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Jeanette HANNAH Charlotte Pischon

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Contents

List of Abbreviations

AP-1	**A**ctivator **P**rotein **1**
CMS	**C**ore-**M**ulti**S**hell-nanocarrier
CMS-ICC	**C**ore-**M**ulti**S**hell-nanocarrier labeled with **I**ndo**C**arbo**C**yanine
C18	$\mathbf{C_{18}}H_{36}$ saturated carbohydrate chain
DAPI	4',6-**DiA**midino-2-**P**henyl**I**ndole
DMSO	**DiM**ethyl **S**ulf**O**xide
FLIM	**F**luorescence **L**ifetime **I**maging **M**icroscopy
DFG	German Research Foundation
hPG	**h**yperbranched **P**oly**G**lycerol
H2DCFDA	6-carboxy-2',7'-**DiC**hlorodi**H**ydro**F**luorescein **DiA**cetate
ICC	**I**ndo**C**arbo**C**yanine
IFN	**I**nter**F**ero**N**
IL	**I**nter**L**eukin
IMQ	**IMiQ**uimod
JNK	c-**J**un **N**-terminal **K**inases
KGM	**K**eratinocyte **G**rowth **M**edium
LCE	**L**ate **C**ornified **E**nvelope
MAP	**M**itogen-**A**ctivated **P**rotein
mPEG	**m**onomethyl **P**oly**E**thylene **G**lycol
MTT	3-(4,5-di**M**ethyl**T**hiazol-2-yl)-2,5-diphenyl**T**etrazolium bromide
MyD88	**My**eloid **D**ifferentiation primary response **88**
NC	**N**ano**C**arrier

NFAT	**N**uclear **F**actor of **A**ctivated **T**-lymphocytes
NFkB	**N**uclear **F**actor **k**appa-light-chain-enhancer of activated **B**-cells
NHK	**N**ormal **H**uman **K**eratinocytes
NP	**N**ano**P**articles
NR	**N**ile **R**ed
PBS	**P**hosphate-**B**uffered **S**aline
ROS	**R**eactive **O**xygen **S**pecies
SDS	**S**odium **D**odecyl **S**ulfate
TAC	**TAC**rolimus
TCSPC	**T**ime-**C**orrelated **S**ingle **P**hoton **C**ounting
TEWL	**T**rans**E**pidermal **W**ater **L**oss
TGFβ1	**T**ransforming **G**rowth **F**actor **β1**
TLR	**T**oll-**L**ike **R**eceptor
TNF	**T**umor **N**ecrosis **F**actor
w%	**w**eight percent

1 Introduction

This study was a project within the German Research Foundation (DFG) collaborate research center (CRC) 1112 studying nanocarrier (NC) for topical application of drugs for their therapeutic use in inflammatory skin diseases like psoriasis and atopic dermatitis.

Topical treatment of skin diseases may have many advantages over systemic treatment: 1) high drug concentrations at the site of action, 2) less exposure of non-target organs to the drug and therefore less systemic side effects and 3) less loss of drug due to metabolism in the liver and other organs. Compared to injections, it is less invasive, painless, and more convenient for the patients. However, topical treatment is also challenging: most importantly, the skin is a very effective barrier, which prevents penetration of a large proportion of otherwise effective drugs. In addition, compared to injection or oral uptake, a topically applied drug typically has relatively little time for sufficient penetration due to mechanical removal and eventual shedding by desquamation. Furthermore, there is low patient compliance with sticky ointments that are still needed to formulate lipophilic drugs.

Psoriasis is a common human skin disease, which is incurable and therefore requires lifelong therapy.[1] The easily visible skin lesions decrease the quality of life and patients often suffer from depression.[2] Available topical creams or ointments are sticky and have to be applied frequently. Furthermore, systemic therapy with immunomodulatory drugs is often required to control severe cases but possesses a high risk for undesired side effects.[1,3] Thus, treatment of psoriasis would profit from improved systems for topical drug delivery and was chosen by the CRC 1112 as one of the two model diseases.

One system that has been proposed to improve topical delivery are NC, where small molecules may incorporate drugs and enable or increase the penetration of drugs or establish a drug depot, minimizing further distribution and systemic side effects. Ideally, they may even deliver their cargo exclusively into diseased skin to the site of action. Thereby the NC could have a therapeutic surplus value by successful topical treatment, targeted to the diseased areas with decreased systemic side effects. In the context of the CRC 1112, several different NC were developed to tackle this task.

The aim of this project was to evaluate the most advanced NC developed within the CRC 1112, the core-multishell-nanocarrier (CMS), on healthy murine skin and a mouse model of psoriasis *in vivo*. Emphasis was placed on local or systemic effects and skin penetration behavior of the carriers themselves, as well as enhancement of cargo drug delivery for a therapeutic surplus value against standard formulations.

Simultaneously, within the CRC the same particles were also investigated *in vivo* in a mouse model of atopic dermatitis by Radbruch et al. (2017).[4] In addition, collaborating groups of the

CRC developed various further NC, which were tested in different models, including excised human skin, pig ear skin, as well as in cell culture and reconstructed skin models.

1.1 Skin – background

Skin architecture

From outside to inside, the skin is composed of a stratum corneum, a viable epidermis, both belonging to the epidermis, a basement membrane, and the dermis and is connected via the subcutis to the underlying tissue.

The epidermis is composed of four basic layers: the stratum corneum, stratum granulosum, stratum spinosum, and stratum basale. The latter three represent the viable epidermis.

The stratum corneum, the outermost layer, fulfills the main barrier function.[5] It is composed of very flat, non-nucleated keratinized keratinocytes, the corneocytes. Their intracellular protein keratin is partially responsible for the toughness of the skin.[6] Multiple lamellar lipid bilayers surround the cells in addition to their cell membranes filling the spaces in between. The lipid composition of these bilayers differs markedly from regular cell membranes and plays a role in barrier function. They contain gycosyl-ceramides, cholesterol, cholesterol-esters, and long-chain fatty acids.[6]

The keratinocytes of the underlying viable epidermis are tightly connected via desmosomes and tight junctions.[7] These keratinocytes originate from basal cells in the stratum basale, the innermost epidermal layer. These germinal cells proliferate to renew the epithelium continuously. On their way to the surface, the cells differentiate to the progressively flattening keratinocytes through the stratum spinosum and stratum granulosum and finally lose their nucleus and become keratinized in the stratum corneum before they are shed.[6]

Approximately 3-5 % of the nucleated cells in the epidermis are Langerhans cells, the specific local dendritic cells.[8] The cell body is preferably located suprabasilar but their long dendrites extend up beneath the stratum corneum,[9] where they take up and process microbial and non-microbial antigens. By secreting cytokines, they contribute to the innate immunity but they are also important for the adaptive immunity by migrating to the regional lymph node and presenting antigens to lymphocytes.[7]

Epidermis and dermis are divided by a basement membrane, which forms a barrier and scaffold for the overlying basal cells.

The underlying dermis contains abundant extracellular matrix molecules produced by fibroblasts forming a mesh important for mechanical strength, elasticity, and resistance to compression and cell anchoring.[7] In addition, nerves, blood, and lymph vessels as well as immune cells such as mast cells, lymphocytes, plasma cells, and dermal dendritic cells are found in the dermis.[6,7]

Skin adnexa include hair follicles with their musculi arrectores pilorum, sweat, and sebaceous glands, contributing to the skin barrier against mechanical injury and playing a role in body temperature homeostasis.[6]

Barrier function

The skin shields the body effectively from mechanical and physical injuries as well as infectious agents but also constitutes a barrier for topically applied drugs or NC. This barrier function is achieved by an interplay of several physical, biochemical, and immunological elements.

The stratum corneum with its multilayer lipid extracellular matrix seems to be the most important barrier for water, hydrophilic substances, and particles. The tight junctions between the viable keratinocytes serve as important barrier as well.[10,11] The next mechanical barrier is the tight mesh of the basement membrane.

