New insights in contact allergy and drug delivery

A study of formulation effects and hapten targets in skin using two-photon fluorescence microscopy

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UNIVERSITY OF
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DOCTORAL THESIS

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New insights in contact allergy and drug delivery
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Abstract

The skin is a remarkable barrier, protecting us from invasion of e.g. harmful microorganisms and UV-radiation. However, the skin is not adopted to resist repeated exposure to the multitude of xenobiotics introduced into modern society. Some of these chemicals are skin sensitizers, and exposure can lead to the development of contact allergy. Contact allergy has significant social and economic consequences, both for the individual and for society. It is therefore important to prevent sensitization. The skin also constitutes a potential route for administration of drugs, and much effort is put into the development of cutaneous and transdermal drug delivery systems.

The work of this thesis aims to improve the understanding of processes related to the interactions between the skin and topically applied compounds, i.e. drugs and skin sensitizers. Specifically, two-photon microscopy has been used to study the cutaneous absorption and distribution of model drugs and a series of model skin sensitizers.

Improved cutaneous absorption was demonstrated using formulations composed of lipid cubic phases. The work also showed elevated sensitization potency of haptens depending on delivery vehicles. Putative mechanistic explanations for the observed effects have been proposed. Specifically, phthalates were shown to increase the sensitization potency of isothiocyanates. The phthalate-induced effect could be linked to a PSU-targeted delivery of the haptens into the skin. It could also be shown that vehicles alter hapten reactivity to *stratum corneum* proteins leading to variations in sensitization potency. Moreover, hapten protein targets in skin have been identified using caged fluorescent model hapten. Specifically, basal cell keratinocytes and the keratins were identified as specific hapten targets in the skin.

In conclusion, the work presented in this thesis contributes to the general understanding of the mechanisms involved in the cutaneous absorption of topically applied drugs and skin sensitizers. It also demonstrates the capabilities of using TPM when investigating the interactions between the skin and xenobiotics.

Keywords: allergic contact dermatitis, bromobimane, confocal microscopy, contact allergy, cubic phases, cutaneous absorption, dermatochemistry, ethosomes, FITC, hair-follicle, hapten, isothiocyanate, lipid vesicles, local lymph node assay, nano, percutaneous absorption, pilosebaceous unit, RBITC, two-photon microscopy, vehicle effects.

List of Publications

This thesis is based on the work presented in the following publications and manuscripts. The publications are reprinted with the permission from the publishers.

Paper I. Lipid cubic phases in topical drug delivery: Visualization of skin distribution using two-photon microscopy. Bender, J., Simonsson, C., Smedh, M., Engström, S., and Ericson, M.B., *Journal of Controlled Release*, 2008. **129**: 163-169.

Paper II. Accumulation of FITC near stratum corneum-visualizing epidermal distribution of a strong sensitizer using two-photon microscopy. Samuelsson, K., Simonsson, C., Jonsson, C.A., Westman, G., Ericson, M.B., and Karlberg, A.T., *Contact Dermatitis*, 2009. **61**: 91-100.

Paper III. A study of the enhanced sensitizing capacity of a contact allergen in lipid vesicle formulations. Simonsson, C., Madsen, J.T., Graneli, A., Andersen, K.E., Karlberg, A.-T., Jonsson, C.A., and Ericson, M.B., *Toxicology and Applied Pharmacology*, 2011. **252**: 221-227.

Paper IV. Caged fluorescent haptens reveal the generation of cryptic epitopes in allergic contact dermatitis. Simonsson, C., Andersson, S.I., Stenfeldt, A.L., Bergström, J., Bauer, B., Jonsson, C.A., Ericson, M.B., and Broo, K.S., *Journal of Investigative Dermatology*, 2011. **131**: 1486-1493.

Paper V. The pilosebaceous unit – a phthalate-induced highway to skin sensitization. Simonsson, C., Stenfeldt, A.L., Karlberg, A.-T., Ericson, M.B., Jonsson, C.A *Submitted for publication*.

Publications not included in the thesis

Two photon microscopy for studies of xenobiotics in human skin Simonsson, C., Smedh, M., Jonsson, C., Karlberg, A.T., and Ericson, M.B., *Optics in Life Science, Proceedings of SPIE,* 2007, 6633.

Two-photon laser-scanning fluorescence microscopy applied for studies of human skin. Ericson, M.B., Simonsson, C., Guldbrand, S., Ljungblad, C., Paoli, J., and Smedh, M., *Journal of Biophotonics*, 2008. **1**: 320-330.

Temporal imaging chamber (TIC) for en face imaging of epidermal absorption in vitro. Simonsson, C., Smedh, M., Jonsson, C., and Ericson, M.B., *Progress in Biomedical Optics and Imaging, Proceedings of SPIE,* 2009, 7367.

Point spread function measured in human skin using two-photon fluorescence microscopy. Guldbrand, S., Simonsson, C., Smedh, M., and Ericson, M.B., *Progress in Biomedical Optics and Imaging, Proceedings of SPIE,* 2009, 7367.

Two-photon fluorescence correlation microscopy combined with measurements of point spread function; investigations made in human skin. Guldbrand, S., Simonsson, C., Goksör, M., Smedh, M., and Ericson, M.B., *Optics Express*, 2010. **18**: 15289-15302.

Ethosome formulations of known contact allergens can increase their sensitizing capacity. Madsen, J.T., Vogel, S., Karlberg, A.T., Simonsson, C., Johansen, J.D., and Andersen, K.E., *Acta Dermato-Venereologica*, 2010. **90**: 374-8.

Ethosome formulation of contact allergens may enhance patch test reactions in patients. Madsen, J.T., Vogel, S., Karlberg, A.-T., Simonsson, C., Johansen, J.D., and Andersen, K.E., *Contact Dermatitis*, 2010. **63**: 209-214.

Modification and expulsion of keratins by human epidermal keratinocytes upon hapten exposure in vitro. Bauer, B., Andersson, S.I., Stenfeldt, A.L., Simonsson, C., Bergström, J., Ericson, M.B., Jonsson, C.A., and Broo, K.S., *Chemical Research in Toxicology*, 2011. **24**: 737-743.

Contribution Report

The author has made the following contribution to the included publications.

Paper I. Contributed to the design of the study, performing the *in vitro* skin penetration and imaging experiments, the interpretation of the results and in writing the manuscript.

Paper II. Contributed to the formulation of the research problem and the design of the study; performed the *in vitro* skin penetration and imaging experiments; contributed to the interpretation of the results and in writing the manuscript.

Paper III. Major contribution to the formulation of the research problem and to the design of the study; performed the experiments, major contribution in the interpretation of the results and wrote the manuscript. Corresponding author.

Paper IV. Contributed to the formulation of the research problem; major contribution in the design of the study; performed the LLNA, the *in vitro* skin penetration experiments, the immunohistochemistry and the imaging experiments; major contribution in the interpretation of the results and in writing the manuscript.

Paper V. Formulated the research problem and designed the study; major contribution in performing the experiments and the interpretation of the results; wrote the manuscript. Corresponding author.

Abbreviations and Symbols

ACD	Allergic contact dermatitis
A:DBP	Acetone:dibutylphthalate
C54	Cysteine 54
DBP	Dibutyl phthalate
dBBr	Dibromobimane
Da	Dalton
DMSO	Dimethylsulfoxide
dpm	Disintegrations per minute
Et:W	Ethanol:water
FITC	Fluorescein isothiocyanate
K5	Keratin 5
K14	Keratin 14
LLNA	Local lymph node assay
LYVE	Lymphatic vessel endothelial hyaluronan receptor
mBBr	Monobromobimane
MHC	Major histocompatibility complex
MO	Monoolein
NA	Numerical aperture
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PSU	Pilosebaceous unit
PT	Phytantriol
RBITC	Rhodamine B isothiocyanate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SI	Stimulation index
SRB	Sulforhodamine B
TPM	Two-photon microscopy

C	Concentration
D	Diffusion constant
δ_2	Two-photon absorption cross-section
f_p	Pulse repetition rate
J	Flux
K	Partition coefficient
K_P	Permeability coefficient
l	Diffusion path-length
λ	Wavelength
P_{ave}	Time-average power
τ	Pulse length

Contents

1 Introduction

All living organisms have an outer protective surface separating endogenous and exogenous compartments. The human skin, a keratinized stratified squamous epithelium, incorporates a multitude of vital physical and biological functions. One of the key functions of the skin is to protect the living interior compartments of the body from invasion by pathogenic microorganisms and harmful UV-radiation [1].

However, the barrier is not foolproof, e.g. it is not evolutionary fit to handle the repeated exposures to many of the now frequently occurring more or less toxic environmental xenobiotics, which have been introduced into modern society. Chemicals are constantly invading the skin and the body. One of the consequences thereof has been an increase in the manifestation of contact allergy, today affecting approximately 15-20 % of the population in the western world [2]. Preventive work is important as contact allergy often has significant socio-economic consequences, both for the individual and society; e.g. numerous cases of contact allergy are work-related and often lead to long sick-leaves and sometimes oblige the patient to change profession.

The efforts to reduce the prevalence of contact allergy include an increase of the understanding of the mechanisms involved in the development of the disease, e.g. skin absorption, chemical reactivity, biotransformations and immunological mechanisms. Much has been learnt, but several key steps in the pathogenesis are still more or less shrouded in mystery. The preventive work also includes the development of efficient and reliable tools for identification of allergens, removal and replacement of identified allergenic compounds in consumer products and development of ‘safe’ formulations, minimizing the absorption and accumulations of potentially harmful components in ‘non-target’ tissue.

However, on the other side is the pharmaceutical industry, struggling with the low permeability of the skin, which makes epicutaneous delivery of drugs especially cumbersome. Nonetheless it is often an attractive alternative to oral or intravenous administration and much effort is being made in the development of more effective formulations, optimizing the absorption profile depending on the target tissue.

This thesis includes five studies, dealing with the cutaneous uptake and distribution of topically applied compounds. One study with the focus on drug delivery, specifically the use of liquid crystalline bicontinuous cubic phases in epicutaneous formulations (Paper I), and four studies investigating different aspects regarding the cutaneous absorption and distribution of skin sensitizers, e.g. factors affecting the uptake and sensitization potency of haptens and hapten targets in the skin (Paper II-V). Specifically, this thesis highlights some of the advantages using TPM when investigating the interactions between the skin and xenobiotics.

The thesis was performed within the Centre for Skin Research Gothenburg (SkinReGU), which is a collaboration between research groups at the Faculty of Science and the Sahlgrenska Academy at the University of Gothenburg and Chalmers University of Technology. It comprises research groups within the fields of dermatochemistry, dermatology, medicinal chemistry, nanotechnology, biophysics, physical chemistry, organic chemistry, surface chemistry, odontology, and pharmaceutics. The center provides a unique interdisciplinary platform for skin related research.

2 The Skin – Anatomy and Function

The skin, or the integumentary system (from Latin *tegere* 'to cover'), is our largest organ. It has a surface area between 1.2-2.2 m^2, an average thickness of 1.5-4 mm and makes up approximately 7% of the total body weigh in the average adult man [3]. The skin can be divided into two major compartments; the epidermis, an avascular stratified squamous epithelium mainly composed of terminally differentiating keratinocytes and the dermis a connective tissue with a large fraction of collagen and elastin fibers providing strength and flexibility (Figure 2.1) [4].

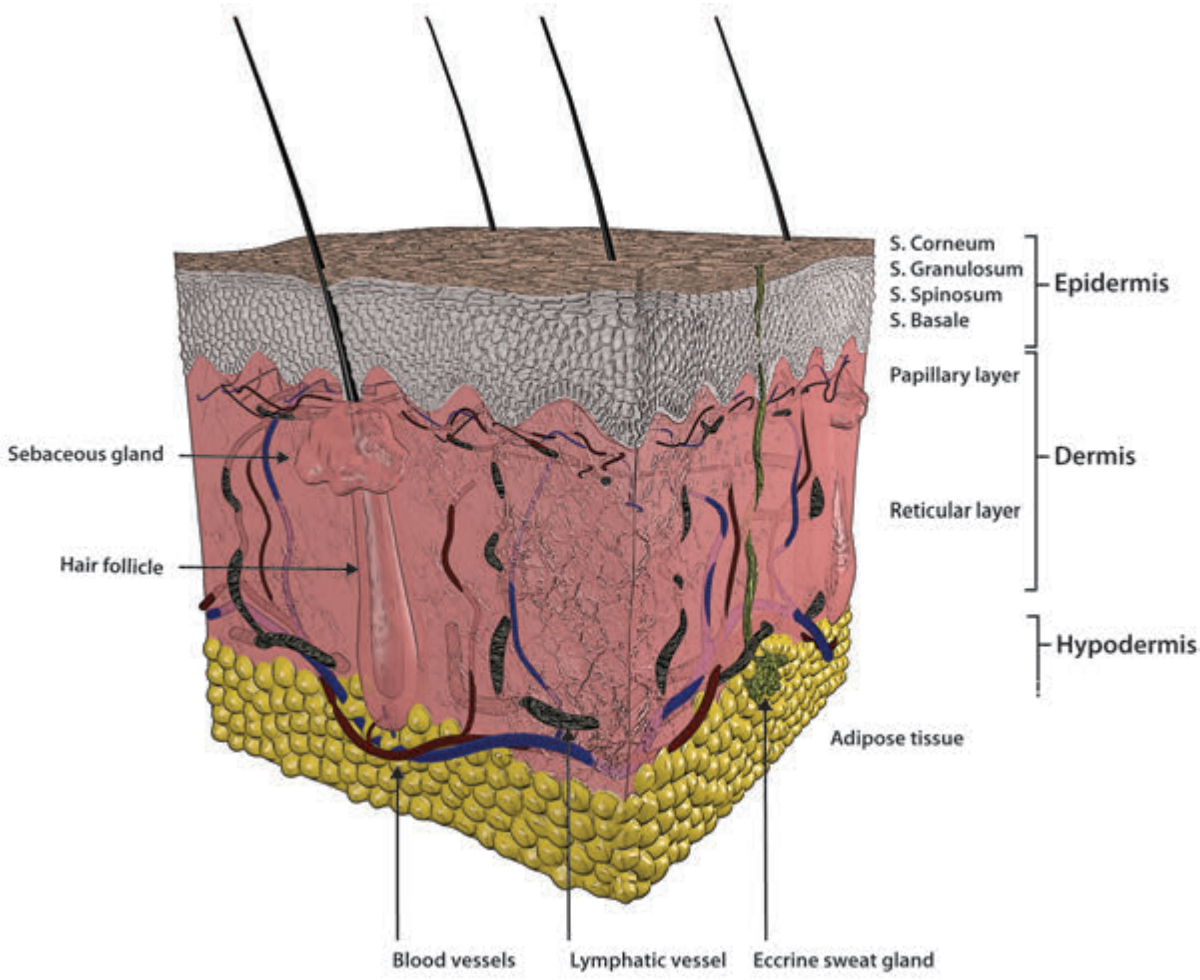

Figure 2.1. Structure of the skin and underlying subcutaneous tissue.

