the viable skin, the published results are mixed, similar to the questions of penetration enhancement. Again, the differences can probably be explained by experimental differences. Table 3 gives an overview of these results. It includes penetration data obtained for other polymeric CMS to increase the number of data points†. All studies used the detection of fluorescently labeled CMS via fluorescence microscopy, except Yamamoto et al. 2016.

* This is also in the range of the TEWL of healthy human patients of approx. 5–12 g/h/m^2 (Frombach et al. 2020).
† To ensure a complete presentation of the data, a PubMed search with the search string (core-multishell [All Fields] AND “core multishell” [All Fields]) was performed (state: 2023.02.13). Of the 147 results, 96 were excluded because they did not examine polymeric core-multishell nanocarriers, and 37 were excluded because they did not contain usable data on CMS penetration, which left 14 publications examining CMS penetration into the skin or cornified oral mucosa.

Table 3. Penetration of CMS through skin and oral mucosal models, literature review

CMS architecture	**Study**	**Epithelial model**	**Incubation time** (regime in case of multiple applications)	**CMS in SC [y/n]**	**CMS in viable skin [y/n]**	**Comments and potential explanation for differences in results**
$_{C18}$**CMS** (hPG-A-C18-mPEG; the prototypical polymeric CMS)	Paper 1 (Radbruch et al. 2017)	Murine skin *in vivo*	1h (2x/d x 5d)	Y	N	Used FFPE material; used cluster-FLIM to confirm
		Murine oxazolone-induced model for AD *in vivo*	1h (2x/d x 5d)	Y	N	Used FFPE material
	Parallel project psoriasis (Pischon et al. 2017)	Murine skin *in vivo*	1h (2x/d x 5d)	Y	(Y)	Minimal oligofocal signal (see discussion); additionally used cluster-FLIM, which did not confirm minimal signal; used FFPE material
		Murine psoriasis model *in vivo*	1h (2x/d x 5d)	Y	(Y)	Minimal oligofocal signal (see above); used FFPE material
	(Alnasif et al. 2014)	Excised human skin *ex vivo*	6 h	Y	N	Used cluster-FLIM to confirm
		Excised human skin *ex vivo*	24h	Y	Y	Long incubation (plus cannot exclude effects of potential occlusion)
		Reconstructed human skin *in vitro*	3 h	Y	N	Tested both a validated commercial model plus an in-house construct
		Reconstructed human skin *in vitro*	6 h	Y	Y	Long incubation (plus cannot exclude effects of potential occlusion)
		Excised human skin *ex vivo*, tape-stripped	6 h	Y	Y	30 tape strips → SC probably mostly gone (on average 3.3±1.1µm SC left)
		In vitro model of generalized peeling skin disease	3 h	Y	N	SC thicker than normal, model for "hyperkeratotic" skin disease (note that thicker SC does not have to mean more barrier)
		Non-melanoma skin cancer	3 h and 6 h	Y	Y	SC looks very disorganized in this model
	(Brodwolf et al. 2020) (note: used cluster-FLIM to confirm)	Reconstructed human skin *in vitro* (with SC)	22 h	Y	Y	Note: this study specifically localized CMS not only in the keratinocyte cytoplasm but also in the nucleus; long incubation (plus cannot exclude effects of potential occlusion)
	(Frombach et al. 2019)	Excised human skin *ex vivo*	4–16h	Y	(Y)	Note: this study found small amounts of focal signal in the epidermis, co-localized with Langerhans cells; it furthermore observed most SC signal in hair follicles; 100% humidity
	(Yamamoto et al. 2016)	Excised human skin *ex vivo*	1.67 h and 16.67 h	Y	N	This study used x-ray microscopy for label-free, semi-quantitative detection of CMS at a subcellular resolution close to electron microscopy (disadvantage: only a very small region of interest can be observed)

	(Jager et al. 2018)	Excised porcine *oral* mucosa	6 h	Y	Y	*Oral* mucosa, not skin → SC barrier differs; only a small amount of penetration past the SC was observed
bCMS (hPG-PCL-mPEG-CMS; PCL shell for cleavability by esterases)	Paper 2 (Radbruch et al. 2022)	Murine model of AD, *in vivo*	1 h (2x/d x 5d)	Y	N	Used FFPE material
	(Du 2018; Du et al. 2016)	Excised human skin *ex vivo*	6 h	Y	N	
hPG-E-C15-mPEG (shells ester-bound to the core for cleavability by esterases)	(Dommisch et al. 2021)	*Ex vivo* porcine *oral mucosa*		Y	N	
		Reconstructed oral mucosa		Y	Y	This model seems to have no SC
	(Graff et al. 2022)	Reconstructed human skin (AD-like, Filaggrin knockout, and IL4/IL13 activation)	6 h (2x, 2d apart)	Y	N	This model simulates some changes as seen in AD
osCMS0 (redox-sensitive) ccCMS (non-sensitive)	(Rajes et al. 2020)	*Ex vivo* human skin	24 h	Y	Y	Long incubation; 100% humidity; not much signal
	(Rajes et al. 2021)	*Ex vivo* human skin model for barrier alteration	24 h	Y	Y	Long incubation at 100% humidity (SC swelling in fact described histologically here) Model likely causes severe SC integrity disruption (16 h pre-incubation with chloroform/methanol, trypsin/serine protease or DPO in acetone/isopropyl myristate); resulting signal only found in some samples
CMS, not specified (likely hPG-A-C18-mPEG)	(Löwenau et al. 2017)	Healthy reconstructed human epidermis	6 h	Y	N	SC looks overhydrated in the figure provided
		UVB radiated reconstructed human epidermis	6 h	Y	Y	The model seems to cause a severe decrease in SC thickness (and histologically looks overhydrated in the non-radiated group)