Besides being a mechanical barrier, the stratum corneum also forms a redox barrier with buffering sulfur-rich layers[12] and vitamin E in sebaceous gland secretions protecting against antioxidant injury.[6] Acids contributing to an average skin surface pH of below 5 as well as lysozymes and antimicrobial peptides or defensins protect to a certain extent against microorganisms.[10,13]

However, the skin surface environment also favors colonization of certain microorganisms. This microbiome also prevents colonization of potentially harmful microorganisms by competing symbiotic, normally harmless bacteria and fungi for space and nutrition on the skin surface. Furthermore, the microbiome is in close contact and interferes with the immune system.[7]

The immune system also plays an important role in the skin's barrier function with immune cells within the skin. Especially Langerhans cells in the epidermis build an immunologic first line of defense as explained above (see 1.1 Skin – background; Skin architecture). In addition, keratinocytes secreting chemokines attracting other immune cells to a site of injury, contribute to the immune barrier against infectious agents.[7]

Murine skin versus human skin

In this study, mice were used because of the availability for *in vivo* studies, the option to model an inflamed skin, and the possibility of histologic examination of organs after experiments on whole organisms in contrast to *ex vivo* skin models. The mouse is a widely used model in research but one has to keep in mind that there are differences in human and murine skin, which can also influence study outcomes regarding skin penetration behavior and immune responses. Histologically visible differences include a thinner skin, with a 29 µm thick epidermis in mice versus a 47 µm thick epidermis in humans and a 9 µm thick stratum corneum in mice versus a 17 µm thick stratum corneum in humans, depending on the body region.[14] This can

lead to a weaker skin barrier in penetration studies depending on the examined substance.[14] Furthermore, commonly used mouse strains express a close coat compared to sparse hair in most human body regions, which complicates topical applications. Sweat glands are only found in footpads in mice and all over the body in humans.[15] These differences influence the choice of models and have to be balanced to practicability. The results have to be compared or controlled between species or mouse strains.

Skin barrier disruption

In penetration studies of drugs or nanoparticles (NP), skin barrier disruption is important to consider. Slight skin barrier disruptions can easily occur, for example, after scratching.[16,17] In inflammatory skin diseases, changes in epidermal thickness, metabolic capacity, microstructure of the stratum corneum, and larger surface integrity can lead to barrier disruptions.[18] An impaired barrier function can lead to increased penetration;[19] this can be intentional in topical therapy targeting only diseased skin regions, but can also be unintentional making barrier disruption models essential in toxicological examinations. In research, several models for skin barrier disruptions have been developed to mimic diseased skin for penetration studies including chemical, mechanical, or inflammatory approaches.

For example, chemical irritants, also used in certain pharmacologic topical formulations as penetration enhancers, temporarily impair the skin barrier to facilitate drug penetration. Mechanical injury of the stratum corneum is often used in *ex vivo* but also in *in vivo* studies, especially via tape stripping, where superficial layers of the stratum corneum are removed by adhesive tape. Simple techniques like massaging can increase penetration by hair movement pumping into hair follicles.[20]

1.2 Psoriasis – a common inflammatory skin disease in humans

The CRC 1112 used psoriasis as a prototype of a common, immune mediated human skin inflammation[1] with impaired barrier, which may result in increased penetration of some NP.[21] On the other hand, barrier alterations could also lead to less penetration of substances, for example, by thickening of viable epidermis and stratum corneum.[22]

Epidemiology and symptoms of psoriasis

Psoriasis has a prevalence of 3 % in the adult population,[23] equally in both sexes, with a bimodal onset of 16-22 or 57-60 years of age.[3]

Well-demarcated, erythematous plaques covered with silver scales are preferably found on the scalp, trunk, buttocks and extremities of the patients with plaque type psoriasis.[3] Histologically these changes are based on a hyperproliferative epidermis with premature and

incompletely cornified keratinocytes.[3] Other forms of psoriasis, including psoriasis arthritis, are of lesser importance for this study.

Pathogenesis of psoriasis

Psoriasis is a multifactorial disease associated with genetic predispositions, epigenetic variations, a dysregulated immune system including autoantigens, and influencing environmental factors.[3,24,25]

Psoriasis related genes are mostly linked to the innate and adaptive immune system.[26] About half of the genetic variations are connected to major histocompatibility complexes, but over 70 other loci have been identified including nuclear factor κβ, Interferons (IFN) or interleukin (IL) 23 signaling.[1]

Environmental factors such as trauma can trigger psoriasis. The trauma initiates typical psoriasis immune signaling pathways by triggering keratinocytes to release antimicrobial peptides like LL37, whose complexes with DNA or RNA bind to toll-like receptors (TLR).[26] Thereby plasmacytoid and myeloid dendritic cells are activated, secreting IFN α and β, activating more myeloid dendritic cells.[26] Psoriasis specific cytokines are produced, IL 12 and IL 23 activate T-helper 17, 1, and 22 lymphocytes, which produce IL 17 A and F, IL 22, IL 21, and IL 36.[26] This culminates in proliferating keratinocytes with increased chemokine production and immune cell infiltration in dermis and epidermis.[26] LL37 is also an autoantigen in some psoriasis patients where recognition by circulating LL37-specific T lymphocytes leads to IL 17 production.[26] Tumor necrosis factor (TNF) α and IFN γ, released by T-helper 1 lymphocytes, also activate keratinocytes to proliferate and produce cytokines and antimicrobial peptides.[26]

Reduced microbiome diversity and altered composition have been identified in patients and inappropriate immune responses against microbiota may lead to lesion maintenance or induction.[26]

Psoriasis pathogenesis has intensively been studied revealing many aspects, but the complete pathogenesis is still not fully understood.

Barrier alteration in psoriasis

In psoriasis, a combination of primary and secondary barrier alterations are suggested.[27] Primary skin barrier dysfunction results from genetic alterations in genes encoding proteins important for barrier function, mostly components of corneocytes, intercellular lipids, or cell interconnection in the stratum corneum. Deletions in late cornified envelope (LCE) genes LCE3B and LCE3C, for example, lead to impaired corneocyte differentiation.[28]

Barrier dysfunction is also in part secondary to immune reactions taking place in psoriatic skin. Enzyme release from leukocytes, for example, can degrade structural elements or infiltrating leukocytes can actively disconnect tight junctions to migrate into the viable epidermis.

For many reported changes regarding barrier function, it is unclear whether they reflect primary or secondary changes including altered lipid composition or decreased expression of the tight junction protein zonulin 1.[29–31]

It remains unclear if the sum of all alterations of the barrier, including in psoriasis, increases or decreases the penetrability for NC or drugs. For the drug tacrolimus (TAC) for example, it is suspected that severe thickening of the epidermis and hyperkeratosis of thick psoriasis plaques hinders TAC penetration and thus its efficacy.[22] This assumption is supported by the fact that TAC alleviates facial psoriasis, which exhibits thinner epidermis.[22]

Standard therapy of psoriasis

Although there is no cure for psoriasis, various treatment options are available and have been complemented by new therapies in recent years. Mono treatments or combinations of topical or systemic immunomodulatory drugs, such as methotrexate, phototherapy, or biologicals, including etanacept, can be administered.[1,3]

These immunomodulatory drugs are effective but can have severe side effects, especially the systemic administered agents can lead to chronic injury of organs biotransforming or excreting these drugs, such as the liver or kidney, or via immunosuppression to infections. However, long-term topical corticoid therapy can also lead to allergy, skin atrophy, or increased susceptibility to skin infection.[32]

Mouse model used in this study: the imiquimod–induced psoriasis-like dermatitis model

Since psoriasis does not occur in species other than humans, there is a need for animal models in research. Several genetically engineered, xenograft and drug- induced models have been developed, but models only resemble some aspects of a real disease.[33] It is important to use a model meeting all the requirements needed for the hypothesis in question.