The skin can be regarded both as a bridge and a barrier between our body and the exogenous environment. It upholds a multitude of vital functions, e.g. it mediates sensory perceptions, regulates body temperature and the endogenous water balance, acts as a blood reservoir and protects the body from harmful UV-radiation and physical trauma [1].

Another key function of the skin is to protect the living interior compartments of the body from invasion by pathogenic microorganisms. This task is fulfilled via the collaboration of a membrane like physical barrier (*stratum corneum*), a biochemical barrier (e.g. hydrolytic enzymes, antibacterial fatty acids and antimicrobial peptides) and an immunological barrier involving the cells of the immune systems [5]. Together, these form a bio-physicochemical first line of defense, which would be impossible to live without. This chapter includes a brief introduction to the anatomy of the skin, with a specific focus on the structures related to the skin barrier functions.

2.1 Epidermis

2.1.1 Cellular composition and structure

Epidermis is composed of terminally differentiating keratinocytes, epidermal dendritic cells or Langerhans cells, melanocytes, and merkel cells [4]. Of these, the keratinocytes are the most abundant cell type making up approximately 95% of the epidermal volume [1]. The keratinocytes forms a stratified squamous epithelium, generally divided into four specific layers based on the degree of cellular differentiation, i.e. the *stratum basale*, the *stratum spinousum*, the *stratum granulosum* and the *stratum corneum* (Figure 2.2) [6]. Langerhans cells, which are the second most abundant cell type in the epidermis, are professional antigen presenting cells, which have an important role in the skin immune defense [7, 8]. Melanocytes are melanin-producing cells residing in the basal cell layer [4]. Melanin is a pigment protecting the nucleus of basal keratinocytes from UV-radiation. Merkel cells, which also reside in the basal cell layer, are sensory receptor cells associated with dermal nerve fibers [4]. The average thickness of human epidermis is approximately 75-150 µm, but varies significantly depending on the body site [3].

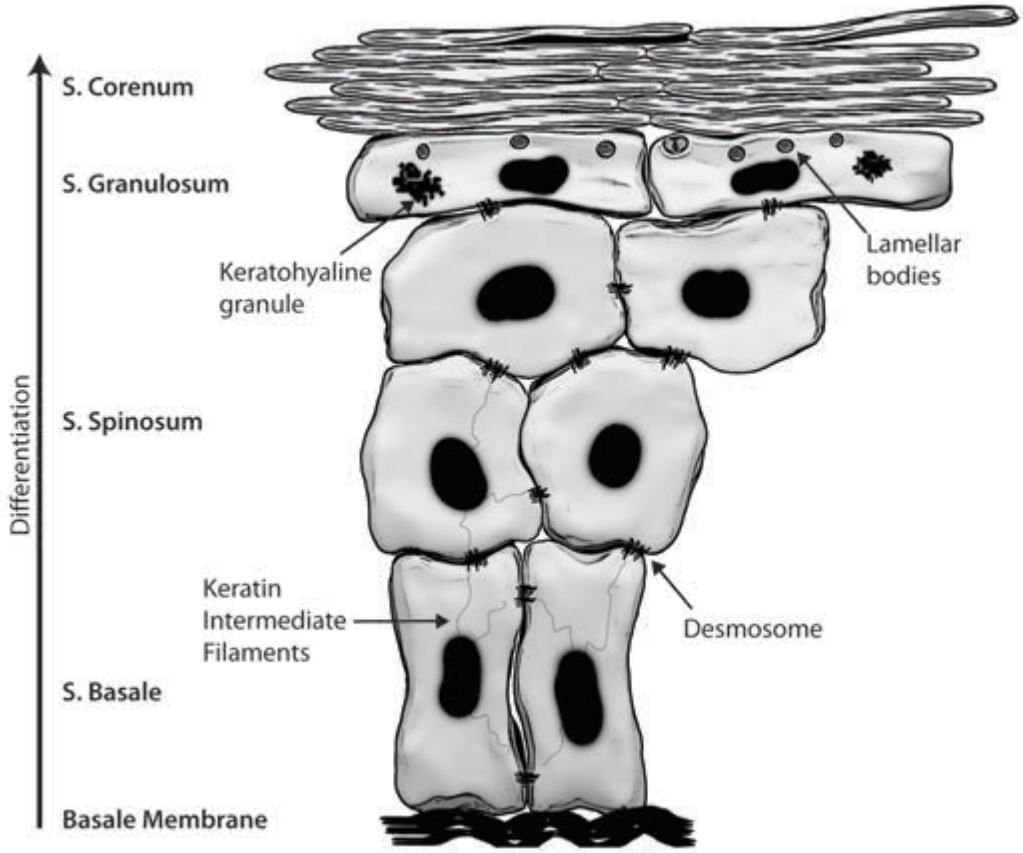

Figure 2.2. The structure of the epidermis, comprising the basal membrane, *stratum basale*, *stratum spinosum*, *stratum granulosum* and *stratum corneum*. Adjacent keratinocytes are connected via desmosomes, which bind to keratin intermediate filaments. Keratinohyaline granules, formed in the spinous layer, contain profillagrin which aggregates the keratins in the stratum corneum. Lamellar bodies contain lipids, which are expelled into the extracellular matrix in the border between *stratum granulosum* and *stratum corneum*.

2.1.2 Viable epidermis

Stratum basale, *spinosum* and *granulosum* are the living layers of the epidermis and are together commonly referred to as the viable epidermis. *Stratum basale* is a single layer of columnar shaped cells connected to the epidermal basement membrane [4]. It includes a subpopulation of mitotic epidermal stem cells [9]. The basal cells undergo continuous cell divisions renewing the suprabasal epidermal cell populations. *Stratum spinosum*, is a five to ten cell layers thick structure composed of cuboidal cells and *stratum granulosum* is composed of two to three layers of squamous cells [3]. Epidermis is an avascular epithelium, and the cells are dependent on the passive diffusive flow of nutrients from the capillaries in dermis. The keratinocytes in viable epidermis are interconnected via desmosomes, adherence junctions, gap junctions and tight junctions [1].

2.1.3 Stratum corneum

Stratum corneum is an approximately 16-20 µm thick layer of dead keratinocytes or corneocytes, embedded in a matrix of lamellar lipid bilayers [6]. It is commonly compared to a brick wall protecting the viable endogenous compartments.

The bricks (corneocytes) are 40-50 µm broad and 1 µm thick, hexagonal, scale-like cells formed by the terminally differentiating keratinocytes, in a type of programmed cell death commonly referred to as cornification [10, 11]. Briefly, as the proliferating basal keratinocytes are detached from the basal membrane and move up into the spinous layer, the cells starts to synthesize new sets of structural proteins, of which some becomes cross-linked beneath the plasma membrane. Cross-linking of proteins under the cell membrane continuous up in the granular layer, building an insoluble protein polymer called the cornified envelope. Concurrently, profillagrin, originating from keratohyalin granules formed in the spinous layer decomposes into fillagrin, which aggregates and cross-links the keratins forming insoluble intracellular macro-fibers, which are covalently attached to the cornified envelope. The corneocytes are continuously exfoliated or desquamated at the skin surface and the epidermis is completely renewed every 25-45 days [3].

The mortar is composed of a matrix of polar lipids, mainly ceramides (45-50 %), free fatty acids (10-15 %) and cholesterol (25 %) [11-14]. These are synthesized from phospholipid precursors in lamellar bodies, originating from the Golgi, which fuses with the plasma membrane in the border between the granular layer and *stratum corneum*. The lipids are organized in multi-lamellar lipid bilayers with alternating lipophilic and hydrophilic domains (Figure 2.1), stabilized via hydrophilic interactions between the polar head-groups and hydrophobic interactions between the long, straight, aliphatic tails of the ceramides and the free fatty acids. A fraction of the ceramides content is also covalently attached to cornified envelope forming a lipid envelope strengthening the cornified envelop [10]. The lipid fraction is of major importance for the skin barrier, e.g. diseases affecting the lipid composition and structure of *stratum corneum* have been shown to alter the barrier properties of the skin [13].

2.1.4 Keratins

Keratin is the main structural protein of the keratinocytes and the most abundant protein in the epidermis [15, 16]. Keratins assembled into fibrous structures called keratin intermediate filaments, extending between the nuclear lamina and cell membrane associated protein complexes called desmosomes (Figure 2.2). Keratin intermediate filaments form the cytoskeleton of the epidermal cells. Keratin intermediate filaments are composed of pairs of different types of keratins. Basal cells mainly express pairs of keratin 5 (K5) and keratin 14 (K14) while spinous and granular cells express keratin 1 and keratin 10 [17-21]. As the cell progress up into the *stratum corneum*, keratin intermediate filaments are aggregated with fillagrin leading to a structural collapse of the cell [22, 23].

2.2 Dermis

The dermis is a connective tissue separated from epidermis by the basal membrane. It can be divided in two separated layers, the superficial papillary layer and the underlying reticular dermis (Figure 2.1). The papillary layer includes the dermal papillae forming peg-like structures penetrating the epidermis. Dermis is mostly composed of collagen and elastin fibers in a polysaccharide matrix. The cellular fraction includes fibroblasts, macrophages, dermal dendritic cells and lymphocytes. The dermis is also rich in vascular channels (blood vessels and lymphatic vessels) and nerve fibers. The dermis is attached to the hypodermis, an adipose tissue, which connects the skin to the internal body structures, primarily the muscles [1, 4].

2.3 Skin appendages

The skin appendages include the hair follicles, the hair, the sebaceous glands and the sweat glands. The appendages originate from epidermal tissue but penetrate deep into the reticular dermis. The hair, hair follicle and hair follicle associated sebaceous glands are generally referred to as the pilosebaceous unit (PSU). The PSU extend all the way down to the hypodermis. In human skin there is an average of 10 – 70 PSUs per cm^2 covering approximately 0.1% of the total skin surface in the average adult man [24]. The PSU cell population includes more than 20 different cell types and it has a relatively large fraction of stem cells and immune cells e.g. Langerhans cells, T-cells and macrophages [9, 25-27]. The follicle wall is composed of an internal and external epithelial root sheath and a basement

membrane called the glassy membrane and it is surrounded by an extensive network of perifollicular capillaries [28]. The follicle associated sebaceous glands produce sebum, a mixture of fatty acids, which are secreted to the skin surface. Sebum function as a natural moisturizer softening the skin and the hair. It is also a bactericidal protecting against pathogen invasion [1]. The skin appendages are regions of partly reduced skin barrier. Their implication in the uptake of topical applied compounds will be discussed further in the subsequent chapters of this thesis.

3 Contact Allergy

Contact allergy is a T-cell mediated delayed type (IV) contact hypersensitivity disease caused by low molecular weight chemical allergens called haptens [29, 30]. The clinical outcome of contact allergy is a skin inflammation with locally confined erythema and oedema referred to as allergic contact dermatitis (ACD) [30]. Contemporary lifestyles has led to an increase in the public exposure to haptens, and contact allergy has become a common health problem, affecting approximately 15 -20% of the population in the western world [2]. Haptens are found in a wide variety of consumer products, e.g. in cosmetics and household products [31, 32]. Occupational related exposure is also frequent [33, 34]. The most common contact allergen today is nickel followed by fragrances [32]. Preservatives [35], UV-filters [36] and epoxy resins [37] are other prevalent haptens. This chapter will give an introduction to the chemical and immunological mechanisms in ACD, methods for predictive testing and factors affecting the sensitization potency of haptens. Also, the fluorescent model haptens used in this thesis will be discussed.

3.1 Pathogenesis

The immunological mechanisms involved in the development of ACD can be divided into two phases, i.e. a sensitization phase and an elicitation phase. The sensitization phase is the first exposure to a hapten leading to a priming and differentiation of effector T-cells and immunological memory. The elicitation phase takes place upon re-exposure to the hapten leading to a hapten-specific T-cell mediated localized inflammation in the affected tissue (Figure 3.1).

3.1.1 Sensitization

The sensitization phase starts with the entry of hapten into the skin, leading to the formation of immunogenic hapten-protein complexes and the release of proinflammatory cytokines by the cells in the skin [38]. Hapten or hapten-protein complexes are recognized and internalized by immature resident epidermal and dermal dendritic cells or recruited dendritic cell precursors.