Penetration into models of healthy skin: No permeation of bCMS into the viable layers of healthy excised human skin was observed (Du 2018; Du et al. 2016), in accordance with the results of the current study (paper 2). For $_{C18}$CMS, more data is available. In one study, no penetration into the viable skin was reported after 6 h on excised human skin and 3 h on reconstructed human skin. However, when these incubation times were prolonged to 24 h and 6 h respectively, penetration was observed (Alnasif et al. 2014). A further study similarly reported penetration using reconstructed human skin with an incubation time of 22 h (Brodwolf et al. 2020). A third study reported some penetration already after 4 h to 16 h of incubation on

excised human skin (Frombach et al. 2019). In that study, our colleagues reported that almost all CMS seemed to remain in the SC. However, they found small amounts of focal signal (Frombach et al. 2019), similar to what we observed on Balb/c skin *in vivo* (Pischon et al. 2017; see section 3.2.2.2). In contrast to our study, our colleagues were able to confirm these spot signals via FLIM analysis and co-localized them not only with Langerhans cells, but also keratinocytes, and very occasionally in the dermis. To confirm these results, they performed flow cytometry after separation of the skin cells. With this method, they found a small amount of CMS signal in up to 9% of epidermal cells (although there was a high variation between the 8 donor samples used; Frombach et al. 2019). Note that that study reported 100% air humidity during the incubation. Studies on other CMS architectures also used relatively long incubation times (Dommisch et al. 2021; Rajes et al. 2021; Rajes et al. 2020). Furthermore, as discussed for cargo delivery/penetration enhancement above, it is possible that most of the studies using Franz cell setups used occluded conditions, even though this was not specifically reported. It seems plausible that under such conditions of occlusion or high environmental humidity a relevant overhydration of the SC could occur, due not only to occlusion itself but also to the prolonged presence of the water-based formulation on the skin. This in turn could cause some increase in penetrability compared to unoccluded conditions in which the water-based vehicle formulation quickly evaporates (see discussion in section 3.1.2.4).

Penetration into models of barrier alteration: Penetration of $_{C18}$CMS was found after subjecting healthy skin to 30 tape strips (Alnasif et al. 2014). Such a large number of tape strips is likely to remove almost all (or all) of the SC, which in fact can be seen in at least one of the figures of that paper. This result primarily shows that the SC seems to be the primary bottleneck for CMS penetration, as we would expect. Importantly, it also confirms the assumption that CMS would penetrate deep into the skin if they were applied to areas of total SC laceration, for example, traumatic wounds. This scenario is far from unrealistic. For example, in AD severe pruritus and scratching, as well as dry skin, can cause areas of excoriation (Ständer 2021). It is important to note that the AD model described here did *not* feature prominent gross excoriation, nor was it seen histologically (papers 1 and 2). Thus, it will underestimate this form of penetration.

Penetration of $_{C18}$CMS into the viable skin was not found in an *in vitro* model of generalized peeling skin disease, but in a model of non-melanoma skin cancer (Alnasif et al. 2014). This is probably explained by the severe alteration of skin and SC architecture in the latter model. A further study similarly found penetration of non-specified but related CMS into UV -radiated reconstructed human skin (Löwenau et al. 2017).

Important further findings from previous reports: Besides gross penetration, there are at least 3 important additional details to be learned from the studies listed above (table 2):

a. Application on human skin *ex vivo* seems to tentatively confirm our assumption that *if* CMS are found in the viable epidermis of healthy skin after relatively short incubation times, they seem to be found in Langerhans cells (Frombach et al. 2019). This could either mean 1.) that they are sampled by the Langerhans cells in the SC and then move into the viable epidermis inside of a Langerhans cell's appendage, or 2.) that they permeate the SC by themselves, and then are taken up by Langerhans cells. Both scenarios seem likely. CMS were also found to be taken up by macrophages (Du et al. 2016). Consequences of CMS uptake by Langerhans cells or other immune cells are not yet understood. However, under cell culture conditions, there seemed to be both lysosomal localization in Langerhans cells, as well as energy-dependent exocytosis of unmodified CMS. CMS uptake did not seem to cause an immune response (Edlich et al. 2018).
b. There is evidence that *if* CMS penetrate into the viable epidermis, they can also be taken up by keratinocytes (Brodwolf et al. 2020; Frombach et al. 2019), via active, probably caveolae-mediated transport (Brodwolf et al. 2020). From there, they could potentially progress to a lysosomal fate, as seen in a keratinocyte culture *in vitro*. However, somewhat more concerning, in the reconstructed human skin *in vitro* model used in this study they seemed to escape from this fate and instead were interpreted to end up not only in the cytoplasm but also in the nucleus (Brodwolf et al. 2020).
c. In the SC, CMS seem to be exclusively located in the extracellular lipid matrix between the corneocytes after short incubation times (1.6 h) when examined by x-ray microscopy. After longer incubation (16.6 h), some signal was found in corneocytes, although it is unclear whether this signal signified an intact particle or degradation product (Yamamoto et al. 2016).

3.2.3. Systemic distribution

After breaching the skin barrier (modeled by s.c. injection), $_{C18}$CMS were observed in the draining lymph nodes, and lower amounts in the liver, spleen, lung, and kidney (paper 1, figure 5). This is not surprising, as these are the main sites of particle clearance by the mononuclear phagocytic system (Murphy and Weaver 2016), and thus where they would be expected to be found. This is somewhat reassuring, as it suggests the immune system may be able to dispose of them. Moreover, $_{C18}$CMS were not found in other organs with the method used (paper 1). To our knowledge, there is no other data on the systemic distribution of CMS in case they reach the subcutaneous tissue. There is, however, evidence that related CMS could show preferential accumulation in tumor tissue (a mouse model of teratocarcinoma; Quadir et al.

2008). It could be speculated, whether they could have a potential tendency to accumulate in areas of increased vessel permeability (“enhanced permeability and retention effect”). Either way, together with the results discussed above, further knowledge is needed on their potential long-term fate once they enter the systemic circulation. In particular, it will be important to explore whether cells of the mononuclear phagocyte system, hepatocytes, or other cells sufficiently dispose of them. In fact, this should potentially be a screening test during the initial toxicologic evaluation of CMS and other particles, particularly those that can be assumed to be too large for renal filtration. It is important to stress that suitable *in vitro*/*ex vivo* models need to be found/created to do so. Since it is not easy to follow these particles in the circulation with high sensitivity, and much less so their potential metabolites, *in vivo* models will likely not provide reliable information. In addition, *in vitro* models can provide information on metabolism by cells of the actual target species envisioned (most likely human or other non-rodent species). They furthermore can be set up much broader to explore a much larger set of models, larger group sizes, a larger range of particle concentrations, and so forth.