In this study, the imiquimod (IMQ)-induced mouse model was used to mimic psoriasis in mice. This model is relatively inexpensive and easy to induce by topical application of IMQ every day on the skin of the mouse. By choosing the area of application, the later inflamed area can be limited to minimize the stress for the mice.

IMQ is a toll-like receptor 7 and 8 agonist used in topical treatment of human genital warts and some skin tumors like basal cell carcinoma.[34] In psoriasis patients, the usage of this drug was found to trigger the formation of psoriasis plaques and repeated application on the skin of healthy mice lead to skin lesions similar to psoriasis.[35]

Morphologic changes resemble those observed in human psoriasis including hyperkeratosis, erythema, scaling, micro abscesses, and infiltration by γδ T cells and T helper 17 lymphocytes.[36] The IMQ-induced psoriasis model also depends on immune pathways shown to be crucial in human psoriasis including IL 22, IL 36 and the IL 23-IL 17 axis.[26,36]

An elevated transepidermal water loss (TEWL) reflects inside-out barrier disruption in this mouse model. Increased drug penetration and thus outside-in disruption was also shown. Decreased amounts of β-catenin and involucrin, important proteins in cell to cell interactions via desmosomes, have been described and could add to this barrier weakening.[37]

The IMQ-induced psoriasis-like mouse model has intensively been used in different studies focusing on human psoriasis pathogenesis as well as in penetration studies and drug efficacy studies because of good pathologic, clinic, and histologic resemblance to the human disease. However, the model's response to anti-psoriatic drugs is not sufficiently studied.

1.3 Tacrolimus – a drug with challenging skin penetration properties

TAC is a macrolide calcineurin inhibitor and is described to be roughly 100 times more potent than cyclosporine A.[38] TAC is immunosuppressive by decreasing transcription of cytokines important for T cell function.

This drug is used systemically after organ transplantations but also topically applied formulations are available for the treatment of inflammatory skin diseases like atopic dermatitis.[39]

In psoriasis, TAC has only been shown to be effective in thin skin regions like the face but not on thick psoriasis plaques.[22] It is suggested that its high lipophilicity and high molecular weight of 822.05 Dalton hinders the penetration through thick psoriasis plaques.[40]

Therefore, TAC is used as a prototype high molecular weight drug, studied in the CRC 1112 whose field of application could be widened if NC could achieve an increased delivery into the skin.

1.4 Nanomaterial and nanocarrier

Nanoparticles, from "nanos" for "dwarf" in Greek, are small particles with a diameter of at least one dimension of 1 nm up to 100 nm.[41,42] Because of their high surface to volume ratio, they exhibit different physicochemical properties compared to their bulk materials.[42,43] For example, quantum effects between the surface and the surrouding molecules may start to have a proportionally larger effect.

Engineered NP are widely used in technology, for example surface coatings, titanium dioxide in sunscreen, or bacteriostatic silver NP used in bandages or to prevent odor in socks.[44,45] NP are also studied for biomedical applications, especially as NC for the delivery of drugs or genes.[46]

Nanocarrier - dermal drug delivery concept

NC are an innovative approach to carry a cargo drug with otherwise suboptimal pharmacokinetic properties to its site of action. In the skin, such a system could mean a tremendous advantage, as most drug formulations based on passive delivery via the skin are typically limited to lipophilic drugs with a molecular weight < 500 Dalton.[47] For each NC, a therapeutic surplus in comparison to standard formulations of drugs has to be shown to legitimize the more expensive production of a medication. This benefit could be achieved by different properties:

The nanoparticulate properties could increase drug penetration through skin. For tacrolimus-loaded lipid NP, for example, an increased skin penetration, skin accumulation, and bioavailability was shown compared to reference drugs.[48,49] NC could even achieve a targeted delivery by restricted penetration into barrier disrupted, inflamed skin areas[50] or accumulation of NC in a specific skin layer with ongoing cargo release from that region.[51] This could also reduce undesired local and systemic side effects by minimizing drug distribution to irrelevant tissues. For example, corticosteroids can locally induce dermal atrophy by inhibiting collagen synthesis or osteoporosis if they are distributed to bones.[52,53]

NC could also protect sensitive drugs on their way to the site of release and action by encapsulation. Alternatively, they can simply be used to give a proper solubility of an otherwise poorly soluble compound in a more convenient topical substrate, like lipophilic drugs in hydrogels, which are more convenient to use for patients compared to sticky ointments.

Nanoparticle toxicology

Besides the therapeutic surplus, biocompatibility of the NC also has to be proven before further development of NC for the use in patients.

Unintended NP, for example in exhaust fumes, ashes, and dust formed in fires or volcano eruptions, are thought to be a threat mainly to the respiratory system because of deep inhalation of particles smaller than 2 µm into the lung alveoli where they are phagocytosed by macrophages.[6] However, topical skin contact is also thought to constitute a potential risk. It was shown that people chronically walking barefoot on volcanic dust can develop podoconiosis with severe lymphedema in limbs due to NP accumulation and induction of lymphangitis.[42]

For intentionally synthetic NC it is therefore important to exclude toxic or negative effects. Even topically applied NP may penetrate through the skin and reach distant sites in the body via the bloodstream, inducing intended or unintended effects.[54]

For example, 70 nm amorphous silica NP were reported to penetrate the skin and were found in regional lymph nodes, hepatocytes, and even the brain and were shown to be cytotoxic for Langerhans cells.[55]

Nanocarrier used in this study: core-multishell-nanocarrier

Architecture of core-multishell-nanocarrier

Dendritic hPG-amid-C18-mPEG core-multishell-nanocarrier are constructed as a copolymer and have an approximate diameter of 16 nm.[4,56–59] They contain a hydrophilic hyperbranched polyglycerol (hPG) core, to which amphiphilic side chains are covalently bound via amid bonds.[4,56–59] The side chains form an inner lipophilic shell with a $C_{18}H_{36}$ saturated carbohydrate chain (C18) and an outer hydrophilic shell of monomethyl polyethylene glycol (mPEG) around the core.[4,56–59] The design resembles a liposome being able to incoorporate different cargos in the different shells. In contrast to a liposome, the single molecule architecture is less prone to destruction by shear force.[4,56–59]

Penetration behavior of and cargo delivery by core-multishell-nanocarrier ex vivo and in vivo

Previous to the study in this thesis (see paper 1 at 2.1), the penetration behavior of CMS had only been investigated *in vitro* and *ex vivo*. CMS did not penetrate into intact reconstructed human skin *in vitro* or human and porcine skin *ex vivo*.[60,61] In contrast, NC penetration was observed after prolonged exposure on *ex vivo* human skin, as well as usage of a non-melanoma skin cancer reconstructed model. Mechanical barrier disruption by tape-stripping of human *ex vivo* skin also resulted in CMS penetration.[60]

Furtermore, CMS was shown to increase cargo delivery of nile red (NR) into epidermis and dermis of intact human skin and porcine ear skin *ex vivo*.[60,61]

Simultaneously to the study in this thesis (see paper 1 at 2.1), also within the CRC 1112, the CMS were examined *in vivo* in an oxazolone-induced mouse model of atopic dermatitis.[4] No penetration into viable layers of the skin in intact as well as barrier altered inflamed mouse skin was shown.[4] No systemic distribution was observed following topical application and no adverse effects of the NC was observed locally in the skin or systemically, even after subcutaneous injection of the NC.[4]

1.5 Scientific questions and hypotheses

1.5.1 Do CMS show any unintended local or systemic effects after topical application on healthy or inflamed psoriasis-like murine skin?

Negative effects of the NC are not expected since available toxicological data to this particle and closely related NC do not show adverse effects *in vitro*.[4,62] (See also toxicological data in paper 1 at 2.1)

1.5.2 Do CMS penetrate into healthy murine skin and if they do, to what extent and how deep?

It is not expected that CMS penetrate into intact murine skin because *in vitro* studies using reconstructed human skin showed only penetration after prolonged exposure times and *ex vivo* studies showed no penetration of the NC in intact human skin or porcine ear skin.[60,61]

1.5.3 Is their penetration behavior influenced by a barrier alteration using the IMQ-induced psoriasis-like mouse model?

CMS penetration into a barrier disrupted skin is expected following published data showing increased NC penetration into tape-stripped *ex vivo* human skin and in an *in vitro* non-melanoma skin cancer reconstructed skin model both resembling a barrier alteration.[60]

1.5.4 Can CMS increase penetration of a cargo into intact murine skin compared to the topically applied cargo substance alone?