Cytokine signalling triggers a migration of haptenated dendritic cells from the peripheral tissue via the afferent lymphatic vessels towards the draining lymph nodes [39-41]. The signaling also elicits a maturation of the dendritic cells into a professional antigen presenting cells with up-regulated expression of major histocompatibility complex (MHC) and co-stimulatory molecules. Naïve T-cells home to the deep cortical unit of the lymph node where dendritic cells present processed hapten-protein complexes (haptenated peptides) on the surface of MHC molecules [42]. If a T-cell has a cognate T-cell receptor and co-receptors (CD4 or CD8) to the MHC-peptide antigen complex it will be activated leading to the proliferation and differentiation into antigen specific effector or memory T-cells. Haptenated peptides presented by dendritic cells on the surface of MHC class I molecules activate cytotoxic T-cells expressing the CD8 glycoprotein co-receptors and haptenated peptides presented on MHC class II molecules activate helper T-cells or regulatory T-cells expressing CD4 glycoprotein co-receptors [43]. Primed effector T-cells leaves the lymph node via the efferent lymphatic and start to circulate the blood, the peripheral tissue and the peripheral lymphoid organs. Sensitization phase takes approximately 10-15 days in man and 5-7 days in mouse [38].

3.1.2 Elicitation

The elicitation phase begins with a non-specific hapten induced secretion of proinflammatory chemokines and cytokines. This triggers and an up-regulation of MHC molecules on the keratinocytes and cutaneous dendritic cells and an extravasation of hapten specific CD8+ cytotoxic effector T-cells. The infiltrated cytotoxic T-cells are activated by haptenated peptides presented on MHC I molecules. Release of new sets of inflammatory cytokines, leads to the infiltration and activation of other cells of the immune system, e.g. neutrophils, natural killer cells and regulatory T-cells [38, 44]. The influx of liquids, proteins and cells from the blood leads to a local erythema and oedema, which generally peak between 48-72 h in man [45] and 24-48 h in mouse [46].

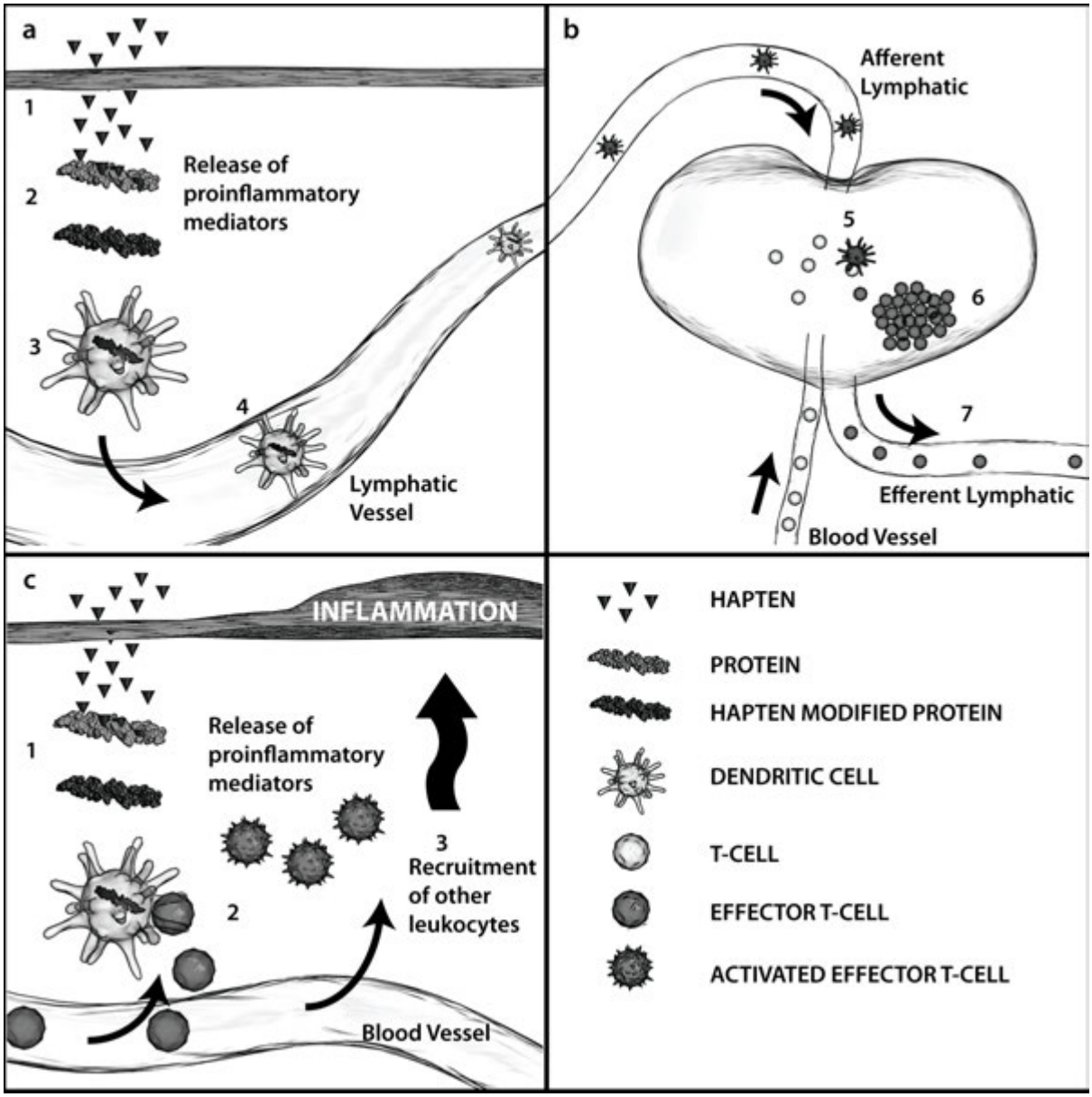

Figure 3.1. An overview of the events during the sensitization and elicitation phase in allergic contact dermatitis. The sensitization phase (**a** and **b)**: Hapten penetrate the skin barrier (1) and interacts with skin resident cells and proteins (2) leading to the formation of immunogenic hapten-protein complexes and the release of proinflammatory mediators. The hapten protein complexes are recognized and internalized by immature cutaneous dendritic cells (3), which migrates towards the skin draining lymph nodes (4), where they present processed hapten-protein complexes to naïve T-cells (5). Activation of T-cells leads to a clonal expansion (6) of effector cells, which leaves the lymph node and enters the systemic circulation (7). The elicitation phase (**c**): Formation of hapten-protein complexes and release of proinflammatory mediators attracts effector T-cells that are activated by skin resident antigen-presenting cells, e.g. dendritic cells (2). Further releases of of proinflammatory mediators attract other leukocytes, e.g. neutrophils and NK-cells, which amplify the inflammatory reaction (3). Adopted from [38].

3.2 Haptens

Haptens are intrinsically too small to be recognized by the immune system and to cause an allergic reactions [47]. To trigger an adaptive immune response, hapten must react with endogenous macromolecules, proteins or peptides, forming immunogenic non-self hapten-protein complexes [48]. Most haptens are electrophiles, e.g. alkyl halides, aldehydes, αβ-unsaturated ketones, esters and amines, hydroperoxides, epoxides, and isothiocyanates [29].

Skin sensitizers are believed to form hapten-protein complexes via polar reactions with nucleophilic amino acids, e.g. lysines, cysteines, histidines, methionines and tyrosines. Examples of reactions which could be relevant, are bimolecular substitutions (S_N2), aromatic substitutions, Michael additions, Schiff base formations and acylations [29]. Haptens can also form protein complexes via radical reactions (e.g. hydroperoxides) [49] or through metal-protein coordination complexes (e.g. nickel and chromium) [50].

The formation of immunogenic hapten-protein complexes is a prerequisite for activation of the adaptive immune system. Still, some non-reactive compounds also cause contact allergy. These are referred to as pre- or prohaptens. These are activated to reactive allergenic intermediates (electrophilic or radical) via autoxidation (prehaptens) [51-53] or metabolic transformations (prohaptens) [54]. Examples of non-reactive chemicals that have been found to form reactive sensitizing intermediates are aliphatic and aromatic amines, azo dyes, catechols, hydroquinones, conjugated dienes, primary alcohols and αβ-unsaturated oximes [29, 55].

In this thesis a series of fluorescent model haptens, i.e. isothiocyanates and bromobimanes, were applied to study different aspects in contact allergy. These haptens are discussed in the following two sections.

3.2.1 Isothiocyanates

Isothiocyanates are strong electrophiles, and are potential sensitizers under the condition that they penetrate the skin barrier. Indeed, several cases of ACD caused by isothiocyanates have been reported. Specifically neoprene materials and adhesive tapes have been shown

to be sources of sensitizing isothiocyanates, e.g. phenyl isothiocyanate is released from rubber materials, as a degradation product from thioureas [56-58].

Isothiocyanates are expected to form hapten protein complexes via conjugation to e.g. lysine and cysteine amino acids or N-terminus of proteins and peptides. However, only the reaction with amines generates stable products. The reaction with cysteine is reversible and thiol adducts can be converted into stable amine adducts under physiological conditions [59] (Figure 3.2). It has been shown that isothiocyanates reacts selectively with terminal amines in proteins [60].

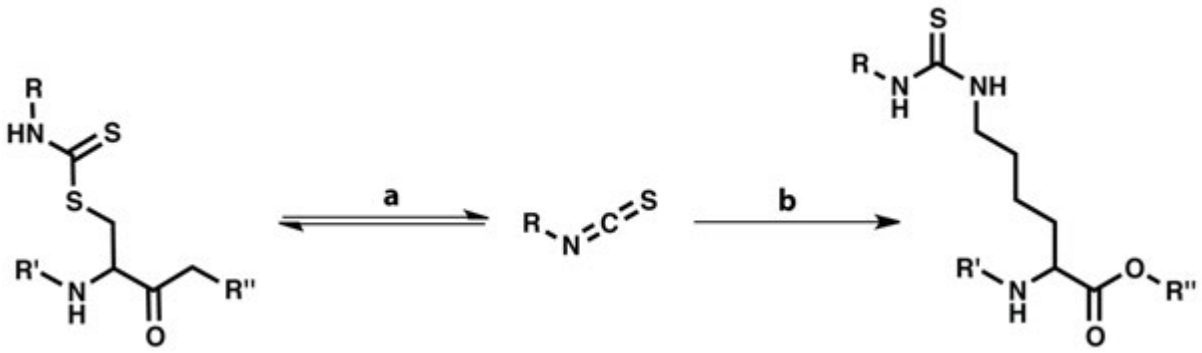

Figure 3.2. Reactivity of isothiocyanates with thiols and amines. Isothiocyanates reacts with thiols (e.g. cysteine, **a**) leading to the formation of dithiocarbamates and with amines (e.g. lysine, **b**) generating thioureas.

Fluorescent isothiocyanates are commonly used as labels for antibodies in immunofluorescence techniques [60, 61]. Fluorescent isothiocyanates have also been used as model haptens in mechanistic studies of contact hypersensitivity [62-64]. In this thesis, fluorescein isothiocyanate (FITC) and rhodamine B isothiocyanate (RBITC) (Figure 3.3) were used as model haptens. FITC is a green fluorophore with excitation maximum near 495 nm and an emission maximum at 520 nm. RBITC is a red fluorophore with excitation maximum at 543 nm and an emission maximum near 580 nm [61]. FITC was used initially but replaced by RBITC, which has less overlap with the skin autofluorescence.

3.2.2 Bromobimanes

Bromobimanes (Figure 3.3) is a group of halogenated thiol reactive caged fluorophores, i.e. they are weakly fluorescent compounds which form highly fluorescent thioether adducts with sulfhydryles via S_N2 displacement of one (mBBr) or two dBBr) bromines [65, 66]. The

fluorescent bimane-derivatives, formed upon alkylation with e.g. proteins or peptides, have a excitation maximum around 390 nm and an emission maximum around 480 nm [67]. Bromobimanes have been used as labelling reagents for identification and quantification of peptides and proteins in cells using various techniques, e.g. gel electrophoresis, liquid chromatography, flow cytometry and fluorescence microscopy [68-78].

In this thesis, the bromobimanes mBBr and dBBr were used as model sensitizers to identify hapten targets in skin, i.e hapten protein complexes were visualized following uncaging of the bromobimanes via conjugation to cutaneous proteins or peptides.

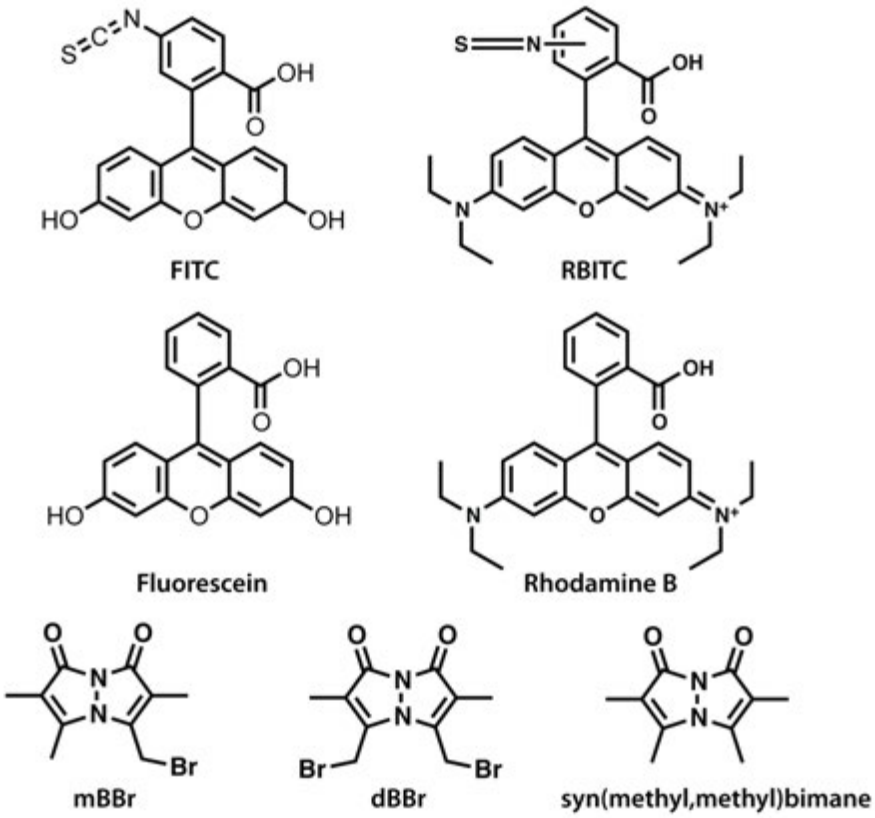

Figure 3.3. Molecular structures of the model haptens fluorescein isothiocyanate (FITC), rhodamine B isothiocyanate (RBITC), monobromobimane (mBBr) and dibromobimane (dBBr) and their non-reactive, non-sensitizing structural analogues fluorescein, rhodamine B and syn-(methyl,methyl)bimane, which were used as control compounds.