3.2.4. Limitations and cluster FLIM results

The most important limitations of the current studies (paper 1 and 2) regarding particle penetration are a potential lack of sensitivity, a lack of quantification, and a lack of knowledge about the lower limit of detection. For the basic detection method used in this project, CMS were covalently tagged with the fluorescent dye indocarbocyanine (ICC; paper 1 and 2, Pischon et al. 2017) and penetration was subsequently manually judged by standard fluorescence microscopy. This is a standard method commonly used for the exploration of similar substances. It is probably suitable to draw conclusions about bulk penetration. However, as discussed already, it may not be suitable to draw conclusions on whether very small amounts of particles penetrate. Note that almost all studies discussed above used the same basic technique to detect CMS (see table 2), although many performed a semi-quantification of the fluorescence signal obtained (Rajes et al. 2021; Rajes et al. 2020; Frombach et al. 2019; Du et al. 2016; Alnasif et al. 2014). This is generally a great advantage compared to the studies of this work. However, it does not seem to have been subjected to statistical tests comparing the signal in a particular skin layer to skin without CMS, so the extent of this advantage is somewhat hard to judge. Alnasif et al. did however describe a lower detection limit of 10µg/ml, although the method which it was derived from was not described (Alnasif et al. 2014).

What could potentially have massively increased this limitation is the use of formalin-fixed, paraffin-embedded (FFPE) material in the current project (paper 1, paper 2, Pischon 2017). During the planning of the project, this method was chosen because it was the standard

procedure at the institute. However, it seems entirely possible that this decision significantly lowered the limit of detection, because some amount of CMS may have been solved from the tissue by the solvents used for preparing FFPE tissue sections (acetone and ethanol). In hindsight, it is clearly a relevant shortcoming that this potential effect was not excluded or quantified. Most of the other studies discussed above used cryosectioning, which does not require the use of solvents. (Sidenote: this limitation only concerns the detection of CMS in this project. For the detection of the model cargo Nile red by fluorescence microscopy, we used cryosections and semi-quantified the results, and for the detection of TAC, TAC was extracted directly from unprocessed tissue and quantified by mass spectrometry, see paper 2.)

To confirm the results obtained for $_{C18}$CMS by regular fluorescence microscopy, we used a second method (paper 1 and Pischon et al. 2017): FLIM. Standard fluorescence microscopy relies solely on the fluorescence intensity at a certain range of wavelengths (for exciting and detected light) to differentiate the tagged $_{C18}$CMS from the background. However, since there is always a small amount of background fluorescence in each range, there is an intrinsic lower limit of detection, not only due to the sensitivity of the detector or eye but simply the background noise. FLIM adds an additional dimension to the signal that differentiates it: the specific fluorescence lifetime curve. This generally makes the signal more specific, which in turn can increase the signal-to-noise ratio, which in turn potentially increases sensitivity. In addition, FLIM was performed via a confocal microscope, which ensures that the signal detected is located in a small voxel in the correct z plane. This removes the potential effect of unequal section thickness, which is a main limitation for semi-quantifying substances in conventional fluorescence microscopy. Furthermore, the fluorescence lifetime curve of the free fluorescence dye tag differs from the CMS-dye covalent construct. This increases the confidence that what is measured is not dye that had detached from the particle and diffused into the skin alone (Alnasif et al. 2014). Finally, we interpreted the signal via a novel fluorescence-lifetime clustering method introduced by our colleagues (Brodwolf et al. 2020). Our colleagues found that the new clustering method seemed to be more sensitive or specific than the standard differentiation of lifetime curves for the task at hand, and was able to differentiate 4 or 5 clusters at photon counts of 40–170 photons per pixel (Brodwolf et al. 2020). Several of the studies discussed above also utilized this technique (Brodwolf et al. 2020; Frombach et al. 2019; Löwenau et al. 2017; Alnasif et al. 2014). It is undoubtedly a great method, and can potentially even be used to derive functional knowledge of the tagged carrier's fate inside a cell (Brodwolf et al. 2020; Balke et al. 2018; Edlich et al. 2018). Nevertheless, no determination of the lower limit of detection for tagged CMS in tissue sections was performed (paper 1 and 2). In addition, the problem of potentially losing part of the CMS amount to solvents remains. This means the FLIM data gives added confidence that there was no bulk penetration and an additional

dimension of detection often not present in other publications examining nanoparticular delivery solutions, but it does not exclude penetration of small amounts either. For further studies, this shortcoming should be addressed.

Note that interestingly, one of the studies discussed above used a completely different detection dimension: differential x-ray absorption of untagged CMS via x-ray microscopy. This technique also allows for semi-quantification, and its results support our findings of no penetration into the viable skin (Yamamoto et al. 2016).

The second extremely important limitation is the use of a murine model (paper 1 and 2), which means that transferability to the healthy human or other target species' skin, as well as naturally occurring AD or psoriasis is not guaranteed, as discussed for cargo delivery penetration/enhancement.

3.2.5. Conclusion regarding CMS penetration

Going further, the discussion above shows that it will be very important to define which question has priority for planning and reporting of penetration studies:

1. If the primary question is whether CMS penetrate into the viable skin in relevant bulk amounts *to literally carry the cargo* there with them: Considering the results discussed above, the author feels that there is currently little reason to assume that this occurs in healthy skin. It also does not seem to be the case in comparatively realistic models of moderate to severe AD-like and psoriasis-like barrier alteration (without severe excoriation/ulceration). If a study is planned to further examine this possibility, it should be discussed whether *in vitro* and *ex vivo* models should be modified towards more realistic treatment and incubation conditions, to avoid overestimation of CMS penetration (as discussed for penetration enhancement above). On the other hand, cryosections should be used instead of FFPE material, or equivalency needs to be established if FFPE use is absolutely required.
2. If the primary question is whether CMS use is particularly *safe* for topical application because they *do not* penetrate in relevant amounts *to cause negative effects*: In this case, of course, it is more important to avoid underestimation of penetration. Thus, it may be better to stick to the long penetration times used in most of the studies above, potentially even with infinite or near-infinite dose and occlusive conditions, which are important for safety pharmacokinetics. It will also be particularly important to a.) better define the detection limits of the methods used, b.) better define the barrier alterations expected, and c.) further explore the fate of the particles, their interaction with the immune system, their

degradation by extracellular tissue enzymes, and their intercellular fate in *in vivo* models of complete SC loss.