CMS are expected to increase cargo penetration into *in vivo* murine skin in concordance with *ex vivo* data using human or porcine skin.[60,61]

1.5.5 Can the IMQ-induced mouse model of psoriasis in BALB/c mice be used to evaluate a therapeutic surplus value of TAC-loaded CMS compared to commercial TAC ointment?

The model is expected to be applicable since other groups have used the model in different mouse strains to test several anti-inflammatory drugs.[40,63–66]

2 Own research publications in scientific journals

2.1 Stratum corneum targeting by dendritic core-multishell-nanocarriers in a mouse model of psoriasis

Authors: Pischon H, Radbruch M, Ostrowski A, Volz P, Gerecke C, Unbehauen M, Hönzke S, Hedtrich S, Fluhr JW, Haag R, Kleuser B, Alexiev U, Gruber AD, Mundhenk L.

Year: 2017

Journal: Nanomedicine: Nanotechnology, Biology, and Medicine

DOI: 10.1016/j.nano.2016.09.004

Bibliographic Source: *Pischon H, Radbruch M, Ostrowski A, Volz P, Gerecke C, Unbehauen M, Hönzke S, Hedtrich S, Fluhr JW, Haag R, Kleuser B, Alexiev U, Gruber AD, Mundhenk L (2017) Stratum corneum targeting by dendritic core-multishell-nanocarriers in a mouse model of psoriasis. Nanomedicine: Nanotechnology, Biology, and Medicine 2017, 13:317-327, DOI: 10.1016/j.nano.2016.09.004*

Declaration of own portion of work in this research publication:

Contributions of H Pischon:

1. Development of the study design including preparation, performance, and evaluation of investigations involving animal experiments, histology, fluorescence microscopy, morphometry, and immunofluorescence

2. Subsequent setup of the entire manuscript with the exception of investigations involving particle synthesis, characterization, and *in vitro* toxicological investigations as well as fluorescence lifetime imaging microscopy

Contributions of the other authors: All co-authors participated in the development of study design, evaluation of experimental results and the setup and review of the manuscript. M Radbruch and L Mundhenk, furthermore, assisted in the performance of the animal experiments. M Unbehauen prepared, conducted, and evaluated NC preparation and characterization. C Gerecke prepared, conducted, and evaluated *in vitro* toxicological analysis. P. Volz prepared, conducted, and evaluated fluorescence lifetime imaging microscopy.

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).

http://www.felasa.eu/recommendations/guidelines/felasa-guidelines-and-recommendations/

2.2 How effective is tacrolimus in the imiquimod - induced mouse model of psoriasis?

Authors: Pischon H, Radbruch M, Ostrowski A, Schumacher F, Hönzke S, Kleuser B, Hedtrich S, Fluhr JW, Gruber AD, Mundhenk L.

Year: 2018

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Contributions of H Pischon:

1. Drafting and development of the study design including animal test proposal, preparation, performance, and evaluation of investigations involving animal experiments, histology, morphometry, as well as preparation and performance of TAC extraction from skin layers for the quantification of TAC

2. Subsequent setup of the entire manuscript with the exception of investigations involving TAC quantification by liquid chromatography tandem-mass spectrometry

Contributions of the other authors: All co-authors participated in the development of study design, evaluation of experimental results and the setup and review of the manuscript. M Radbruch and L Mundhenk, furthermore, assisted in the performance of the animal experiments. F Schumacher prepared, conducted, and evaluated the quantification of TAC by liquid chromatography tandem-mass spectrometry (LC-MS/MS).

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).

http://www.felasa.eu/recommendations/guidelines/felasa-guidelines-and-recommendations/

3 Concluding discussion and outlook

3.1 Effect of core-multishell-nanocarrier on healthy or inflamed skin

No adverse effects of CMS were observed locally in skin or in any distant organs tested after repeated topical application. Furthermore, in the IMQ-model with an altered skin barrier, no worsening of the inflammation was found in the CMS treated groups. (See paper 1 at 2.1)
This confirms the hypothesis 1.5.1 (see 1.5 scientific questions and hypotheses), as expected, since toxicological prestudies *in vitro* in cell culture of fibroblasts and primary normal human keratinocytes did not report toxicity in the literature.[4,62]
In addition to the *in vivo* data, even reanalysis *in vitro* using CMS doses higher than usually used in toxicological tests did not aggravate primary normal human keratinocytes. (See paper 1 in 2.1)
In vivo experiments are necessary for toxicity testing of promising NC at advanced stages of development. Advantages over *in vitro* tests include the possibility to test for possible toxic metabolites of NC derived by degradation by enzymes and pH on the skin surface as well as intracellular metabolization by immune cells. For CMS, potentially formed toxic metabolites could be aminated polyglycerols.[67,68] This aspect might be missed in cell culture toxicology tests using only keratinocytes or tumor derived cell lines with changed cell biology due to their neoplastic transformation.
Further evidence for good biocompatibility of these particles comes from the *in vivo* experiment by Radbruch et al. (2017), which was performed simultaneous within the CRC 1112. In this experiment no adverse effects were observed after topical application on healthy murine skin and an oxazolone-induced model for atopic dermatitis with a different pathogenesis[4] compared to the psoriasis-like dermatitis model used in the study in this thesis (see Paper 1 at 2.1). In fact, not even repeated subcutaneous injection over 5 days led to local or systemic changes.[4]
In contrast to the study in this thesis (see Paper 1 at 2.1) using BALB/c mice, Radbruch et al. (2017) used SKH1 mice.
Both study protocols, of Radbruch et al. (2017) and Paper (see 2.1), with daily NC application on 5 consecutive days and subsequent sampling are not suitable to evaluate possible long-term effects, or genotoxicity of CMS. Slow accumulation over time could lead to adverse effects not observed in this study. Even potential hypersensitivity reactions with newly formed antibodies or newly primed T lymphocytes could be missed due to the short time span between the first contact and a recontact after possible antibody production against the NC.[7] This would have to be evaluated in further *in vivo* experiments with applications over a much longer period and analysis of further readout parameters.

Of note, this study was not primarily designed as toxicological study of CMS and the small number of mice per group limits its sensitivity to test for adverse effects.

3.2 Local and systemic distribution of core-multishell-nanocarrier in intact and barrier disrupted skin

Local penetration behavior of CMS in intact skin

As expected following topical application of CMS on intact murine skin, no penetration into viable layers of the skin was observed; instead, the particles accumulated in the stratum corneum and superficial hair follicle infundibula. (See paper 1 at 2.1)

In the literature, *ex vivo* studies also described no penetration of CMS into intact human skin or porcine ear skin.[60,61] *Ex vivo*, CMS penetrated only after prolonged exposure times into human skin, as well as in a non-melanoma skin cancer reconstructed model.[60]

This experiment was performed in Franz cells, common in *ex vivo* penetration studies, in which the skin is placed between a donator and acceptor chamber, which is filled with medium fluid. Over the incubation time of 24h, enabling CMS penetration, the skin was not only exposed to the applied CMS in the donator chamber but likely also overhydrated by the fluid of the acceptor compartment, leading to a swollen skin with a decreased barrier function. Therefore experiments in Franz cells, with long incubation times like 24h could lead to false positive penetration.[69–71]

Lademann et al. published a pumping mechanism for solid NP of a certain size into the hair follicle by hair movement.[72] It seems unlikely that the soft, deformable CMS would penetrate into hair follicles by this mechanism. Indeed, no particles were transported deep into the hair follicles despite massaging of the skin. Of course, evaluation of this mechanism is limited in this experiment, since the mice were depilated and thus lacked hairs reaching over the skin surface, which could have enabled the transport. However, the results are in concordance with the *in vivo* study by Radbruch et al. (2017) on hairless SKH1 mice, where sparse long hair is present, closer resembling human body skin, still no deep penetration was achieved.[4]

Ex vivo experiments using porcine ear skin[61], which is the model where the pumping mechanism was first described yielded similar results.[20] Therefore, this mechanism seems unimportant for the CMS.