3.3 Predicting sensitization potency

When suspected, contact allergy can be diagnosed in a patch test performed by dermatologists [51]. Positive patch test reactions can have significant socio-economic consequences, both for the individual and society; e.g. numerous cases of contact allergy are work-related and often lead to long sick-leaves and sometimes oblige the patient to change profession. Preventive work, e.g. identification of haptens and removal and replacement of allergenic compounds from the market, is therefore of great importance to reduce the prevalence of contact allergy. Next to human volunteers [79-81], animal models e.g. the Guinea Pig Maximization Test [82, 83], and the murine Local Lymph Node Assay (LLNA) [84-86] are probably the most reliable assays for predictive screening of contact allergens. Presently it is the murine LLNA that is the most commonly adopted method. The LLNA was applied to investigate the sensitization potency of the model haptens used in this thesis.

3.3.1 The Local Lymph Node Assay

The murine LLNA (Figure 3.4) is a validated standard test for predictive screening and identification of sensitizing chemicals [87]. In the LLNA, sensitization potency of a chemical is evaluated by measuring a dose dependent proliferative response in the cells of the skin draining cervical lymph node cell population. The LLNA assays presented in this thesis were performed according to OECD recommendations. Briefly, 18 mice are divided in six groups with three mice in each group. Each of five groups is then exposed to a single specific concentration of the hapten in a test vehicle for 3 consecutive days (day 1-3). Concurrently, the 6^{th} group (the control group) is exposed to the vehicle without the hapten. The formulations are applied topically on the dorsal side of the ears. Three days after the last application (day 6) mice are injected with ^{3}H-methyl thymidine and are sacrificed. Cervical lymph nodes are excised, single cell suspensions are prepared and further treated before analysis by β-scintillation counting (day 7).

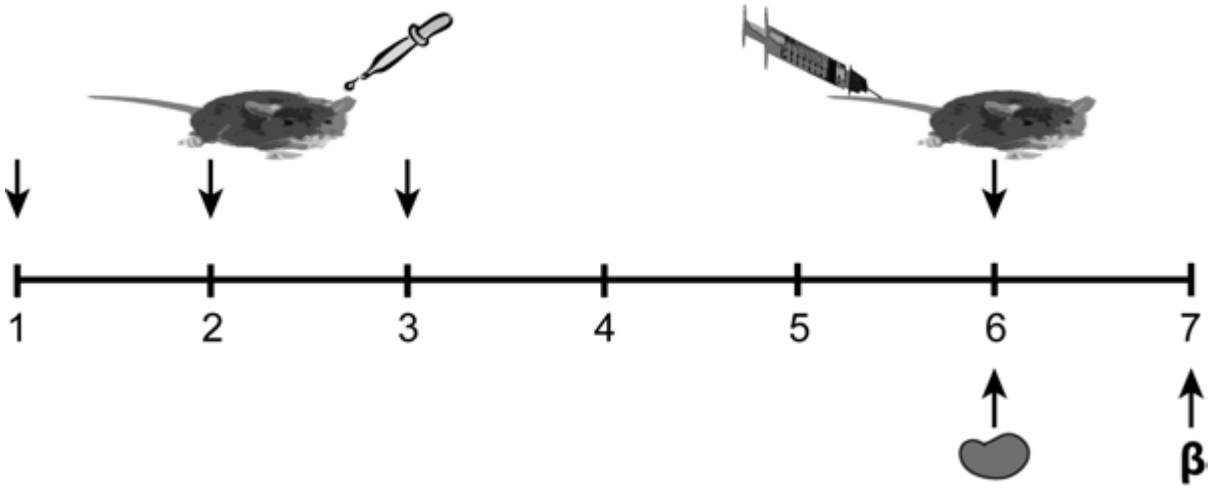

Figure 3.4. The murine local lymph node assay (LLNA). Day 1-3: Mice are treated with test formulations once a day. Day 3-6: Incubation. Day 6: Mice are injected with ^{3}H-methyl thymidine, cervical lymph nodes are excised and single cell suspensions are prepared. Day 7: Analysis of single cell suspensions by beta-scintillation counting and calculation of stimulation index and EC3 value.

The β-scintillation counter measures the amount of ^{3}H-methyl thymidine incorporated in DNA of the lymph node cells from each treatment group, and is expressed as disintegrations per minutes (dpm). A stimulation index (SI) is calculated according to Equation 1, where n is the number of lymph nodes in each group.

$$SI = \frac{\left({}^{dpm}/_{n}\right)_{Treated}}{\left({}^{dpm}/_{n}\right)_{Control}} \qquad \textbf{Equation 1}$$

The sensitizing capacity of chemicals tested in the LLNA is classified based on EC3 values. The EC3 value is the concentration at which the lymph node cell proliferative response is increased by a factor of three compared to a vehicle exposed control group, i.e. a SI equal to 3. A hapten can be classified as extreme (EC3 < 0.1%, w/v), strong (EC3 < 1 %, w/v), moderate (EC3 <10 %, w/v) or weak sensitizers (EC3 < 100%, w/v) according to a generally adopted classification system [88].

The LLNA was employed to determine the sensitizing capacity of the model haptens used in Paper II, IV and V. A modified non-pooled LLNA was used in Paper III to perform a statistical analysis of the variation in the sensitization potency of a hapten when applied in different

vehicles. The statistical LLNA experiments were performed as the standard assay described above with the following exceptions: only three groups (one control and two test groups) with up to nine mice per group were used, the two test groups received the same concentration of the hapten but in different vehicles and excised lymph nodes from each mouse were treated and analyzed separately. All animal assays included in this thesis were approved by the local ethics committee.

3.3.2 Alternative non-animal based assays

In the past guinea pigs were frequently used for the identification of contact allergens. Recently, guinea pig based assays became more or less replaced by the murine LLNA, described above. Due to public concerns the use of animal based toxicity assays, e.g. in the screening of products for identification of skin sensitizers, is now restricted by new legislations within the European Union. An ban on animal testing of cosmetic products was implemented in 2009 and there is a pending marketing ban on animal tested cosmetic products, starting from 2013 according to the seventh amending directive [89] to the European cosmetic directive [90]. Thus, there is an urgent need for the development of robust alternative methods, i.e. *in vitro*, *in chemico* or *in silico* models for predictive screening.

Although, *in vitro* methods have been shown to be relatively successful in some cases, e.g. to predict local toxicity, predicting the sensitization potency of chemicals remains complicated. This is due to the many mechanisms involved and their complexity. Although much effort has been made, a considerable amount of work is still a head before acceptable alternative methods can be adopted, which could replace the murine LLNA. Example of parameters which may influence on the sensitization potency of a hapten and must be considered in the design of an *in vitro* test are autoxidation, skin penetration, bioavailability, biotransformations, hapten-protein reactivity and epitope recognition by T-cells. A thorough understanding of these processes and their influence on the sensitization potency of haptens are important when designing alternative screening methods.

As it is difficult to include all the above-mentioned parameters in a single assay it is generally assumed that a toolbox of alternative methods will have to be used to replace e.g. the murine LLNA. Until now, numerous different approaches have been investigated, e.g. *in*

vitro cell based assays [91, 92], *in chemico* peptide reactivity assays [93, 94] and *in silico* expert systems [95, 96]. A peptide reactivity assay and two the cell based assays, i.e. the Myeloid U937 skin sensitization test and the human cell line activation test are presently up for pre-validation by the European Centre for the Validation of Alternative Methods [97]. However, it has been pointed out that the deadlines for replacement of the animal based assays with alternative methods are unrealistic, especially in quantitative risk assessment of contact allergens [98].

4 Cutaneous absorption

Successful and safe delivery of topical drugs and cosmetics relies on effective formulations, which maximize drug concentrations in target tissue. Some compounds are intended to stay on the skin surface (sunscreens and barrier creams) [99-101] and some in the *stratum corneum* (moisturizers). Other should reach the viable epidermis of dermis (local analgesics, antifungal agents and drugs used in photodynamic therapy) [102-104], be taken up systemically (nitroglycerine, and nicotine) [105] or are specifically targeting the PSUs (retinoids) [106]. Target specific delivery is not always an easy task, due to the complexity of the skin and the skin barrier. Several different parameters will have to be considered and optimized to achieve maximum effect of the active components while reducing negative side effects in non-target tissue. Cutaneous absorption is also highly relevant factor in risk assessment, e.g. in the identification of skin sensitizers, as the sensitization potency partly depends on the bioavailability of the hapten. This chapter will comprise an overview of some of the processes related to uptake of xenobiotics by the skin (with respect to the physical barrier) and factors affecting the uptake. It will also give an introduction the topical delivery systems used in the studies presented in Paper I and III. For clarification, cutaneous absorption will refer to the penetration of a topically applied compound into a specific layer of the skin and percutaneous absorption refers to the uptake by the skin and the resorption by the circulatory system, i.e. the lymph and the blood, in the dermis. Also, in the discussion, the definition topical application or delivery will here only refer to external epicutaneous administration.

4.1 Skin penetration pathways

The efficiency of a topical drug or the sensitizing potency of a hapten depends on the ability of the compound to cross the *stratum corneum* and enter the viable compartments of the skin and the circulation. The uptake over the *stratum corneum* can proceed via three general pathways (Figure 4.1), i.e. the intracellular route over the corneocytes, the tortuous extracellular pathway via the lipid matrix, and the shunt pathway through the skin appendages (PSU and sweat glands) [107].

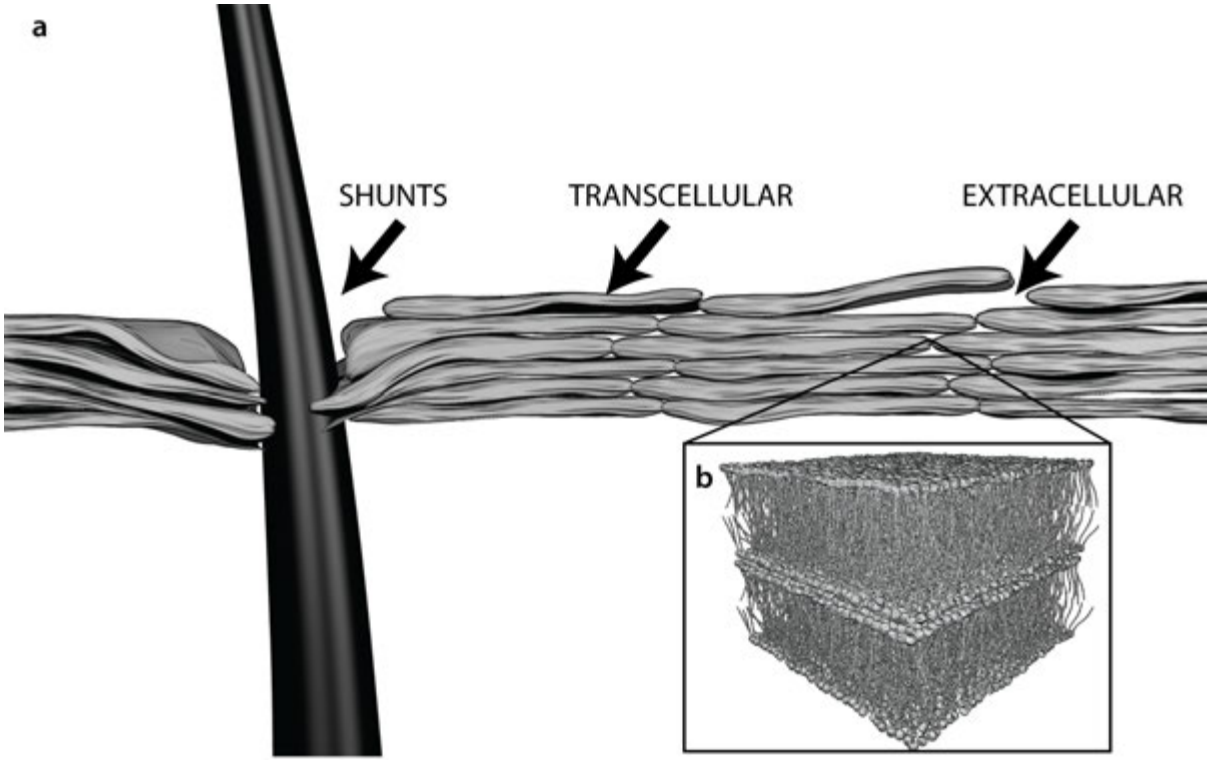

Figure 4.1. Potential penetration pathways through the *stratum corneum*. Topically applied compounds can enter the skin via shunts (e.g. the pilsosebaceous units and sweat glands), via the transcellular route over the corneocytes or via the tortuous extracellular pathway in the lipid matrix (**a**). A three-dimensional illustration of the lipid matrix, composed of bilayers of polar lipids, i.e. ceramides, fatty acids and cholesterol (**b**).