The latter focus would of course also be important if the primary question is whether CMS have a potential advantage for topical delivery of highly lipophilic drugs in wound gel formulations or for delivering cargo to Langerhans cells. (For further discussion, see the following section on biocompatibility.)

3.3. Biocompatibility *in vivo* – effects of $_{C18}$CMS and bCMS on healthy and inflamed tissue

Do CMS cause adverse effects in vivo, locally, or systemically, under the conditions of these studies?

As expected, no histopathologic or clinical adverse effects were observed after topical application of $_{C18}$CMS on healthy skin and oxazolone-induced dermatitis (paper 1), as well as bCMS on oxazolone-induced dermatitis (paper 2). Inflammation did not seem to have been increased by the particles. Furthermore, no adverse effects were detected after subcutaneous injection of the full dose which would otherwise be applied topically.

This had been hypothesized, as both particles' architectures had been subjected to *in vitro* tests for toxicity as parts of previous studies, which concluded that there was sufficient biocompatibility (Du et al. 2016; Wolf et al. 2009). In addition, as part of the current studies, we performed further *in vitro* toxicity tests before the studies commenced. For $_{C18}$CMS, these included tests for cell viability (Cell Counting Kit-8 assay), cytotoxicity (bis-AAF-R110 assay), apoptotic activity (caspase 3/7 assay), and oxidative stress (H2-DCF-DA) on cultures of human immortalized keratinocytes (HaCaT), with exposition doses of 50, 100, 200, 500, and 700 µg/ml $_{C18}$CMS (paper 1). For bCMS this comprised an MTT assay, also performed on HaCaT cells, with a dose of 500 µg/ml (paper 2). For bCMS, a bovine corneal opacity and permeability test (BCOP) was also previously performed (Du 2018).

Nevertheless, the current observations are important, as this is one of the first studies to examine $_{C18}$CMS *in vivo*, and the first study doing so for bCMS.

A limitation of these current observations is that they comprised treatment periods of 5 days only (paper 1, paper 2, Pischon et al 2017), which are considered exploratory. Short-term, regulatory toxicologic studies usually start at 14 d treatment protocols, as it was previously performed for one of the "hPG-E-C15-mPEG" CMS (Hönzke et al. 2016b).

Furthermore, previous *in vitro* results did show potential signs of cytotoxicity after prolonged incubation times (Wolf et al. 2009). More importantly, after the initial studies on $_{C18}$CMS, Hönzke et al. performed more rigorous and systematic *in vitro* testing, which included examining some of the potential *degradation products* of $_{C18}$CMS. Doing so, they found that while hPG and mPEG exhibited no cytotoxicity as expected, the parts of the C18 alkyl "inner shell" component had some toxicity. In addition, the full C18-mPEG co-polymer showed potentially relevant toxicity (Hönzke et al. 2016b). A potential explanation may be that the amphiphilic chain molecule could function as a type of surfactant. This molecule also caused significant production of reactive oxygen species (ROS), as well as genotoxicity in the COMET test. According to the authors of that study, the latter may be a consequence of ROS production. The authors recommend commencing with one of the other CMS architectures assessed, the ester-based hPG-E-15-mPEG CMS (Hönzke et al. 2016b). This type of CMS is a modified version of the $_{C18}$CMS studied here, in which the amide bond attaching the shell to the core is replaced by an ester bond (Hönzke et al. 2016b). The goal is that these particles can be more easily degraded by esterases in the body. bCMS are a similar, novel concept with the same goal, as described in the introduction. Nevertheless, this is a reminder that degradation products must be considered during toxicologic evaluation.
Of course, the relevance of any toxicity observed with these methods must always be evaluated in conjunction with expected exposure scenarios. This makes it more important to learn more about the penetration behavior of the particles themselves under different conditions, as discussed above.

Finally, the effects of CMS on the specific immune system, either of the particles themselves or a potential "Trojan horse" effect of haptens hitchhiking on a penetration enhancement effect, cannot be judged by the studies of the current project (paper 1, paper 2, Pischon et al. 2017, most importantly because the 5 d treatment period is too short for sensitization. As discussed in section 3.2.2.3, previous *in vitro/ex vivo* studies found potential uptake not only by keratinocytes (Brodwolf et al. 2020; Frombach et al. 2019), including with potential lysosomal escape to the cytoplasm and nucleus (Brodwolf et al. 2020), but also uptake by professional antigen-presenting cells (Frombach et al. 2019; Edlich et al. 2018; Du et al. 2016). This is indirectly supported by the current studies, as $_{C18}$CMS were found in the organs responsible for particle clearance by the mononuclear phagocytic system after systemic distribution (paper 1). On the one hand, such uptake could be beneficial for degradation and avoidance of accumulation over time. On the other hand, there is some evidence for exocytosis of unmodified CMS after initial uptake by Langerhans cells (Edlich et al. 2018). Overall, this means that further *in vitro* studies on interaction with human cells or cells of other species,

particularly cells of the mononuclear phagocyte system, as well as on a potential effect on hapten penetration would be interesting (also see section 3.4).