Uptake of CMS into Langerhans cells has been reported in cell culture *in vitro*.[73] Also in paper 1, (see 2.1) a rare uptake of CMS by Langerhans cells is suggested. This was a rare observation of 4 colocalizations of a fluorescent signal with immunofluorescently marked Langerhans cells in 31 analyzed skin sections. For validation purposes of these signals, a fluorescence lifetime imaging microscopy (FLIM) examination could be helpful, particularly with the novel clustering technique used in paper 1. (See 2.1) Using FLIM, the fluorescent signal

could be identified with greater specificity and discriminated from autofluorescence or other artifacts, as the fluorescent tag indocarbocyanine (ICC) exhibits a unique fluorescent lifetime signature, which differs from fluorescing substances with the same emission spectrum. (For further details, see paper 1 at 2.1) The confocal character of FLIM also allows for the localization of the fluorescent signal to the intracellular compartment or the extracellular space next to the immunofluorescently stained Langerhans cell. For technical reasons, this was impossible for the slide in which the signals were observed. Even in a high number of examined consecutive slides, no further colocalizations of fluorescent signals with Langerhans cells in the viable epidermis could be found. To validate an uptake of topically applied CMS by Langerhans cells *in vivo*, more extensive studies have to be conducted to repeat the rare observation including for example FLIM analysis.

Local penetration behavior of CMS in barrier altered skin models

Surprisingly, the barrier alteration induced by IMQ in the psoriasis-like dermatitis model had no effect on the CMS penetration behavior. Even on ulcerated areas with serocellular crusts, no particles were found in deeper, viable layers of the skin. (See paper 1 at 2.1)

In the oxazolone-induced atopic dermatitis model, representing a different barrier alteration by inflammation by Radbruch et al. (2017) showed similar results without CMS penetration *in vivo*.[4]

This lack of penetration in both models is in contrast to the literature showing penetration into barrier altered non-melanoma skin cancer reconstructed models or *ex vivo* human skin with a barrier disruption by tape stripping.[60] This highlights the importance of testing in different barrier altered models and the final proof in human skin diseases. Maybe the inflammatory skin models resemble barrier function of human inflammatory skin diseases like atopic dermatitis and psoriasis better than the *in vitro* and *ex vivo* models with a more artificial barrier disruption, which enabled NC penetration.[60] In contrast to the decreased outside-inside barrier function by tape stripping, the various complex alterations in human psoriasis, including hyperkeratosis and epidermal thickening, do not have to sum up to an outside-inside barrier impairment but could also hinder the penetration of substances or particles.[22] TEWL is a widely used parameter to measure a barrier dysfunction in clinic and research. It is based on increased permeability of water from inside to outside, which was elevated in both induced inflammatory mouse models. However, that does not necessarily have to reflect outside-inside barrier function.[4] (See paper 1 at 2.1) Furthermore, the barrier function has to be evaluated for each substance or particle individually, even though predictions can be made regarding size, surface charge, and hydrophilicity.[47]

It must be kept in mind that the fluorescent ICC tag bound covalently to the CMS to make particle tracing in tissues possible could potentially influence the penetration behavior of the NC by steric changes.[74] (See paper 1 at 2.1) Furthermore could a cargo drug loading into the NC change penetration behavior slightly. A cargo could influence steric properties of the NC regarding deformation or interplay with tissue. To evaluate these aspects additional *in vitro* studies with a more systemic approach comparing unloaded and loaded NC with different cargos in the same model are needed. In addition, common covalently bound fluorescent tags should be compared to less convenient but less influential radioactive tags[75]. However, these experiments exceed the scope of this work.

The hypothesis that CMS penetrate into skin of the barrier altered IMQ-induced mouse model is not supported by these results. Further *ex vivo* studies using human psoriasis lesional skin could help to elucidate real penetration behavior in the planned field of application as a mechanism for targeted drug delivery.

Systemic distribution of CMS

Not surprisingly, considering that CMS do not penetrate into skin, CMS were not found in any draining lymph nodes or distant organs. (See paper 1 at 2.1) Distant organs screened included spleen, liver, lung, brain, thymus, heart, stomach, small and large intestine, mesenterial lymph nodes, pancreas, thyroid glands, adrenal glands, kidneys, testicles, and bone marrow.

Radbruch et al. (2017) identified organs prone to CMS accumulation after simulated full penetration through skin by repeated subcutaneous injection of CMS.[4] The particles were found in regional draining lymph nodes, in some glomeruli in the kidneys, in the liver, most likely in Kupffer cells, pulmonary alveolar macrophages, and spleen macrophages without any pathologic effects.[4] The latter three belong to the mononuclear phagocyte system, filtering agents from circulating blood.[4]

In the study in this thesis CMS were, as mentioned above, not found in these identified accumulation prone regions[4] after topical application on intact or barrier altered skin. (See paper 1 at 2.1)

3.3 Cargo delivery of core-multishell-nanocarrier

Although CMS did not penetrate into viable skin layers themselves, they have the ability to increase the penetration of loaded cargo, nile red (NR), into intact murine skin compared to NR in a cream. (See paper 1 at 2.1)

So the data support the hypothesis 1.5.4.

Literature also showed increased NR delivery by CMS into *ex vivo* porcine and human intact and tape stripped skin, as well as in *in vitro* human reconstructed skin.[60,61]

The original concept (see also above at 1.4 Nanomaterial and Nanocarrier; Nanocarrier dermal drug delivery concept) of NC was that they would penetrate into skin and bring their loaded cargo along. In the deeper skin layers, this cargo would be released and binds to target structures or diffuses further to their side of action. Since CMS do not seem to penetrate skin but still deliver their cargo, the question arises how they do that.

One hypothesis is that they penetrate with the cargo into the stratum corneum, from where they slowly release the cargo as a depot, holding the cargo longer in the stratum corneum increasing the time to penetrate deeper without being washed or rubbed off. A depot effect is to some extent also achieved by a cream or an ointment, accumulating in the stratum corneum lipids.[76]

Another hypothesis is that CMS work as penetration enhancers by lowering the skin barrier function, which is mainly a property of the stratum corneum. The flexible, dendritic structure of CMS with long amphiphilic side chains forming the shells (also see Architecture of core-multishell-nanocarrier at 1.4 Nanomaterial and Nanocarrier) could lead to intercalation in lipid bilayers in the stratum corneum. Thereby, the order of the extracellular stratum corneum lipids could be disturbed, potentially leading to a barrier disruption and increased cargo penetration.[4]

Penetration enhancers are already used in pharmacology and in cream to achieve sufficient penetration of drugs. A prototype is dimethyl sulfoxide (DMSO), which has been widely studied and can lead to severe pathohistologically visible changes in skin.[77] CMS do not lead to changes visible by light microscopy, which could be an advantage. Further experiments are needed to evaluate CMS mode of action, which is beyond the scope of this project. To elucidate the function of CMS as penetration enhancer an *in vitro* study could be conducted comparing loaded CMS to a mixture of unloaded CMS with free cargo and to the cargo substance alone. A comparison of CMS to standard penetration enhancers would be needed to show a surplus to already used penetration enhancers.

The increased delivery of the loaded cargo has to be proven for real drugs loaded into the carriers. This could be done *ex vivo*, complemented in a second phase with an *in vivo* experiment using inflammation models to show a therapeutic surplus value of the CMS.