Of these, the extracellular pathway via the lipid matrix is generally regarded as the most important. Intracellular transport is limited due to the physical strength of the cornified envelope and the dense keratinization of the corneocytes. Shunts may offer a relatively rapid uptake through the *stratum corneum* and into the deeper skin layers; however these are generally considered to play minor role in the total flux due to the relatively limited surface area of the appendages, <1% of the skin surface [108, 109]. However, the significance of shunt diffusion is debated. It has been suggested that transport via the shunts could predominate in some specific cases, e.g. in the cutaneous absorption of very polar compounds, compounds with relatively high molecular weight or larger particles [109]. Ultimately it is the physiological state of the skin barrier, chemical and physical properties of the applied compound and the formulation in which it is applied on the skin that determine the penetration pathways through the *stratum corneum* as well as the amount the applied dose absorbed.

4.2 Factors affecting the absorption of topically applied compounds

The cutaneous and percutaneous absorption of a topically applied compound depends on several different, chemical (compound specific) and physiological (skin dependent) factors (Table 4.1). These factors may also indirectly affect the potency of a skin sensitizer. Molecular weight and logP are important molecular properties regulating cutaneous and percutaneous absorption. A low molecular weight and a logP around 2 is often regarded as optimal for effective transdermal delivery [110]. Compounds with a molecular weight larger than 1000 Da are not assumed to penetrate healthy skin [111]. Molecular weight, logP can be used as predictors in *in silico* calculations of skin permeability coefficients (K_P) *in silico [112, 113]*.

Another important chemical parameter is binding. Conjugation to proteins in epidermis or dermis may reduce the cutaneous absorption or lead to an accumulation of the compound in a specific skin layer [114]. This is a parameter, which has shown to be specifically relevant when investigating the cutaneous absorption of haptens, as will be demonstrated in this thesis. Examples of other chemically related factors are concentration, dose, hydrogen donor and acceptor strength, pKa and vapour pressure. Physiological factors are also important to consider. For example, local variations in e.g. *stratum corneum* thickness and hair follicle density may influence the degree of absorption. Skin diseases or physical damage to the skin barrier can also affect the uptake[114].

Table 4.1 A summary of chemical, environmental and physiological factors affecting the cutaneous absorption of xenobiotics. Adopted fom [114].

Chemical factors	Environmental factors	Physiological factors
Charge	Concentration	Age
Chemical reactivity	Contact area	Blood flow
H-bond donor/acceptor	Contact time	Disease or damage
logP	Dose	Gender
Melting point	Formulation/Vehicle	Hair follicle density
MW	Occlusion	Metabolism
pKa	pH	Species
Vapor pressure	Temperature	Skin thickness

4.3 Fick's law of diffusion

Percutaneous absorption can be regarded as multi-lamellar membrane transport process, including a multiple partitioning and diffusion coefficients in the different skin layers, controlling the overall flux. S*tratum corneum* diffusion coefficient is commonly considered to be the rate-limiting step in the cutaneous absorption of xenobiotics [115]. One exception could be very lipophilic compounds were the partitioning from the *stratum corneum* to the more hydrated environment in the viable epidermis could be the rate limiting step. In a general approximation, *stratum corneum* is regarded as an inert homogenous membrane, and percutaneous absorption is treated as a classical membrane transport phenomena, i.e. a passive diffusion driven by a concentration gradient [116]. Consequently, cutaneous absorption can be analyzed according to the general Law of mass transfer and Fick's Laws of diffusion. Fick's first law of diffusion (Equation 2) states that the flux (J), i.e. the number of absorbed molecules per unit area and unit time is proportional to the concentration gradient (ΔC) over the skin.

$$J = K_P \Delta C = \frac{KD\Delta C}{l} \qquad \textbf{Equation 2}$$

The proportionality constant (K_P), called the permeability coefficient, is a measure of the diffusion rate (cm/h) of a topically applied compound through the skin. The permeability coefficient is the product of the partition coefficient (K) between the vehicle and *stratum corneum* and the diffusion coefficient (D) in the *stratum corneum* divided by the diffusion path-length. However, the skin is a complex, heterogeneous, biologically active structure and several deviations from this approximation are likely to be observed.

4.4 Topical delivery systems

Effective, target specific uptake of drugs is one of the big challenges in the pharmaceutical industry. While oral administration is by far the most commonly used method it is not always the most appropriate. For example, oral administration is not suitable if the drug is sensitive to metabolic transformations in the liver or in the gastro-intestinal tract. Oral

administration is neither the best choice when a prolonged continuous release is desirable [117]. In some cases epicutaneous administration might be good alternative, especially in local therapy of skin related diseases. However, epicutaneous delivery is often hampered by the low permeability of the skin barrier. Thus, much effort is being made in the development of efficient topical formulations.

Epicutaneous formulations must be adapted depending on the target tissue, e.g. maximizing the percutaneous absorption of systemically active drugs or target specific skin compartments in the treatment of skin related diseases. Similarly, formulations of cosmetic product must be optimized in order to improve their efficiency and to minimize the risk of unwanted side effects. Often cosmetic products, compared to drugs, are intended to act on the surface of the skin, alternatively in the uppermost layers of the stratum corneum. Penetration into the viable compartments of the skin or systemic uptake is generally undesirable. Development of formulations reducing the uptake while maintaining the specific cosmetic effect could lead to safer consumer products. Interestingly, although having opposite aims, with the pharmaceutical industry wanting to improve the absorption whereas cosmetic industry wants to reduce the absorption, pharmaceutical and cosmetic cutaneous formulations are often based on same formulation strategies using emulsion based delivery systems [118].

4.4.1 Cutaneous penetration enhancers

Many different penetration enhancing formulations and strategies has been investigated in order to improve the absorption of topically applied drugs. Chemical penetration enhancers are pharmacologically inactive compounds, applied in order to increase uptake of drugs from a topical formulation [119]. Some chemical enhancers are thought alter drug solubility in either the formulation or in the *stratum corneum* changing the stratum corneum-vehicle partitioning coefficient. Other enhancers are thought influence the drug diffusion in *stratum corneum*, e.g. by disruption and fluidization of the ordered lamellar lipid bilayer structure or by *stratum corneum*-lipid extraction [109, 120]. The exact mechanism of many penetration enhancers remains speculative. Physical enhancers, e.g. dermal patches with occlusive effect [105, 121, 122], iontophoresis, i.e. electric potential driven transport of charged molecules over *stratum corneum* [123, 124] and physical disruption of the *stratum corneum*, e.g. using micro-needles [125], can be used to improve transdermal delivery of drugs.

4.4.2 Lipid based formulations

When amphiphilic molecules are suspended in aqueous solutions, they self-assemble in order to minimize the contact between hydrophobic parts of the molecules and the solvent system. The phase separation leads to the formation of various structures, e.g. spherical or cylindrical micelles, lamellar phases, bicontinuous structures or vesicles. The structure that is formed depends on several different factors, e.g. the molecular structure of the surfactant and its critical packing parameter, the solvent system, the temperature and the concentration[126].

The simplest forms of self-assembly are spherical micelles, generally formed in relatively dilute concentrations of surfactant in aqueous solutions (at the critical micelle concentration, CMC) [126]. At increasing concentration micelles can grow into thread like cylindrical micelles which eventually overlap and form a liquid crystalline bicontinuous phase, composed of two continuous hydrophilic solvent phases and a lipophilic surfactant phase [126]. Two different lipid-based delivery systems were studied in the work connected to this thesis. The potential of bicontinuous cubic phases in topical delivery of drugs were investigated in Paper I, and effect of lipid vesicles on the sensitization potency of a hapten was investigated in Paper III and these are discussed in more detail below.

4.4.3 Bicontinuous cubic phases

Bicontinuous cubic phases (Figure 4.2) have been shown to be promising drug delivery system [127]. One of their main advantages is the ability to dissolve both hydrophilic and lipophilic compounds. This is due to the bipolar structure of the system [118]. Bicontinuous cubic phases have also proven to be effective formulations in epicutaneous delivery of drugs. Monoolein (MO) and phytantriol (PT) (Figure 4.2) are polar lipids forming reverse bicontinuous cubic phase in water. The MO and PT based cubic phases consists of a continuous lipid bilayer (3-4 nm) and two water channels (5-6nm) [118]. Bender el al. have previously shown that topical delivery of aminolaevulinic acids, used in photodynamic therapy, can be improved when applied in MO and PT based cubic phases compared to a commercial ointment composed of a micro-emulsion in mice [128]. However, the mechanism was unknown and was therefore further investigated in Paper I.

4.4.4 Lipid vesicles

Lipid vesicles are colloidal particles formed by amphiphatic molecules, e.g. phospholipids, suspended in aqueous solutions. The lipids are organized in bilayers, which close on themselves forming unilamellar or multilamellar (depending on the formulation and preparation), spherical shaped structures with aqueous cores (Figure 4.2). The size of the vesicles may range from approximately 20-25 nm up to a few microns [129]. The first Paper describing the formation of phospholipid vesicles, liposomes, were published in 1965 [130]. Investigations of the use of liposomes in topical formulation started in the beginning of 1980 [131]. Since then there has been an increasing interest for lipid vesicles in skin related products, from both the pharmaceutical and the cosmetic industries. Like the cubic phase, lipid vesicles can dissolve high concentration of both lipophilic and hydrophilic compounds. It has also been shown that lipid vesicles can improve local delivery and sustained release of topical drugs [131-137]. Furthermore, vesicles made of phospholipids have high biocompatibility and are therefore considered relatively safe to use [138].

As lipid vesicles are included in many formulations, we wanted to study how these affect the sensitization potency of haptens. The lipid vesicles used in Paper III were prepared by suspending 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, Figure 4.2) in a 40 % ethanol in water solution. Lipid vesicles prepared using ethanol in the aqueous phase are commonly referred to as ethosomes. This type of vesicles were first introduced by Touitou et al [139]. Nano-scale vesicles (< 100 nm) were prepared using extrusion technique [140] and micro-scale vesicles were prepared by the thin film hydration method [141].

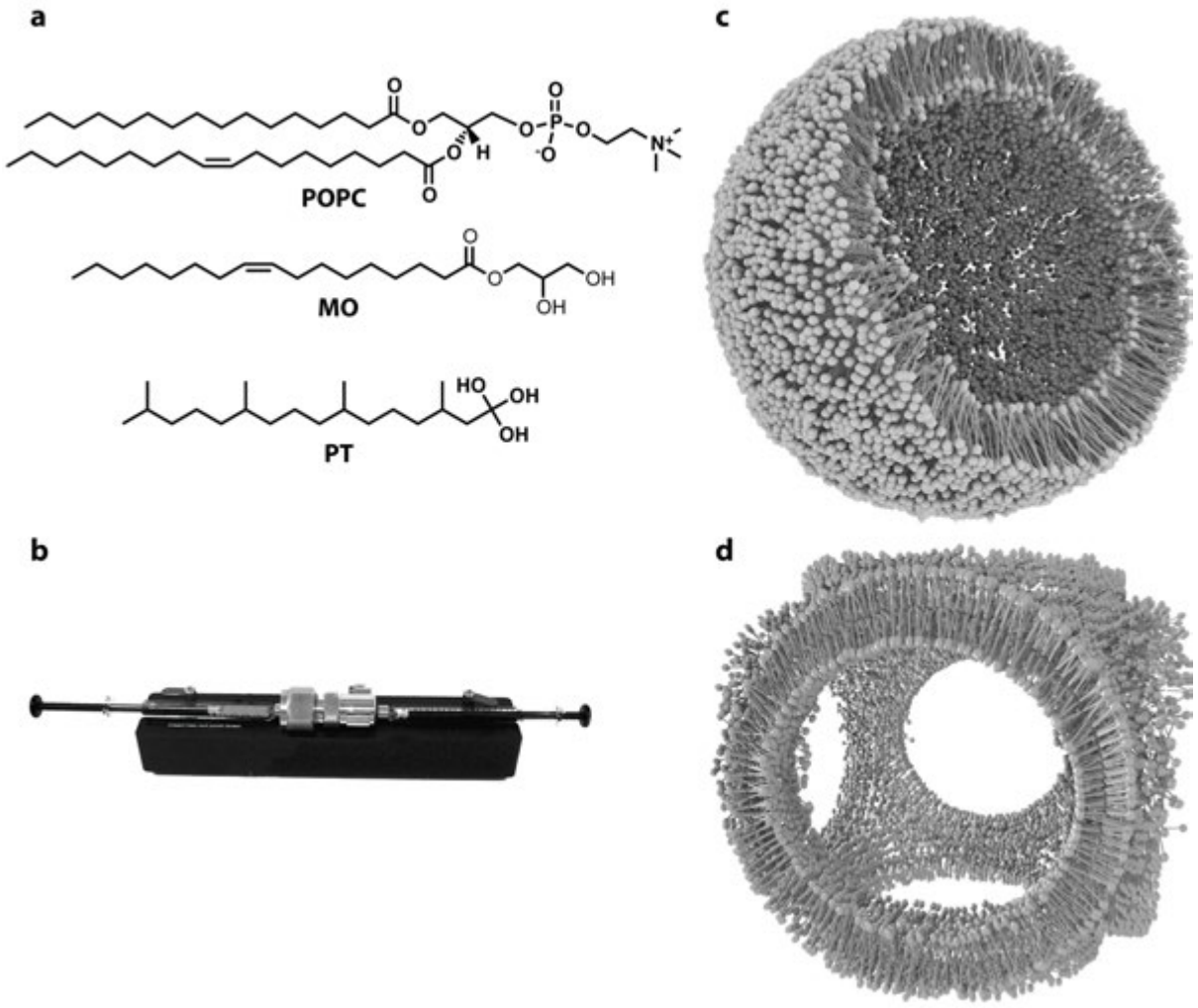

Figure 4.2. Molecular structures of Monoolein (MO), phytantriol (PT), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, **a**). An extruder used for the preparation of nano-scale vesicle systems (**b**). A three-dimensional illustration of a lipid vesicle (**c**). A three-dimensional illustration of a bicontinuous cubic phase (**d**).