3.4. The path forward and suggestions for further research

As discussed, there are many open questions, particularly about which factors may influence CMS penetration enhancement. Going forward, they should be systematically investigated. This should be done *in vivo*/*ex vivo*. At this stage of CMS development, taking into consideration the potential benefits of topical CMS formulations and the availability of other formulation options (e.g., lipid nanoparticles), this seems ethically imperative. The author believes that it is also the most effective way. In addition to the reasons discussed above, there is yet another, very practical reason: With *in vitro*/*ex vivo* experiments, the group sizes, number of iterations, and additional factors that can be assessed and controlled for are only dependent on the available time and other resources. On average, *in vivo* skin penetration studies are not only more time-consuming in planning and implementation, but every additional test always requires very careful ethical weighing. Performing a large number of iterative tests with fine-grained modification of parameters between experiments is much harder or impossible without relevant ethical implications. Thankfully, various groups in the CRC have done remarkable work in developing and refining *ex vivo/in vitro models* for skin barrier-altering conditions (e.g., Graff et al. 2022; Rancan et al. 2021; Frombach et al. 2020; Döge et al. 2016; Hönzke et al. 2016b; Alnasif et al. 2014), approaches to study the interaction of nanocarriers with individual cells (e.g., Brodwolf et al. 2020; Yamamoto et al. 2019, to which we were privileged to add a modest contribution), and to model their interaction with the skin (e.g., Schulz et al. 2017). This, together with the general advances in model development, political initiatives in the European Union and elsewhere, and even increasing commitment by large pharmacological companies to move away from animal testing in the future, means that it will become more and more practicable.

The author would like to suggest the following follow-up experiments. This could both help solve some of the questions raised above and explore whether such modifications/add-ons to current *in vitro*/*ex vivo* models could generally add to their predictive value.

1. Using a short, non-occluded “exposure time” before removal of the formulation and a longer “incubation time.” As discussed above, this seems particularly important to compare the behavior of volatile formulations to non-volatile ones under real-life treatment conditions. This may be necessary to exclude the possibility that CMS simply lack any penetration effect under non-occluded conditions (see discussion of penetration enhancement in section 3.1.2.4 and figure 1). This can be followed by long incubation times to allow for slow permeation of the drug from the superficial SC through the skin, as it would in real life. If necessary, for the model, this incubation time can then be occluded or use a high

humidity environment. It seems like this would more closely resemble real-life topical application.

2. Exploring the carrier effect not only on penetration after a single dose but also modeling repeated application, steady state, “depots,” and similar scenarios. For example, it may be worthwhile to try to “pre-load” a piece of excised skin with TAC, incubating it in a TAC solution until tissue concentrations are reached that have been measured in human patients after repeated dosing. Then the TAC-loaded carrier could be applied (exposure, followed by remnant removal), and after an additional incubation period, it could be measured whether the difference to the control is still as pronounced as it was after a single application.

   Somewhat opposed to the point made above for the apply-and-wipe method, this may also be an argument to simply add additional experimental groups with classical steady state, infinite dose conditions in addition to finite dose groups (Selzer et al. 2017, Selzer et al. 2013). Finally, it may be necessary to use more sophisticated *in vitro* models that allow for prolonged treatment scenarios. For example, organ-on-chip skin models could be used which can be treated repeatedly and include artificial equivalents of circulation (Tavares et al. 2020).
3. Exploring the carrier’s effect on the outflux kinetics from different skin layers compared to a receptor medium. For example, TAC-loaded CMS could be loaded into the SC, and a typical “release” study could be performed by submersing the SC in media usually used for release studies from particles. If the SC is investigated, it may be possible or even best to leave the rest of the skin attached, as most separation methods could introduce artifacts. Potentially, receptor media that approximate a drug’s overall free energy in a specific skin layer could eventually be found. This approach also seems particularly important to investigate the hypothetical “SC depot” effect, which has frequently been named as a potential benefit of nanocarriers. Moreover, it seems particularly important for highly lipophilic drugs like TAC, where the transition from the “lipophilic” SC into the “hydrophilic” viable epidermis may be quite important for the kinetics and potentially rate-limiting (Flaten et al. 2015).

In addition, it seems very important to create more finely-grained time-resolved kinetic profiles of the carriers *in vitro*/*ex vivo*, instead of relying on one or two time points. This then could also be used to explore diffusivity and free energy profiles, which seems particularly important (e.g., Schulz et al. 2017).

Moreover, when evaluating penetration enhancement effects on drugs that are known to penetrate without the enhancer (even if the amount of penetration is considered sub-optimal), and if penetration enhancement is the primary goal, it may have to be shown *in vitro*/*ex vivo*

that the enhancer results in improved penetration compared to simply increasing the strength or dose of the standard formulation (unless it is clear that the latter is not effective, feasible or advisable under real-life conditions). To do so, *in vitro/ex vivo* experiments may have to include additional control or follow-up groups, in which the concentration of the drug applied in the standard formulation is sequentially increased to try to match the skin concentrations achieved with the penetration-enhancing formulation. Subsequently, the relevance of the observed benefit must be critically evaluated. This seems ethically imperative before performing further *in vivo* studies.