Further studies should focus on the mode of action of CMS using different techniques. Several collaborations have been started to find changes in skin treated with CMS. TEM is used to visualize possible ultrastructural changes in the stratum corneum. In cooperation within the CRC Raman spectroscopy is used to study the order of lipids within the stratum corneum after CMS incubation.

3.4 Applicability of the imiquimod-induced psoriasis-like dermatitis model for *in vivo* testing of the topical, anti-inflammatory drug tacrolimus or nanocarrier for its delivery

Choosing the right model for an experiment is essential to generate reliable results, but can be difficult if there is a lack of information in literature for either detailed characterization or comparison of models. Prediction or comparison of results with other studies elaborate similar hypotheses is limited by usage of model, mouse strains, protocols, and conducted readout.
According to Nestle and Nickoloff the ideal model for psoriasis should fulfill the following criteria:[78] 1) epidermal changes including hyperproliferating keratinocytes with altered differentiation patterns, 2) papillomatosis, 3) intralesional inflammatory cells including T lymphocytes, dendritic cells, monocytes, macrophages, neutrophils, and mast cells, 4) pivotal functional role of T lymphocytes, 5) vascular changes characterized by tortuous capillaries and increased numbers of endothelial cells, and 6) response to anti-psoriatic drugs.
The IMQ-induced psoriasis-like mouse-model fulfills criteria 1, 3, 4 and 5 but van der Fits et al. already stated that the response to anti-psoriatic drugs still has to be shown.[36]

In paper 2, (see paper 2 at 2.2) topical treatment of the IMQ model with TAC was conducted using three different treatment protocols. In humans the effectiveness of TAC has been shown for facial psoriasis; in the plaque-type psoriasis it is hypothesized that poor skin penetration could inhibit topical efficacy.[22] This poses a potential application for NC, if they are able to increase dermal cargo delivery.
Topical TAC treatment once daily, starting on the fourth day of dermatitis induction, could not exert TAC-specific anti-inflammatory efficacy. (See paper 2 including supplemental material at 2.2)
Also in a preventative approach (see section “second treatment protocol” in Paper 2 at 2.2) with treatment starting on the first day of dermatitis induction, no TAC-specific anti-inflammatory efficacy was observed.
There is a conflict to reported successful treatment of the IMQ model with TAC.[40,63–66] On one hand, some induced the psoriasis-like dermatitis in different mouse strains or used slightly different protocols, which might have an impact on the experimental outcome, complicating a direct comparison of results.[64,79] On the other hand, some studies were missing vehicle controls only comparing to untreated controls[63–66] or showed only minimal improvement of rather subjective clinical scores compared to vehicle controls.[40] In those cases, drug unspecific skincare effects of the vehicle or random individual variations of dermatitis induction or maintenance severity favoring TAC groups might have been over interpreted as TAC-specific

effects. This could point towards the possibility that TAC might not be effective in the IMQ model at least using BALB/c mice.

Hypotheses why TAC-specific effects in the IMQ model could not been shown here include the following (see paper 2 at 2.2):

The barrier alteration of the model or application regimes could have impaired TAC penetration in effective amounts into the skin. There are no data on effective amounts extractable from skin layers in successfully treated human psoriasis or murine models of psoriasis. Nonetheless, after topical application of commercially available ointment with the highest TAC concentration in the second treatment protocol, a TAC concentration of 300 ng/cm² in the dermis could be achieved. This is in the same magnitude as TAC extracted from skin of successfully treated humans with atopic dermatitis.[80] Therefore, it can be assumed that TAC penetration was sufficient to its site of action, the lymphocytes in the dermis.[81]

Furthermore, the drug could target pathways not pivotal for the phenotype development in the IMQ model.

TAC is a calcineurin inhibitor.[38] Its immunosuppressive effect is due to interaction of several pathways leading to decreased transcription of cytokines important for T cell function. By inhibiting the calmodulin-dependent phosphatase calcineurin, nuclear translocation of nuclear factor of activated T lymphocytes (NFAT) and nuclear factor kappa-light-chain-enhancer of activated B-cells (NFkB) is blocked and transcription of IL 2 but also IL 4, 10, and 17, TNF α, and IFN y is decreased. Lack of these cytokines influences T helper and T killer lymphocyte differentiation, activation and survival. TAC also blocks the activation of mitogen-activated protein (MAP)-kinase-kinase-kinases like MAP3K11 and MAP3K7, leading to decreased c-Jun N-terminal Kinases (JNK) and p38 activation, which modulate activator protein (AP) 1 family related gene expression. TAC also inhibits IL-2-dependent T cell proliferation via increasing transforming growth factor β1 (TGFβ1) expression.[39]

The IMQ-induced psoriasis-like dermatitis is a primarily T lymphocyte driven immune response due to TLR 7 and 8 activation. (See also Model used in this study: Imiquimod–induced psoriasis-like dermatitis at 1.2 Psoriasis – a common inflammatory skin disease)

TLR 7 and 8 are pattern recognition receptors expressed in endosomes in plasmocytoid dendritic cells, monocytes, macrophages, B lymphocytes and eosinophils. Their activation signals via the adaptor protein myeloid differentiation primary response 88 (MyD88) and transcription factors like NFkB and activator protein 1 (AP-1) family members are activated leading to increased expression of pro-inflammatory cytokines and chemokines including IFN α and β. AP-1 is activated via MAP-kinase signaling pathway including p38.[82] Some pathways, which activate NFkB and AP-1 via p38 can be blocked by TAC.[39]

IMQ induces plasmocytoid dendritic cell maturation,[83] T helper 1 lymphocyte response[84] and inflammatory cytokine production by keratinocytes.[36,85,86]
TLR-independent effects include adenosine receptor signaling interference leading to similar inflammatory effects.[87] In this pathway, calcineurin is involved, which is inhibited by TAC.
IL 22, IL 36, and the IL 23-IL 17 pathways play pivotal roles in the IMQ-induced phenotype development.[26,36] IL 17A is predominantly produced by different T lymphocytes including T helper 17 lymphocytes.[88] It can also be produced by neutrophils, but since lymphocytes are the main component of leukocyte infiltration in this model this seems less important.[89] IL 17 production via NFAT is also blocked by calcineurin inhibition of TAC.
Since IMQ is T lymphocyte driven and TAC interferes with proper T lymphocyte inflammatory responses, it suggests itself that TAC would interfere with dermatitis induction in this mouse model.
Finally, it is also conceivable that the TAC efficacy on IMQ-induced dermatitis is mouse strain dependent. In this study, BALB/c mice were used, since van der Fits et al. introduced the IMQ-induced mouse model in this strain and it was further used in literature for this model.[36] Some studies reporting TAC specific effects induced the IMQ model in C57BL/6 mice.[64,79] Mouse strain dependent differences in experimental outcome are not unusual.[79] Both C57BL/6 and BALB/c mice are inbred strains with a very narrow, yet different genepool background. Different responses of the immune system have been described.[79] In contrast to these strains, the IMQ-model was not inducible at all in the hairless, but immunocompetent and therefore for dermatology studies convenient SKH1 mouse strain (see paper 2 at 2.2). This strongly suggests a high dependence on the right genetic background.

To evaluate why this model might not be treatable with TAC or why it might not be possible in this mouse strain is beyond the scope of this work but the elucidation could be valuable for research. Further elucidation could serve important information about the inflammatory pathways of this model or their dysfunction in this mouse strain, both further identifying limitations and possible applications for evaluation of specific questions. This could even help to understand pathogenesis of human psoriasis better and possibly result in new targets for clinical treatment.
However, the IMQ-induced psoriasis like dermatitis model was not applicable to evaluate a therapeutic surplus value of TAC-loaded CMS over TAC ointment, at least using these protocols in BALB/c mice.

In contrast to the first two treatment protocols just discussed, in the third treatment protocol (see paper 2 at 2.2) test substances were applied twice daily leading to no or only minimal psoriasis-like phenotype expression. Twice daily, topical test substance application somehow

disturbed the model induction via topical IMQ application in all groups. This observation was independent of the type of test substance, including hydrogel and ointment without active compounds and TAC ointment. Therefore, the absence of skin inflammation induction cannot depict a TAC-specific anti-inflammatory effect.