4.5 Methods to study cutaneous and percutaneous absorption of topically applied compounds

There is a rather extensive collection of tools and techniques, which can be used to analyze the cutaneous and percutaneous absorption of topically applied compounds. The choice should ideally be made based on the aim of the study, and a combination of two or more complementing techniques will generally give the best picture of the reality. However, compromises might have to be done depending on e.g. the available equipment and materials, ethical considerations and technical expertise.

In clinical studies, tape stripping and dermal micro-dialysis are commonly used techniques to measure local concentrations of topically applied drugs [142, 143] while blood and urine analyses are used to analyze the systemic absorption [144, 145]. Pharmacodynamic activity can also be used to assess the bioavailability of some drugs. However, it is not always suitable or practically possible to perform analysis on patients. As an alternative, laboratory animals can be used or measurements can be performed *in vitro* using excised or reconstructed skin or synthetic membranes in diffusion cells. Another alternative is calculation of permeability and diffusion constants *in silico*, based on the intrinsic molecular properties of the investigated compound, e.g. molecular weight and logP [112, 113].

Diffusion cells (Figure 4.3) are one of the most frequently used *in vitro* methods to analyze the cutaneous uptake of topically applied compounds [146]. Briefly, a skin sample is mounted between a donor and receiver compartment. The donor compartment is filled with a receiver solution and the investigated formulation is applied on the skin in the donor compartment. The receiver solution is sampled at specific time intervals (static diffusion cells) or continuously (flow through diffusion cells) and the concentration of the compound is analyzed using a suitable method depending on the compound investigated. The rate of change in the concentration in the receiver solution can be used to calculate the flux (J), diffusion constant (D) and the permeability constant (P) for the compound in the membrane according to Fick's first law (Equation 3). Assuming infinite dose conditions and steady state diffusion, ΔC can be substituted with the concentration in the vehicle, C_V.

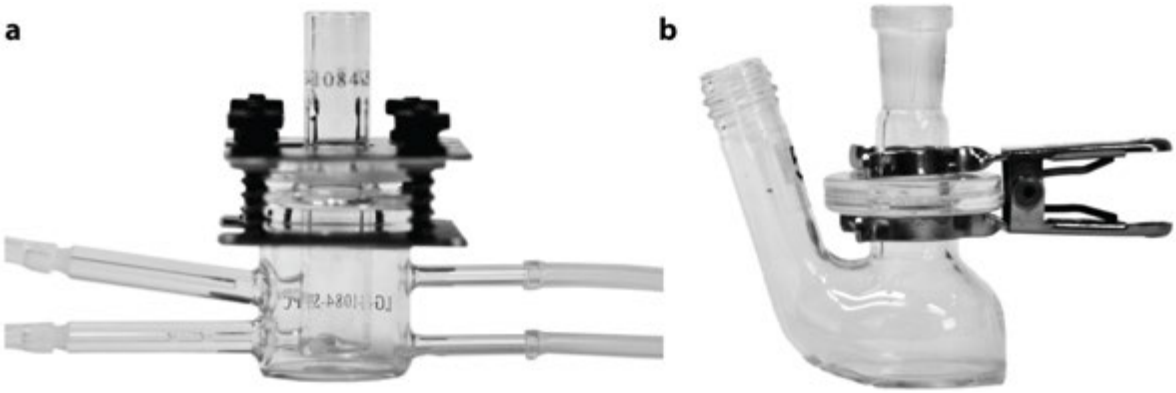

Figure 4.3. Tools for *in vitro* analysis of percutaneous absorption. A flow through diffusion cell (**a**) and a static diffusion cell (**b**).

Permeability coefficients derived from analysis using diffusion cells are often used as a relative measurement of the bioavailability of topically applied compounds in drug delivery and toxicological studies. Diiffusion cells measures only the amount of compound going through the skin, an approximation of the systemic bioavailability. It does not provide any information regarding local tissue concentrations. Calculated permeability coefficients obtained by measuring the flux of two different compounds or a single compound from two different formulations, do not necessarily reflect the relative drug concentrations in the skin. Tape stripping and skin extraction can be used to obtain additional information regarding local tissue concentrations, i.e. using the Saarbrücken penetration model [147]. Neither tape stripping nor skin extraction techniques have been used in this thesis and will therefor not be discussed in more detail here.

Although the methods discussed in this chapter successfully predict the cutaneous or percutaneous bioavailability of topically applied compounds, they have a common limitation, i.e. they provide a relatively restricted insight into what is actually happening in the skin. In addition, using tape stripping and skin extraction, the vertical resolution of the cutaneous drug distribution is relatively poor and the methods do not take into account variations in the horizontal distribution. Optical techniques, e.g. fluorescence and electron microscopy, can therefor give a better picture of intracutaneous processes related to the uptake of xenobiotics as will be discussed in the next chapter.

5 Laser Scanning Microscopy

Electron microscopy provides outstanding resolution, and can be used to study e.g. skin morphology and vehicle-induced ultra-structural modification of the skin barrier [6, 148-150]. However it is limited to analysis of fixed sectioned samples. Fluorescence microscopy, e.g. laser scanning confocal microscopy and non-linear two-photon laser scanning microscopy (TPM), has a lower resolution, but afford direct 3D visualization of processes and structures in intact unfixed tissue, in or close to *in vivo* conditions. Both confocal and TPM can be used for tissue imaging; however, TPM is in many respects a more powerful technique for for non-invasive visualization. In this thesis both techniques has been applied and will thus be reviewed below. As TPM has been in focus of the present work, more emphasis will be given to this technique.

5.1 Laser scanning confocal microscopy

Confocal microscopy is a well-established imaging technique, and an important tool in e.g. studies of biological systems. Detailed information regarding the basics of laser scanning confocal microscopy can be found elsewhere [151]. Briefly, in fluorescence confocal microscopy signal is generated via single-photon excitation of fluorophores. The sample is irradiated with light having an energy corresponding to, or close to, the one-photon absorption maximum of the compound to be imaged. When irradiated molecules are excited from a ground electronic state (S_0) to an excited electronic state (S_1). The energy difference between S_0 and S_1 corresponds to the energy of the illumination light. The signal is generated when the molecules return to their ground electronic state and emits a photon (Figure 5.1).

In a conventional fluorescence microscope the entire field of view of the sample is illuminated and imaged simultaneously. This has some advantages, e.g. faster image acquisition. An important drawback is a rather poor depth discrimination of the signal. In a laser scanning confocal system depth discrimination is achieved by point scanning the

illumination beam over the sample. The fluorescence signal from the focal volume returns via the objective lens and the scanning system and is focused on a detector. A pinhole placed in front of the detector function as a spatial filter, excluding out of focus light.

Optical sectioning is a non-invasive technique to dissecting and visualizing the interior of e.g. biological samples. Briefly, a z-stack of images are collected by scanning the fluorescence in the x,y-plane at successive sample depths. The image stack can then be combined into a 3D (x, y, z) representation of the sample. In this work, a Zeiss 510 Meta and a LSM 710 system has been used, and tissue preparation has involved cryosectioning and immunofluorescence techniques.

5.2 Laser scanning two-photon microscopy

TPM is fast growing technique within the field of biomedical imaging and it has been adopted in a wide range of research areas. TPM is especially suitable for live cell and *in vivo* imaging and has been used to visualize e.g. lymphocyte mobility [152] and dendritic cell networks in intact lymph nodes [153], T-cell priming by dendritic cells in lymph nodes [154], blood flow dynamics deep into the brain of living rats [155] and the dynamics and functions of Langerhans cells in lymph nodes [156]. TPM has also been adopted to study skin morphology and the interactions between the skin and topically applied compounds and formulations, e.g. to investigate the effect of penetration enhancers in topical formulations [157, 158] the uptake of nano-particle borne drugs [159] and the interaction of vesicle formulation with human skin [149] . TPM has also been evaluated as a tool for non-invasive optical diagnostic of skin cancer [160]. The construction of a two-photon is similar to a confocal microscope, i.e. it is a laser scanning system in which images are acquired by raster scanning the sample. A major distinction between the two techniques is the generation of signal, i.e. the excitation process.

5.2.1 Two-photon excitation

As described above, in a confocal microscope fluorescence signal is generated via one photon excitation, i.e. with light having an energy corresponding to the potential between the electronic states S_0 and S_1 of the investigated compound. In TPM, the same signal is induced via a two-photon excitation process, i.e. transfer from S_0 to S_1 proceeds via a

simultaneous (10^{-16} s) absorptions of two photons, each having approximately half the energy of the potential between S_0 and S_1 (Figure 5.1).

Two-photon excitation was theoretically predicted by Maria Göppert-Mayer in 1931 [161] and practically demonstrated by Kaiser and Garret in 1961 [162]. However, the technique remained in the hands of the 'spectroscopist' until 1992 when Denk and coworkers realized the first imaging system based on two-photon excitation by combining a laser scanning systems and a high intensity pulsed infrared laser [163].

The probability of a two-photon excitation depends on the intensity of the excitation light. Under normal light conditions, it is unlikely that a molecule absorbs two photons within the same quantum moment, e.g. the probability for a one- and a two-photon excitation of a molecule of Rhodamine B in bright sunlight, is approximately once every second respectively once every 10 million years [164]. The output power from a conventional continuous wave laser, used in a standard confocal imaging system, is around 100 000 times to low to generate measurable amounts of two-photon induced fluorescence [164].

In TPM, the rate of the two-photon excitation is increased to measurable levels, by focusing a high intensity pulsed laser beam with a high numerical aperture objective. A pulsed laser enables the use of a very high peak power while keeping the average power low enough not to damage the sample. For example, a pulsed titanium:saphire laser is normally operated at approximately 80 MHz with a pulse duration time of around 100 fs. Thus the laser is on only approximately 8 µs per second keeping the average output from the laser on the same level as a conventional continuous wave laser. As intensity depends on the surface (photon×s^{-1}×cm^{-2}), focusing the laser beam with a high NA objective (~10^{-9} cm^2) generates enough photons in the focal volume [165].

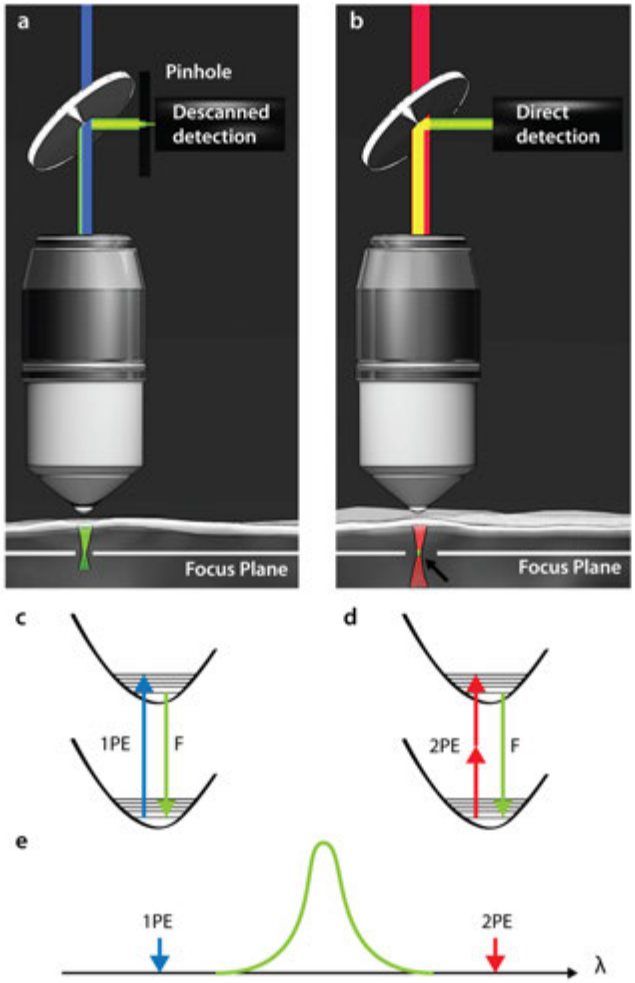

Figure 5.1. Principles of optical sectioning in confocal and two-photon microscopy. In confocal microscopy, the fluorescence is generated using UV-Vis excitation light. The signal is collected via the objective lens and the scanning unit (not shown) and focused on a detector. A pinhole placed in front of the detector function as a spatial filter, excluding out of focus light (**a**). In TPM, fluorescence is induced using near infrared light and optical sectioning can be achieved without the pinhole due to the intrinsically confined excitation volume (green dot, arrow, **b**). Jablonski diagram, showing the generation of fluorescence (F) via a single-photon excitation (1PE, **c**) and a two-photon excitation (2PE, **d**). A single-photon excitation of a fluorescent compound leads to a Stokes shift and a fluorescent signal at a longer wavelength. In TPM the emitted light has a shorter wavelength compared to the excitation wavelength (**e**).

The probability of a two-photon excitation can be calculated according to Equation 3, where δ_2 is the two-photon cross section, P_{ave} the time averaged power of the excitation light, f_P the frequency of the pulsed laser, τ the pulse duration time, NA the numerical aperture objective and λ the wavelength of the excitation light [166].

$$n = \frac{\delta_2 P_{ave}^2}{\tau_p f_p^2} \cdot \left(\frac{NA^2}{2hc\lambda}\right)^2 \qquad \textbf{Equation 3}$$

5.2.2 Optical sectioning

Compared to confocal microscopy, optical sectioning is improved in TPM due to superior penetration of the near infrared illumination light, which is the so called 'optical window' of tissue [165, 167]. As the two-photon excitation depends on the absorption of two photons there is a quadratic, nonlinear, relationship between the fluorescence emission and the intensity of the illumination light. Thus the rate of the two-photon excitation decreases rapidly outside the focal volume. This means that all signals come from molecules in the focal volume and optical sectioning without using a pinhole is possible. Hence, all signal, also scattered photons from the focal volume can be detected, improving the sensitivity and light collection efficiency [168].