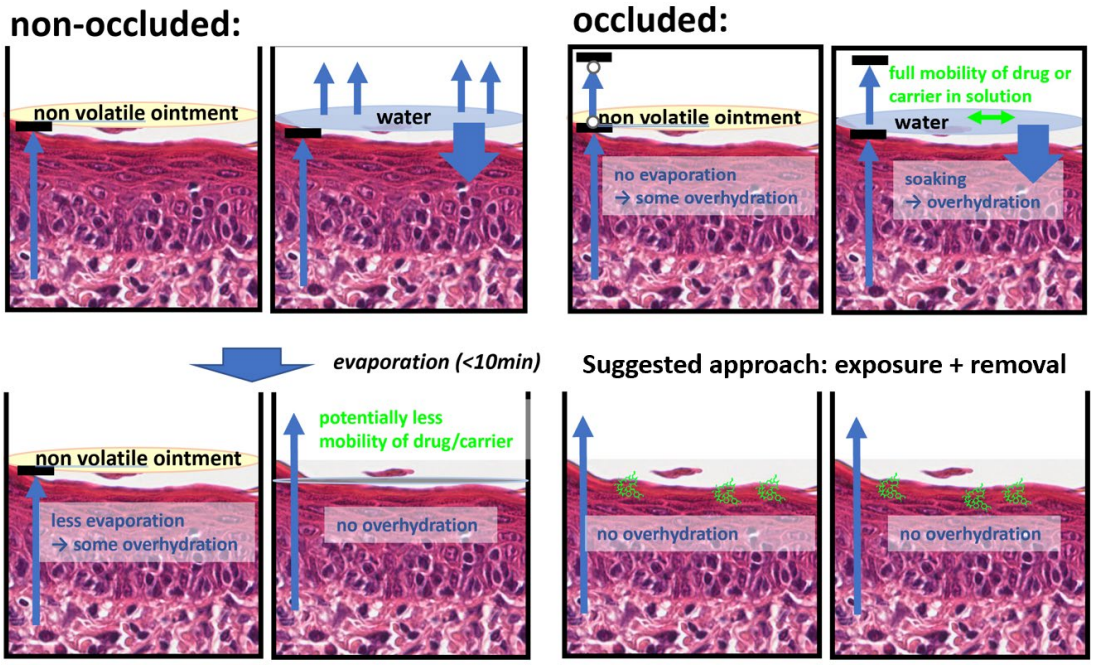

Figure 1 – Suggestion for "exposure time" during penetration experiments

A further, important question that also needs to be addressed in the author's opinion, is whether effective topical nanocarriers cause a "Trojan horse" risk. If they increase the penetrability of the SC for intended cargo substances, it seems plausible that they also increase it for haptens or other unwanted guests molecules. For CMS, it has been argued that loading into the carrier is necessary for the penetration enhancement effect. However, a.) it seems that this assumption is based on a few, limited studies (e.g., Saeidpour et al. 2017), b.) even if true, it does not seem impossible that substances load themselves into the carrier, e.g., on the surface of the SC, and c.) there is actual evidence that CMS may even increase the permeation of small peptides through the SC without them being loaded (Do et al. 2014). It thus should be considered whether a "Trojan-horse-for-haptens" test should be established as a new part of each systematic characterization, e.g., in the workflow described by Hönzke et

al. (2016b). In the simplest form, this could consist of a regular penetration study in which a test hapten (yet to be determined) is co-applied.

In addition, for the cases where loading is potentially not necessary, it would be important to determine whether, and how quickly, penetration enhancement is reversible. In fact, if the interaction of the carrier, especially their amphiphilic side arms, with the SC is assumed, it seems important to generally explore what type of changes this induces in the SC.

Of less importance, but nevertheless interesting: it could be explored whether flexing of the skin during movement has a relevant effect on the diffusion of nanocarriers into or through the skin. A simple device to do so could, for example, be a small water-filled balloon underneath a skin equivalent or *ex vivo* tissue, that is periodically slightly inflated and deflated.

A further, very specific, aspect to be examined is whether the choice of vehicle delivering the loaded CMS has a relevant effect on penetration enhancement. For example, HEC is a biocompatible and common choice, but it may have influenced the release after drying of the formulation as discussed above and have less desirable non-primary properties compared to other gel-forming agents. For example, agents with emollient properties that leave even less undesirable remnants on the skin may be considered.

In addition to these practical suggestions, it is very important that regulatory bodies and university ethics boards only allow *in vivo* experiments to commence if *in vitro* data can be provided that shows the hypothesized effect under exactly the same experimental conditions, including substance extraction, detection methods, etc. There should be a mandatory step that explores whether a result obtained, e.g., on human skin *ex vivo* translates to the non-target species skin (e.g., mouse) that is planned to be used in the study, but under *ex vivo* conditions. It should be possible to obtain the necessary excised skin samples by sharing them in the scientific community, without the need for additional animals.

Finally, studies aiming for penetration enhancement need to more precisely define the exact end goal envisioned, e.g., how much penetration of a certain drug would have to be increased precisely, in which disease, to reach which specific, *relevant* benefit (e.g., necessary target concentrations impossible to reach with simpler means than the novel formulation, relevantly less egress to the systemic circulation, relevant depot effects, relevantly quicker action, relevant increase in the availability of otherwise prohibitively expensive drugs, etc.). As others have commented, it is easy to overinterpret marginally improved outcomes as confirmation to commence with development (e.g., Lammers 2019), while losing sight of what is actually needed.

3.5. Conclusions

The project underlying this dissertation includes the first *in vivo* study investigating the penetration enhancement effect of polymeric core-multishell nanocarriers (CMS) after topical application. A penetration enhancement effect of biodegradable hPG-$PCL_{1.1K}$-$mPEG_{2k}$ CMS (bCMS) for tacrolimus (TAC), which had previously been described, could not be confirmed under the conditions studied. In fact, the results suggest a decrease in penetration into the skin. Nevertheless, despite a lack of enhancement, drug delivery to the skin and efficacy of the formulation could be confirmed.

bCMS and hPG-amid-C18-mPEG CMS ($_{C18}$CMS) nanocarriers themselves both penetrated into the stratum corneum. No bulk penetration into the viable skin could be observed. No adverse effects were observed.

The causes for the observed discrepancies to previous *in vivo*/*in vitro* results will require a systematic analysis. No *in vivo* studies investigating penetration enhancement effects of CMS, nanogels or potentially similar delivery solutions should commence, before 1. a systematic root cause analysis has shown what caused these discrepancies, 2. all relevant aspects discussed above have been modeled *in vitro* for the specific nanocarrier-drug combination in question, and 3. an evidence-based estimate is available on whether the expected penetration enhancement would lead to a relevant benefit for the specific use case.

4. Summary/Zusammenfassung

4.1. Summary

Polymeric Core-Multishell-Nanocarriers for Topical Drug Delivery to the Skin: Nanocarrier Distribution, Cargo Delivery and Efficacy for the Treatment of Inflammatory Skin Conditions

Moritz Radbruch

Polymeric Core-Multishell-Nanocarriers (CMS) are a family of molecules designed to function as universal drug carriers. Their architecture resembles unimolecular micelles/liposomes, with shell-like, hydrophobic, and hydrophilic domains. Studies using *ex vivo/in vitro* conditions have shown that they can be used to topically deliver drugs to the skin, including strongly hydrophobic drugs in water-based formulations. Interestingly, these studies reported that CMS not only successfully delivered cargo substances, but in fact increased their concentrations in the target area, the viable skin. Along with the relatively good biocompatibility reported, these properties make them interesting as tools for the treatment of inflammatory skin conditions and other topical applications.