A lack of model induction could most conveniently be explained by lack of IMQ penetration due to interaction with the other applied formulations. This could especially arise by two mechanisms:

a) If a formulation is applied before application of IMQ, it could physically hinder IMQ penetration into the skin. For example, application of ointment could lead to deposition of additional lipids in the stratum corneum and thus increase hydrophobicity of this layer. Remnants of hydrogel could also remain in the stratum corneum and hinder IMQ penetration. However, dermatitis evolved in the first treatment protocol with test substance application approximately 4 h before application of IMQ. This is the same period of time between the first test substance application and the IMQ application in the third protocol, thus this alone cannot explain the lack of model induction.

b) The amount of time IMQ remained on the skin could be too short for sufficient penetration of IMQ. This could happen if a test substance is applied after IMQ, because removal of remaining IMQ cream from the skin surface was needed prior to every application of test substance. However, dermatitis also evolved in the second treatment protocol with removal of IMQ cream and test substance application approximately 2 h after IMQ application. This is the same period of time between the IMQ application and the second test substance application in the third protocol, thus this alone cannot explain the lack of model induction.

Only in the third treatment protocol, being a mixture of the first two protocols with IMQ application 4 h after and 2 h before test substance application, IMQ effects were inhibited.

It is suspected that the additive effect of test substance remnants, hindering penetration, and premature removal of IMQ from the skin prevented model induction even if neither effect alone prevented it.

Another possible explanation would be that IMQ did penetrate sufficiently, but unspecific skin care effects of any hydrogel or ointment application block the phenotype expression. Skin care effects could cause a protection of the skin barrier. A disturbance of the skin barrier possibly is a necessary secondary, worsening aspect of phenotype development.

Whichever explanation may reflect the truth, the third protocol, as conducted, is unsuitable to test any applied substances for anti-inflammatory efficacy or penetration behavior in altered skin barrier (see paper 2 at 2.2). Perhaps a longer time period between the IMQ and the second test substance application could improve model induction, but this is speculative.

3.5 Conclusions

NC have to fulfill three major requirements for the usage for drug delivery in inflammatory skin diseases: 1) they have to be biocompatible, 2) they need to improve drug delivery to the site of action and 3) this has to lead to a therapeutic surplus value over conventional drug formulation.

To conclude, the CMS themselves do not penetrate into murine skin, intact or inflamed, and do not show any adverse effects. Thus, CMS seem to fulfill requirement number 1, but long-term toxicological studies in larger numbers of individuals and clinical trials have to complement these results.

The increased cargo delivery into skin, shown for the model drug NR, is promising for the further development as NC. Nevertheless, superior cargo delivery has to be tested for individual pharmacologically active drugs like TAC to fulfill the second requirement.

A therapeutic surplus value still has to be shown for the usage of CMS compared to commercial formulations of the drugs.

The IMQ-induced psoriasis-like mouse model seems not applicable for that task, since no TAC-specific drug efficacy could be shown in BALB/c mice.

In summary, the CMS are promising candidates for a further development regarding biocompatibility and improved drug delivery but a therapeutic surplus value needs to be shown.

Further studies should be conducted to uncover the cargo delivery mechanism of CMS. This could improve refinement or invention of new NC or help to find other fields of application for the CMS.

In addition to the findings regarding NC, three important observations were made:

a) TAC may not be effective in the IMQ-induced psoriasis-like mouse model in BALB/c mice.
b) Suitable vehicle controls, often omitted in previous reports are imperative, because vehicles may have a strong influence on the model.
c) SKH 1 hairless mice should not be used for the IMQ model, since it is not inducible in this strain following standard protocols.

Why the psoriasis-like dermatitis could not be alleviated with TAC as well as why the model could not be induced in SKH1 mice remains unclear. Further studies to elucidate this could be beneficial to gain more insights into the pathogenesis of the model or the immune system of the mouse strains giving new applications or limitations for both, or possibly help to better understand the pathogenesis of human psoriasis leading to new therapeutic targets.

4 Summary

Core-Multishell-Nanocarrier for Topical Drug Delivery in a Psoriasis Mouse Model

Jeanette HANNAH Charlotte Pischon

Psoriasis is a common, chronic, multifactorial, human skin disease, characterized by well-demarcated, raised, erythematous plaques covered with silvery scales.[1,3] It is incurable and often requires long period therapy with immunomodulatory drugs, which can lead to side effects, especially when administered systemically in more severe cases.[3] Nanocarrier are engineered particles of a size between 1 nm and 100 nm at least in one dimension. For skin, nanocarrier are designed to increase the delivery of drugs or genes through the skin barrier, target the drug to a specific layer, or prevent systemic distribution and thereby negative effects in distant organs.

In paper 1 of this thesis, core-multishell nanocarrier were investigated topically on mouse skin *in vivo*. This nanocarrier had been designed like a uni-molecular micelle[4] with a hydrophilic core, an inner lipophilic shell, and an outer hydrophilic shell to make the particle water-soluble and provide space for drugs of different lipophilicity to be loaded into the core or the inner shell. Originally, they had been thought to penetrate through the skin and release their cargo at its site of action. Literature already had stated that several nanocarrier or nanoparticles do not penetrate into skin, whereas others do and even others penetrate only through a disrupted barrier.[19,21] The core-multishell nanocarrier used here did not penetrate into viable layers of intact or inflamed skin using the imiquimod-induced psoriasis model in BALB/c mice. Instead, they accumulated in the stratum corneum. Previous *in vitro* data had shown core-multishell nanocarrier penetration into tape stripped human skin *ex vivo*.[60] The accumulation in the stratum corneum could possibly be used as a depot for a retarded and prolonged release of drugs.

Parallel to the penetration study no adverse effects were observed locally or systemically. This is in concordance with literature also stating no negative effects even after repeated subcutaneous injection of this carrier.[4]

The topical application of nile red loaded core-multishell nanocarrier revealed superior cargo delivery into the viable epidermis compared to a nile red cream. Further elucidation of the mechanism by which the core-multishell nanocarrier enhances the penetration of nile red, and proof of concept for real drugs are needed. However, the core-multishell nanocarrier remains as a promising candidate for further development for therapy of skin diseases including testing

of a therapeutic surplus value of drug loaded nanocarrier compared to commercial topical drug formulations.

No tacrolimus-specific anti-inflammatory effects could be shown in the imiquimod-induced psoriasis-like dermatitis model using BALB/c mice, despite penetration of the drug into the dermis.

Further research is needed to elucidate the reason for the lack of that efficacy and the conflict to literature and the dependence of tacrolimus efficacy in this model on mouse strains.[40,63–66] However, this model was not applicable to evaluate therapeutic superiority of core-multishell nanocarrier for tacrolimus delivery compared to tacrolimus ointment.

The importance of choosing an appropriate model for the specific question, using multiple objective readout parameters to avoid over-interpretation of small variations, and testing against all needed control groups, including a vehicle control, in addition to untreated controls, is highlighted.

5 Zusammenfassung

Core-Multishell-Nanocarrier für den topischen Wirkstofftransport

in einem Psoriasis Mausmodell

Jeanette HANNAH Charlotte Pischon

Psoriasis ist eine häufige, chronische, multifaktorielle Hauterkrankung des Menschen, welche durch scharf begrenzte, erhabene, erythematöse Plaques, die mit silbrigen Schuppen bedeckt sind, charakterisiert ist.[1,3] Psoriasis ist unheilbar und oft ist eine Langzeittherapie mit immunmodulatorischen Medikamenten nötig, welche vor allem in schweren Fällen bei systemischer Applikation zu unerwünschten Nebenwirkungen führen können.[3] Nanocarrier sind synthetische Partikel von einer Größe zwischen 1 nm und 100 nm in mindestens einer Dimension. Für die Anwendung auf der Haut wurden Nanocarrier entwickelt um, Wirkstoffe oder Gene vermehrt durch die Hautbarriere zu transportieren, bestimmte Schichten anzusteuern oder eine systemische Verteilung des Wirkstoffes, und dabei Nebenwirkungen in Organen, zu verhindern.