Out of focus photobleaching and phototoxic effects are also reduced due to the inherently confined excitation volume and the infrared light is less harmful to living cells and tissue [169]. Moreover, as fluorescent molecules has relatively broad and overlapping two-photon absorption spectra it is often possible to excite several different compounds with the same laser, while keeping the full visible spectral region free for the detection of the emission light [170]. Thus TPM permits straightforward multicolor imaging, e.g when imaging the distribution of a topically applied compound in combination with endogenous fluorescent signals or second harmonic generations [171].

In this work two different TPM systems have been used. A Bio-Rad Radiance 2100MP Rainbow confocal scanning system coupled to an inverted Olympus IX71 microscope and a femtosecond pulsed titanium:sapphire laser (Tsunami®, Spectra-Physics) was used in Paper I-IV. The system used in paper V was a Zeiss 710 laser scanning system operated with a Axio Examiner upright microscope and a MaiTai DeepSeeTM femtosecond pulsed laser.

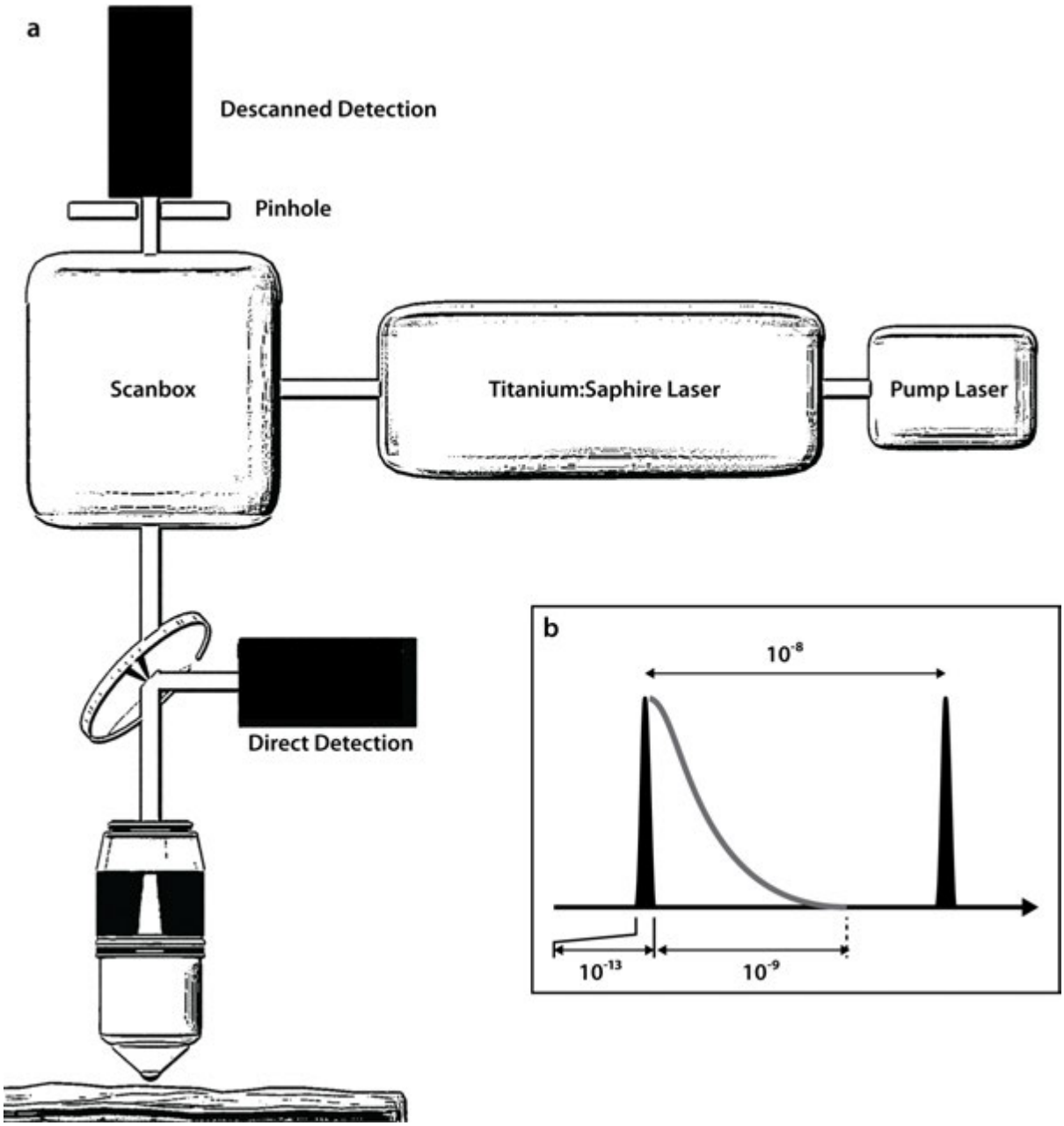

Figure 5.2. Schematic illustration of a two-photon microscope including a pump laser, a pulsed titanium:sapphire laser, a scan box with scanning mirrors, the microscope with objective lens, direct detectors and descanned detectors (**a**). Timescales for the generation fluorescence using a pulsed titanium:saphire laser, i.e. pulse duration time = 10^{-13} s, fluorescence = 10^{-9} s and pulse repetition rate = 10^{-8} s (**b**).

6 Aims and objectives

This thesis explores different aspects of the cutaneous absorptions and distribution of drugs and skin sensitizers. Specifically, TPM has been applied to get mechanistic insights into processes related to the uptake of xenobiotics and ACD. The specific aims in this thesis were:

1. To investigate the uptake and distribution of a hydrophilic model drug following topical application in bicontinuous cubic phases.

2. To compare the cutaneous uptake and distribution of a hapten and a non-sensitizing structural analogue.

3. To investigate the sensitization potency of haptens, when applied in combination with nano- and micro-scale lipid vesicles

4. To study lipid vesicle induced effect on the skin penetration and percutaneous absorption of haptens in relation to sensitization potency.

5. To identify hapten targets in skin using caged fluorescent compounds.

6. To investigate phthalate induced enhancements in the sensitization potency of isothiocyanates.

7 Summary of Papers

7.1 Lipid cubic phases in topical drug delivery: Visualization of skin distribution using two-photon microscopy (Paper I)

It has been shown that MO and PT (Figure 4.2) based lipid cubic phases increases aminolevulinic acid (drug used in photodynamic therapy of skin cancer) induced generation of protoporphyrin in mice [128]. The aim in Paper I was to investigate the uptake and distribution of a hydrophilic fluorescent model drug, sulforhodamine B (SRB, Figure 7.1) in human skin, following topical application in the same, MO and PT based formulations.

The experiments were performed *in vitro* using excised human skin, and the cutaneous absorption of SRB was visualized using TPM. The uptake from the cubic phases was compared with a commercial ointment and an aqueous solution. Briefly, full-thickness excised human breast skin was incubated for 24 h with test formulations in static diffusion cells. The formulations were then removed and the skin was mounted in imaging chambers and imaged by TPM.

The cubic phases was shown to be the most effective formulation, improving the uptake of SRB, as compared to the ointment and the aqueous solution (Figure 7.1), corroborating the previous results by Bender et al [128] It was also revealed that SRB penetrated the skin via the extracellular lipid matrix from all four formulations. However, the distribution of SRB in the extracellular domain differed between the cubic phases, the ointment and the water formulation. Two separate hydrophilic penetration routes were identified, an extracellular and an intercluster pathway. Extracellular uptake was predominant when SRB was applied in the water formulation and in the commercial ointment as seen by a homogenous polyclonal distribution in the *stratum corneum* (Figure 7.1). Strongly fluorescent, relatively broad transversal SRB labeled regions could be detected in some samples exposed to the commercial ointment. These were also detected in samples exposed to the MO and PT based cubic phases and is believed to correspond to deposition of the formulation in skin wrinkles.

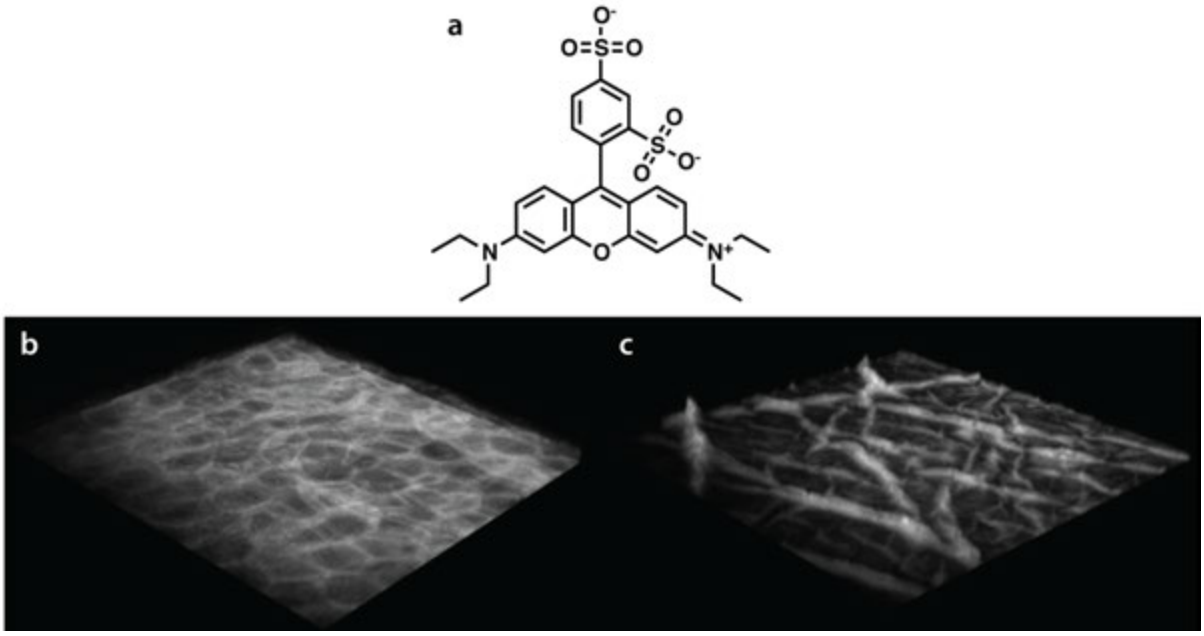

Figure 7.1. Cutaneous distribution of sulforhodamine B (SRB, **a**), following topical application in an aqueous solution (b) and a MO-based lipid cubic phase (**c**). Image plane 323 × 323 µm.

When SRB was applied in cubic phases, it was also found in a finer network of micro-fissures, enclosing clusters of corneocytes and thinner thread like structures. Similar micro-structures, separating clusters of 3 to 10 corneocytes, have previously been described as a potential penetration pathway from lipid based formulations in mouse skin, and has been referred to as the intercluster pathway [149, 172]. When SRB was applied in the cubic phases it penetrated relatively deep into these fissures although their dimension decreased with depth. SRB was detected below 30 µm in 50% of the samples treated with the MO-based cubic formulations and in approximately 70% of the samples exposed to the PT-based cubic phases. Consequently, the intercluster pathway might be a less resistant route for relatively deep deposition of hydrophilic drugs in the skin. It is possible that this pathway is favored by some lipid-based delivery systems.

In conclusion, the previously reported improved delivery of hydrophilic drugs using cubic phases was confirmed. In addition, skin distribution data obtained by TPM indicates that SRB is taken up via micro-fissures in the *stratum corneum*, which could act as a reservoir for continuous release of drugs to the surrounding lipid domain of the *stratum corneum* and to the deeper skin layers.

7.2 Accumulation of FITC near stratum corneum – visualizing epidermal distribution of a strong sensitizer using two-photon microscopy (Paper II)

To trigger an immunological response a hapten must penetrate the skin and form immunogenic hapten-protein complexes (Figure 3.1); however, where exact this happens and the nature of relevant hapten targets has not been elucidated. The aim in Paper II was to compare the cutaneous uptake and distribution of a reactive hapten and a non-reactive non-sensitizing structural analogue. The two investigated compounds were, FITC and fluorescein (Figure 3.3). The sensitization capacity of FITC and fluorescein was investigated in the murine LLNA and the cutaneous and percutaneous absorption were investigated *in vitro* using excised human skin, combining diffusion cells, confocal and TPM.