The work described here is part of a project that aimed to reproduce and further investigate these findings under conditions of inflammatory skin diseases *in vivo*. The project was part of a Collaborative Research Center of the German Research Foundation that aimed to develop and investigate a range of nanocarriers for topical delivery to the skin.

The specific CMS architectures investigated here were hPG-C18-mPEG CMS ($_{C18}$CMS) as well as hPG-PCL-mPEG CMS (bCMS). Both architectures have been developed at the Institute of Chemistry and Biochemistry, Freie Universität Berlin. $_{C18}$CMS represent the prototypical CMS architecture, best characterized for topical delivery. bCMS are easily cleaved by esterases to improve long-term biocompatibility. As a model for a prototypical inflammatory skin condition, an oxazolone-induced mouse model with characteristics of atopic dermatitis (AD) was used. The cargo investigated was tacrolimus (TAC). TAC is a potent anti-inflammatory drug and one of the two main pharmacological treatment options for AD. With a relatively large molecular mass of 822 Da, it is considered at the threshold of substances that can penetrate into the skin in relevant amounts for topical treatment.

In the first part of the project, the penetration of $_{C18}$CMS into the skin, their potential systemic distribution, and the effect of oxazolone-induced inflammation on the penetration were investigated by fluorescence microscopy. Furthermore, the potential effects of the carriers on

clinical and histological parameters were evaluated. In the second part of the project, the delivery of TAC by bCMS into inflamed skin, resulting systemic drug concentrations, as well as the clinical and histologic anti-inflammatory efficacy, were investigated.

Under the conditions used, bCMS did not seem to increase the skin concentrations of the cargo drug in the viable skin compared to the standard ointment formulation. What is more, concentrations measured in the systemic circulation were significantly lower. This in fact suggests that bCMS decreased overall skin penetration. This differs from previous reports obtained using *ex vivo/in vitro* conditions, which consistently found penetration enhancement for TAC in bCMS and for various other cargo substances in multiple CMS architectures. Nevertheless, drug delivery to the skin and the anti-inflammatory efficacy of the formulation could be demonstrated.

Although $_{C18}$CMS penetrated into the stratum corneum, no penetration was observed into the viable layers of healthy skin. This penetration behavior of the carrier was not affected by oxazolone-induced dermatitis. The same was observed for bCMS in inflamed skin. These results seem to be partially in line with previous *ex vivo/in vitro* results under certain conditions, such as relatively short incubation times and relatively mild barrier alterations but differ from results under other conditions, such as longer incubation times and models of more severe barrier alterations.

When modeling complete penetration of $_{C18}$CMS through the viable skin, the fluorescently labeled carriers were found in the main sites of the mononuclear phagocyte system and particle clearance, i.e., the local lymph nodes, spleen, lung, liver, and kidney. No adverse effects were observed histologically.

Overall, the results seem to confirm that CMS can be used for topical delivery and treatment of inflammatory skin conditions with TAC. However, CMS may not necessarily enhance penetration or efficacy. Moreover, the results suggest a potential systematic difference between *ex vivo/in vitro* and *in vivo* conditions. On the one hand, this cautions that murine models may be unsuitable to investigate the penetration enhancement effect of CMS. On the other hand, it suggests parameters that could potentially be optimized in *ex vivo/in vitro* models to increase predictability. Of these, particularly relevant parameters seem to be incubation times, exposure periods with subsequent removal of formulations, occlusion/hydration status, effects of repeated applications, steady-state conditions and drug depots, effects that depend on tissue layers other than the stratum corneum, and time-resolved kinetics. This may be particularly important when comparing water-based, volatile formulations with less volatile ointment formulations, and for drugs with comparatively high steady-state concentrations in

the skin. With respect to the 3R principles, the results strongly suggest that no further *in vivo* studies should be performed to investigate the penetration enhancement effects of CMS or similar delivery solutions before 1.) a systematic analysis has shown what caused these discrepancies, 2.) all relevant aspects discussed have been modeled *in vitro* for the specific nanocarrier-drug combination in question, and 3.) an evidence-based estimate is available on whether the expected penetration enhancement would lead to a relevant benefit for the specific use case.

4.2. Zusammenfassung

Polymere Core-Multishell-Nanocarrier zum topischen Wirkstofftransport durch die Haut: Nanocarrier Verteilung, Transport und Wirksamkeit bei der Behandlung entzündlicher Hautveränderungen

Moritz Radbruch

Polymere Core-Multishell Nanocarrier (CMS) sind eine Familie von Molekülen, die als universelles Transportsystem für pharmakologische Wirkstoffe entwickelt wurden. Ihre Architektur gleicht unimolekularen Mizellen / Liposomen, mit schalenförmig angeordneten hydrophoben und hydrophilen Domänen. *Ex vivo / in vitro* Studien zeigen, dass sie für die topische Applikation von Wirkstoffen auf der Haut genutzt werden können. Dies gilt insbesondere auch für die Applikation von stark hydrophoben Wirkstoffen in Wasser-basierten Formulierungen. Interessanterweise deuten diese Studien darauf hin, dass CMS geladene Wirkstoffe nicht nur erfolgreich an die Haut abgeben, sondern die Penetration der Wirkstoffe in die Haut sogar verstärken. Zusammen mit einer relativ guten Biokompatibilität machen diese Eigenschaften CMS interessant für die Behandlung von entzündlichen Hauterkrankungen.

Die vorliegende Arbeit war Teil eines Projektes zu der Fragestellung, ob diese Ergebnisse in entzündeter Haut unter *in vivo* Bedingungen reproduziert werden können. Dieses Projekt wiederum war Teil eines Sonderforschungsbereichs der Deutschen Forschungsgemeinschaft mit dem Ziel, verschiedenste Nanocarrier für den topischen Wirkstofftransport in die Haut zu untersuchen.