In der ersten Publikation aus dieser Dissertation wurden Core-Multishell Nanocarrier *in vivo* topisch auf Maushaut getestet. Dieser Nanocarrier wurde in Analogie zu einer ein-molekularen Mizelle[4] mit einem hydrophilen Kern, einer lipophilen inneren Schale und einer hydrophilen äußeren Schale entwickelt, um den Partikel wasserlöslich zu machen und Wirkstoffe verschiedener Lipophilität im Kern und der inneren Schale transportieren zu können. Ursprünglich sollten die Nanocarrier in die Haut eindringen und ihre Ladung an dessen Wirkungsort freigeben. In der Literatur ist bereits zu finden, dass verschiedene Nanocarrier und Nanopartikel nicht in Haut eindringen, wobei andere dieses tun und wieder andere nur in barrieregestörte Haut eindringen.[19,21] Die hier genutzten Core-Multishell Nanocarrier drangen nicht in vitale Hautschichten von gesunder oder entzündeter Haut des Imiquimod induzierten Psoriasis ähnlichen Mausmodels in BALB/c Mäusen ein, sondern akkumulierten im Stratum corneum. Vorangegangene *in vitro* Studien hatten bereits ein Eindringen von Core-Multishell Nanocarriern in humane Haut *ex vivo* nach *Tape stripping* gezeigt.[60] Diese Ansammlung der Nanocarrier im Stratum corneum könnte möglicherweise als Depot für eine retardierte und damit verlängerte Wirkstofffreisetzung genutzt werden.

Gleichzeitig konnten in den Penetrationsexperimenten keine lokalen oder systemischen negativen Effekte der Core-Multishell Nanocarrier beobachtet werden. Das steht in Einklang

mit der vorhandenen Literatur, welche sogar nach wiederholter subkutaner Injektion dieser Nanocarrier keine negativen Effekte beschreibt.[4]

Die topische Applikation von mit Nilrot beladenen Core-Multishell Nanocarriern zeigte einen vermehrten Ladungstransport in die vitale Epidermis im Vergleich zu Nilrot in einer Crème. Weitere Aufklärung des Mechanismus, mit dem die Core-Multishell Nanocarrier das Eindringen von Nilrot verstärken, und ein Wirksamkeitsnachweis für echte Wirkstoffe benötigen weiterführende Untersuchungen. Allerdings bleiben die Core-Multishell Nanocarrier vielversprechende Kandidaten für die Weiterentwicklung der Nanocarrier zur Therapie von Hauterkrankungen einschließlich des Nachweises eines Therapievorteils wirkstoffbeladener Core-Multishell Nanocarrier gegenüber kommerziellen topischen Formulierungen.

Es konnten keine Tacrolimus spezifischen antiinflammatorischen Effekte im Psoriasis ähnlichen, Imiquimod induzierten Dermatitis Modell in BALB/c Mäusen gezeigt werden, trotz Eindringen des Wirkstoffes in die Dermis.

Weitergehende Untersuchungen sind notwendig, um den Grund für die fehlende Effizienz, dessen Widerspruch mit vorhandener Literatur und eine mögliche Abhängigkeit der Tacrolimus Wirksamkeit in diesem Modell von Mausstämmen zu erforschen.[40,63–66] Unabhängig davon ist das Imiquimod induzierte Psoriasis Modell mit den hier verwendeten Protokollen in BALB/c Mäusen nicht zur Testung eines möglichen Therapievorteils von Core-Multishell Nanocarriern zum Tacrolimus Transport gegen Tacrolimussalbe geeignet.

Des Weiteren wird die Wichtigkeit betont, ein passendes Modell zur Forschungsfragestellung auszuwählen und mehrere objektive Parameter auszuwerten, um Überinterpretation von kleinen Schwankungen gegen alle notwendigen Kontrollgruppen inklusive Vehikelkontrollen zusätzlich zu unbehandelten Kontrollen zu vermeiden.

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7 List of additional own publications regarding this topic

Other research papers in scientific journals

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Radbruch M, **Pischon H,** Ostrowski A, Unbehauen M, Haag R, Mundhenk L., Gruber AD. Nanocarrier auf atopischer Dermatitis im Mausmodell: Die Hautbarriere hält. 59. Jahrestagung der DVG-Fachgruppe Pathologie 2016 Fulda. Oral talk abstract in: *Tierarztl Prax Ausg K*. Stuttgart, Schattauer, 2016; 44(3): A34

Pischon H, Radbruch M, Ostrowski A, Unbehauen M, Haag R, Gruber AD and Mundhenk L. Dermale Penetration von Core-Multishell-Nanocarriern im Psoriasis-like Mausmodell. 59. Jahrestagung der DVG-Fachgruppe Pathologie 2016 Fulda. Poster abstract in: *Tierarztl Prax Ausg K*. Stuttgart, Schattauer, 2016; 44(3): A37-A38

Radbruch M, **Pischon H**, Ostrowski A, Unbehauen M, Volz P, Alexiev U, Haag R, Gruber AD, Mundhenk L. The stratum corneum is the target of CMS nanocarriers after topical application in healthy and inflamed mouse skin. International Conference on Dermal Drug Delivery by Nanocarriers 2016 Berlin. Oral talk abstract in proceedings: http://www.sfb1112.de/Internationale-Tagung-des-SFB-1112/Abstracts/index.html

Pischon H, Radbruch M, Ostrowski A, Unbehauen M, Gerecke C, Kleuser B, Haag R, Alexiev U, Mundhenk L, Gruber AD. CMS nanocarriers possess high biocompatibility in vitro and in mice following topical application on healthy and inflamed mouse skin. International Conference on Dermal Drug Delivery by Nanocarriers 2016 Berlin. Poster abstract in proceedings: http://www.sfb1112.de/Internationale-Tagung-des-SFB-1112/Abstracts/index.html

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Radbruch M, **Pischon H**, Ostrowski A, Unbehauen M, Volz P, Alexiev U, Haag R, Gruber AD, Mundhenk L. Tracking polymeric core-mutlishell-nanocarrier and its cargo after topical application in vivo. DRS Biomedical Sciences PhD Student Symposium 2016 Berlin. Oral talk abstract in: 9. Doktorandensymposium & DRS Präsentationsseminar "Biomedical Sciences", Fachbereich Veterinärmedizin, Freie Universität Berlin: 16. September 2016, Programm & Abstracts. Berlin, Mensch und Buch Verlag, 2016; p 60. *ISBN 978-3-86387-744-6*

Fürstenau J, **Pischon H**, Dietert K, Radbruch M, Gruber AD, Mundhenk L. CLCA2 is overexpressed in psoriatic but not in atopic dermatitis mouse skin models. 34rd Annual Meeting of the European Society of Veterinary Pathology & 27th Annual Meeting of the European College of Veterinary Pathologists 2016 Bologna, Italy. Poster abstract in: *J Comp Pathol*. 2017; 156(1): 137. DOI: 10.1016/j.jcpa.2016.11.251

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Radbruch M, **Pischon H**, Giulbudagian M, Du F, Yamamoto K, Klossek A, Rühl E, Calderon M, Haag R, Gruber AD, Mundhenk L. Neue Nanocarrier in der Haut: besondere Möglichkeiten, besondere Risiken. 60. Jahrestagung der DVG-Fachgruppe Pathologie 2017 Fulda. Oral talk abstract in: *Tierarztl Prax Ausg K*. Stuttgart, Schattauer, 2017; 45(3): A19-A26. DOI: 10.1055/s-0038-1625020

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10 Declaration of Originality

Hereby, I declare that the present thesis has been prepared by myself. I assure that I exclusively used the mentioned sources and facilities.

Berlin, 21.11.2018 Jeanette HANNAH Charlotte Pischon