Untersucht wurden hPG-C18-mPEG CMS ($_{C18}$CMS) sowie hPG-PCL-mPEG CMS (bCMS). Beide CMS wurden von Forschungsgruppen des Instituts für Chemie und Biochemie der Freien Universität Berlin entwickelt. $_{C18}$CMS sind die prototypischen CMS und waren am weitesten charakterisiert für die topische Applikation. bCMS weisen eine bessere Abbaubarkeit durch Esterasen auf, was ihre Langzeitgewebeverträglichkeit erhöhen soll. Als Modell für eine entzündliche Hautveränderung wurde ein durch Oxazolon ausgelöstes Mausmodell genutzt, was Charakteristika von atopischer Dermatitis (AD) aufweist. Der zu transportierende Wirkstoff war Tacrolimus (TAC). TAC ist ein wirksames, anti-inflammatorisches Medikament und eine der zwei wichtigsten medikamentösen Behandlungsoptionen bei AD. Mit einer relativ großen Molekülmasse von 822 Da ist es darüber hinaus nah an der Grenze, ab der anzunehmen ist, dass Substanzen die Haut nicht mehr in relevanten Mengen überwinden.

In der ersten Hälfte des Projektes wurde die Penetration von $_{C18}$CMS in gesunde Haut, ihre potentielle systemische Verteilung sowie ein potentieller Effekt der Oxazolon induzierten Dermatitis auf die Penetration der Carrier mit Hilfe von Fluoreszenzmikroskopie untersucht.

Des Weiteren wurden potentielle Effekte der Carrier auf klinische und histologische Parameter untersucht. In der zweiten Hälfte des Projektes wurde der Transport von TAC durch bCMS in die entzündete Haut sowie die resultierende systemische Wirkstoffkonzentration mit Hilfe von Massenspektrometrie untersucht. Des Weiteren wurden die daraus resultierende klinische und histologische Wirksamkeit sowie die Penetration von bCMS in die Haut untersucht.

bCMS schienen unter den untersuchten Bedingungen die Penetration von TAC in die Haut nicht zu erhöhen. Die gemessenen systemischen TAC Konzentrationen waren darüber hinaus signifikant verringert. Dies deutet darauf hin, dass CMS die Penetration in die Haut insgesamt möglicherweise sogar hemmen. Dies unterscheidet sich von Beobachtungen anderer Studien unter *ex vivo / in vitro* Bedingungen. Diese zeigten durchweg eine Penetrationsverstärkung, sowohl für TAC in bCMS als auch für andere Modellsubstanzen in verschiedenen CMS Architekturen. Nichtsdestoweniger konnten sowohl der Wirkstofftransport in die Haut als auch daraus resultierende anti-inflammatorische Wirksamkeit gezeigt werden.

$_{C18}$CMS selbst fanden sich im Stratum corneum, eine Penetration in die lebenden Schichten der Haut wurde nicht beobachtet. Dieses Verteilungsmuster wurde durch die Oxazolon-induzierte, entzündliche Hautveränderung nicht beeinflusst. Dieses Verteilungsmuster konnte auch für bCMS in entzündeter Haut bestätigt werden. Das Verteilungsmuster stimmt teilweise mit vorherigen *ex vivo / in vitro* Ergebnissen unter bestimmten experimentellen Bedingungen überein (relativ kurze Inkubationszeiten und relativ milde Barriereveränderungen), aber divergiert von Studien unter anderen Bedingungen (längere Inkubationszeiten und stärkere Barriereveränderungen).

Bei künstlich modellierter, kompletter Penetration von $_{C18}$CMS durch die Haut konnten die Carrier wie erwartet in Hauptlokalisationen von Partikelfilterung und des mononukleär-phagozytären Systems gefunden werden (lokale Lymphknoten, Milz, Lunge, Leber, Nieren). Es wurden histologisch keine adversen Effekte beobachtet.

Die Ergebnisse scheinen zu bestätigen, dass CMS für einen effektiven topischen Wirkstofftransport von TAC in entzündete Haut genutzt werden können. Allerdings verstärken CMS dabei anders als erwartet möglicherweise nicht die Penetration von TAC. Des Weiteren deuten die Ergebnisse auf einen potentiellen systematischen Unterschied zwischen *ex vivo / in vitro* und *in vivo* Bedingungen hin. Einerseits kann dies ein Warnhinweis sein, dass Mausmodelle möglicherweise nicht geeignet sind, um penetrationsverstärkende Effekte von CMS zu untersuchen. Andererseits deuten die Ergebnisse auf Parameter hin, die möglicherweise an *ex vivo / in vitro* Modellen modifiziert werden könnten, um bessere Voraussagen zu ermöglichen. Besonders wichtig erscheinen dabei Inkubationszeiten,

Einwirkzeiten mit nachfolgender Entfernung der Formulierung, Okklusion / Hydrierungsstatus, Effekte von wiederholter Applikation, Steady-State Zuständen und Wirkstoffdepots, Effekte von tieferen Hautschichten sowie Kinetikstudien. Diese Faktoren sind möglicherweise besonders relevant bei Vergleichen zwischen Wasser-basierten, flüchtigen Formulierungen und weniger flüchtigen Salbenformulierungen sowie bei Wirkstoffen mit vergleichsweise hohen Steady-State Konzentrationen in der Haut. Im Sinne der 3R Prinzipien weisen die Ergebnisse darauf hin, dass keine weiteren *in vivo* Studien zur Untersuchung eines Penetrations-verstärkenden Effekts von CMS (oder ähnlichen Wirkstofftransportern) unternommen werden sollten, bevor 1. der Grund für den beobachteten Unterschied zu *ex vivo / in vitro* Studien gefunden wurde, 2. alle relevanten Aspekte potenzieller Studien für die spezifische Kombination aus Carrier und Wirkstoff vorher *ex vivo / in vitro* untersucht wurden und 3. eine evidenzbasierte Schätzung vorliegt, ob der voraussichtliche penetrationsverstärkende Effekt einen relevanten Vorteil für die geplante Anwendung hat.

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9. Declaration of Originality

I hereby declare that this dissertation was prepared by me and that I exclusively used the sources and facilities referenced. I also declare that I have not submitted the dissertation in this or any other form to any other institution as a dissertation.

Berlin, 14.05.2024 Moritz Jan Florian Radbruch