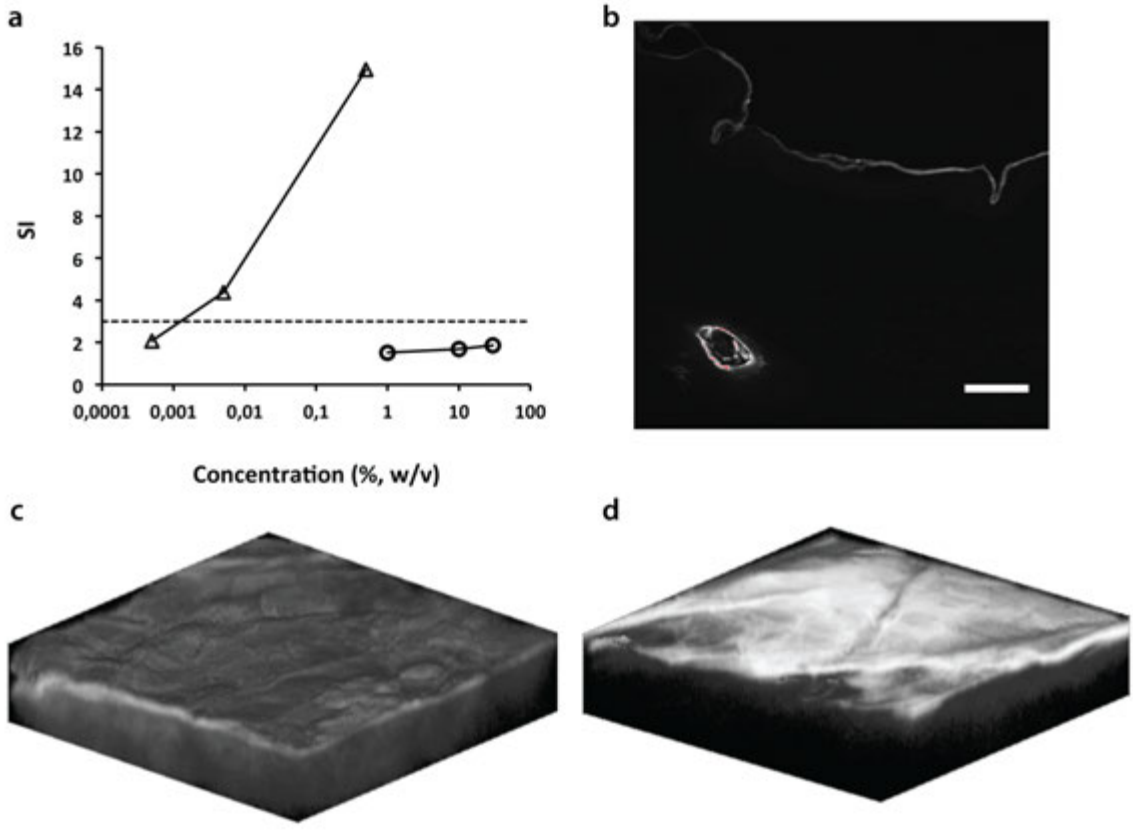

Figure 7.2. LLNA dose response curves for FITC (**Δ**, EC3 = 0.002%) and fluorescein (**O**). The latter was found to be a non-sensitizer up to the highest tested concentration of 25% (**a**). Confocal image of FITC exposed cryosectioned skin sample (**b**, scale bar = 50 µm). Non-invasive TPM visualization of the cutaneous distribution of fluorescein (**c**) and FITC (**d**) in excised human skin (image plane = 179 × 179 µm).

The results revealed that FITC was an extreme sensitizer whereas fluorescein was a weak or non-sensitizer up to the highest tested concentration (30%, w/v) in the LLNA (Figure 7.2). Though an extreme sensitizer, only small amounts of FITC seemed to penetrate the viable compartments of the skin (Figure 7.2). While fluorescein readily penetrated the skin, FITC accumulated at the surface, in the *stratum corneum*. A similar distribution could be seen in confocal images of cryosectioned FITC and fluorescein treated samples. Interestingly, FITC was also detected relatively deep into the skin inside hair follicles, in the cryosectioned samples.

It is likely that the reduced uptake of FITC is due to conjugation to macromolecules in the stratum corneum. Based on these results one could speculate that the limited fraction of the applied dose reaching the viable tissue is enough to trigger an immunologic reaction. Alternatively, it is possible that Langerhans cells in the boundary between the viable epidermis and *stratum corneum* could intercept hapten protein complexes formed in the *stratum corneum*. A third alternative is that uptake via the hair follicles could play an important alternative way for some haptens to enter the skin.

In conclusion, it was found that, although FITC is an extreme sensitizer in the LLNA, it does easily penetrate the skin barrier. Based on these results it is unclear if only low amount of compound reaching the viable epidermis is enough to trigger an immune reaction or if alternative mechanisms, e.g. follicular uptake, can explain the sensitization potency of FITC.

7.3 A study of the enhanced sensitizing capacity of a contact allergen in lipid vesicle formulations (Paper III)

The introduction of nano-sized materials in topical products has lead to many important improvements but also some questions regarding adverse health effects. Recent research indicates that the sensitization potency of haptens increases when applied in vesicle based delivery systems [173, 174]. The aim in Paper III was to study the variations in the sensitization potency and skin penetration of the fluorescent model hapten RBITC (Figure 3.3) when applied in combination with micro- and nano-scale vesicles (ethosomes) and in an ethanol in water (Et:W) control solution. The sensitization potency was studied *in vivo* using

a modified non-pooled murine LLNA and the skin penetration and percutaneous absorption was analyzed *ex vivo* using diffusion cells in combination with TPM. Nano-scale vesicles (< 100 nm) and micro-scale vesicles (> 1 μm) were prepared and analyzed as described in chapter 4.

The modified LLNA with non-pooled lymph nodes (Figure 7.3) revealed an almost 5-fold increase in the lymph node cell proliferative response when RBITC was applied in a vesicle formulation (3579 ± 1074 dpm) compared to when it was applied in a Et:W solution (643 ± 334 dpm). There was also a significant, but small, increase in the lymph node cell proliferative response when RBITC was applied in Et:W compared to the negative control (409 ± 139 dpm), i.e. an empty vesicle formulation.

When the sensitization potency of RBITC was investigated in two structurally different vesicle systems, i.e. in nano-scale and micro-scale vesicles, it was slightly increased ($p < 0.05$) in the micro-scale vesicles (4913 ± 1551 dpm) compared to the nano-scale vesicles (3146 ± 837 dpm). A potential explanation for the difference between the micro- and nano-scale vesicle formulation could be variations in the concentration of POPC rather than the structural differences of the vesicles. It was found that approximately 20 % of the lipid fraction was lost during the extrusion process, thus the actual lipid concentration was 20 % lower in the nano-scale formulation compared to the micro-scale formulation tested in the LLNA. This assumption is supported by previous results by Madsen et al showing that the sensitizing potency of isoeugenol is related to the concentration of POPC in the formulation [174]. The overall conclusion from the LLNA experiments was that the POPC-based vesicle formulations significantly increase the sensitization potency of RBITC, but that the size or the structure of the vesicles has a less significant effect.

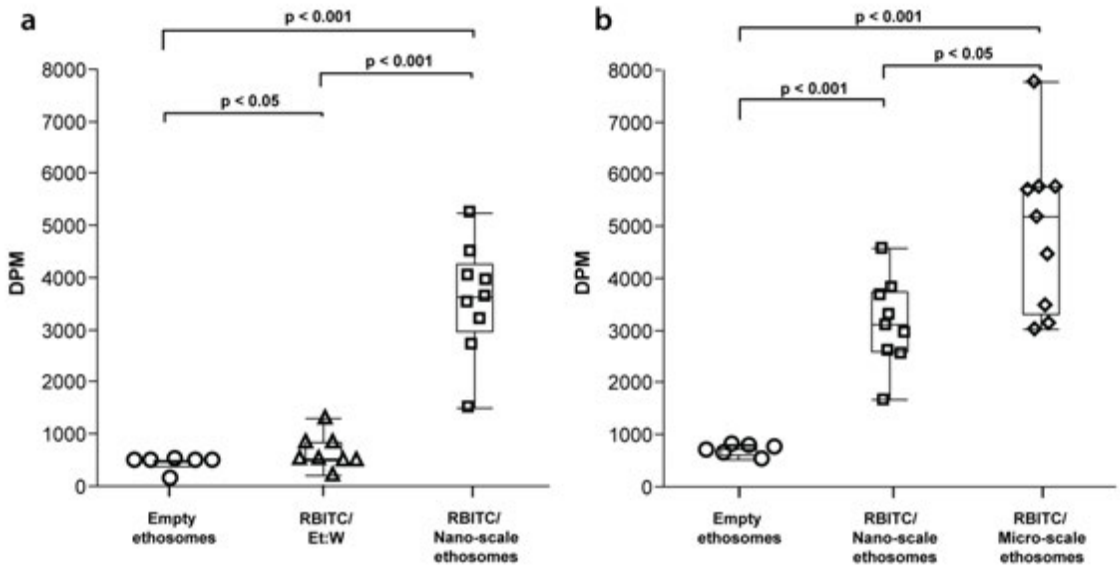

Figure 7.3. Non-pooled local lymph node assays, demonstrating the relative sensitization potency of RBITC in a nano-scale ethosomal formulation compared to an Et:W solution (**a**) and nano-scale compared to a micro-scale ethosomal formulation (**b**).

Initial measurement of the percutaneous absorption of RBITC did not indicate any difference between the vesicle based formulation and the Et:W solution. However, TPM revealed that both nano-scale and the micro-scale lipid vesicles increased the epidermal uptake of RBITC compared to the Et:W solution (Figure 7.4). Specifically, the signal was significantly increased between a depth of 32 to 88 µm, when RBITC was applied in nano- and micro-scale ethosomes compared to the Et:W solution. The result indicates that the observed variations in the sensitization potency of RBITC between the vesicle formulations and the Et:W solution is at least partly a consequence of variations in the cutaneous uptake of the hapten.

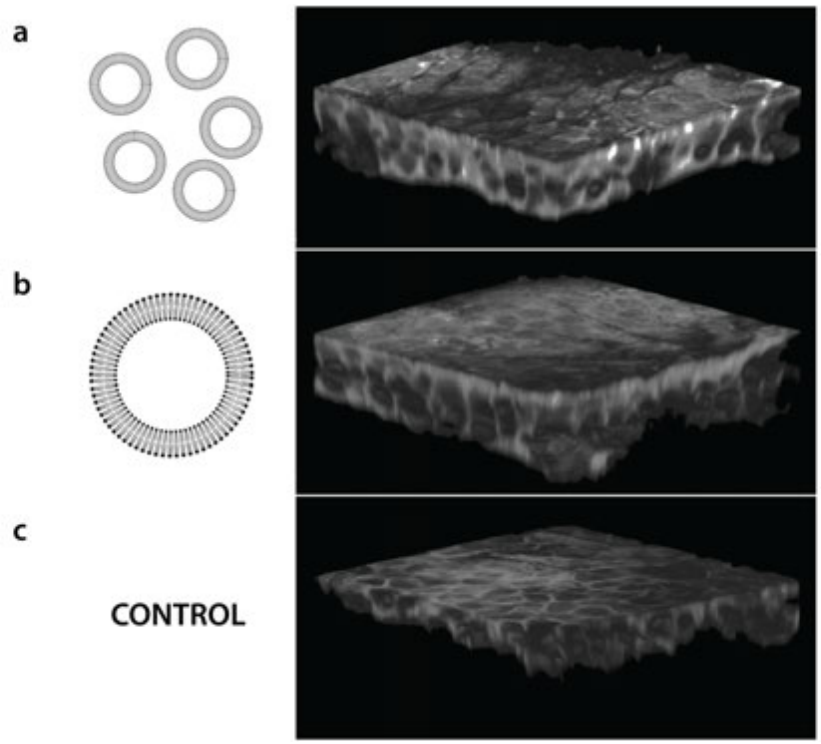

Figure 7.4. Epidermal distribution of RBITC following topical application in a nano-scale vesicle formulation (**a**), in a micro-scale vesicle formulation (**b**) and in an ethanol:water control solution (**c**).

There was no difference in the cutaneous absorption from the two vesicle formulations. A likely explanation for this is that the vesicles are disrupted on the skin surface and that the size therefor only has a minor effect. It is possible that the free lipid molecules act as a penetration enhancers, i.e. by interacting with the lipid bilayers altering the solubility or diffusion coefficient of RBITC in the SC or by increasing the vehicle to *stratum corneum* partitioning coefficient.

In conclusion, the results presented in Paper III, shows that the sensitization potency of RBITC increases when applied in nano and micro-scale ethosomes compared to an Et:W solution. In combination with the results by Madsen et al this implies a general increase in the sensitization potency of haptens applied in vesicle formulations. However, nano-scale vesicles do not seems to have any increased adverse effect compared to larger micron-scale vesicles. The sensitization enhancing effect of the vesicle formulations is likely related to an improved penetration of the haptens from these vehicles. Still, further studies are needed before a more general conclusion can be drawn regarding the risk of being sensitized in combination with exposure to vesicle based formulations.

7.4 Caged Fluorescent Haptens Reveal the Generation of Cryptic Epitopes in Allergic Contact Dermatitis (Paper IV)

The location of hapten protein targets (e.g. intra cellular, membrane bound or extra cellular proteins) and the molecular identity of the immunogenic hapten-protein complexes and corresponding epitopes have not been elucidated. In Paper IV, a group of caged fluorescent compounds, i.e. bromobinanes (Figure 3.3), were topically applied and followed using a combination of fluorescence microscopy, proteomics and mass spectrometry, with the aim to identify their protein targets.

It was presumed that the thiol reactive bromobimanes were potential sensitizers. However, before any further studies could be conducted, this had to be verified experimentally. Thus, skin sensitization potency of mBBR, dBBR and a non-reactive negative control compound, syn-(methyl,methyl)bimane, was tested in the LLNA (Figure 7.5 a). The LLNA result revealed that both mBBR (EC3 = 0.12%) and dBBR (EC3 = 0.17%) were strong sensitizers while syn-(methyl,methyl)bimane was a non-sensitizer up to a concentration of 2.5%.

The localization of hapten targets was initially visualized by TPM, which revealed that the bromobimanes reacted with proteins or peptides in *stratum corneum* (Figure 7.5). With a relatively high concentration of the hapten in combination with a high abundance of nucleophilic amino acids, peptides and partially degraded proteins in the *stratum corneum* [175, 176] it is not surprising to find extensive hapten-protein conjugations in this skin layer. However, the immunological implication for the formation of hapten protein complexes in *stratum corneum* is unclear. TPM imaging also revealed that the bromobimanes penetrated and reacted with cells in the viable epidermis. A bit surprising, the bromobimanes did not primarily react with the suprabasal cells, as seen by a relatively weak fluorescence signal in the stratum spinosum. Instead, TPM images indicated that basal cells were the primary targets in the viable epidermis. This result was supported by confocal imaging of cryosectioned bimane treated skin samples.

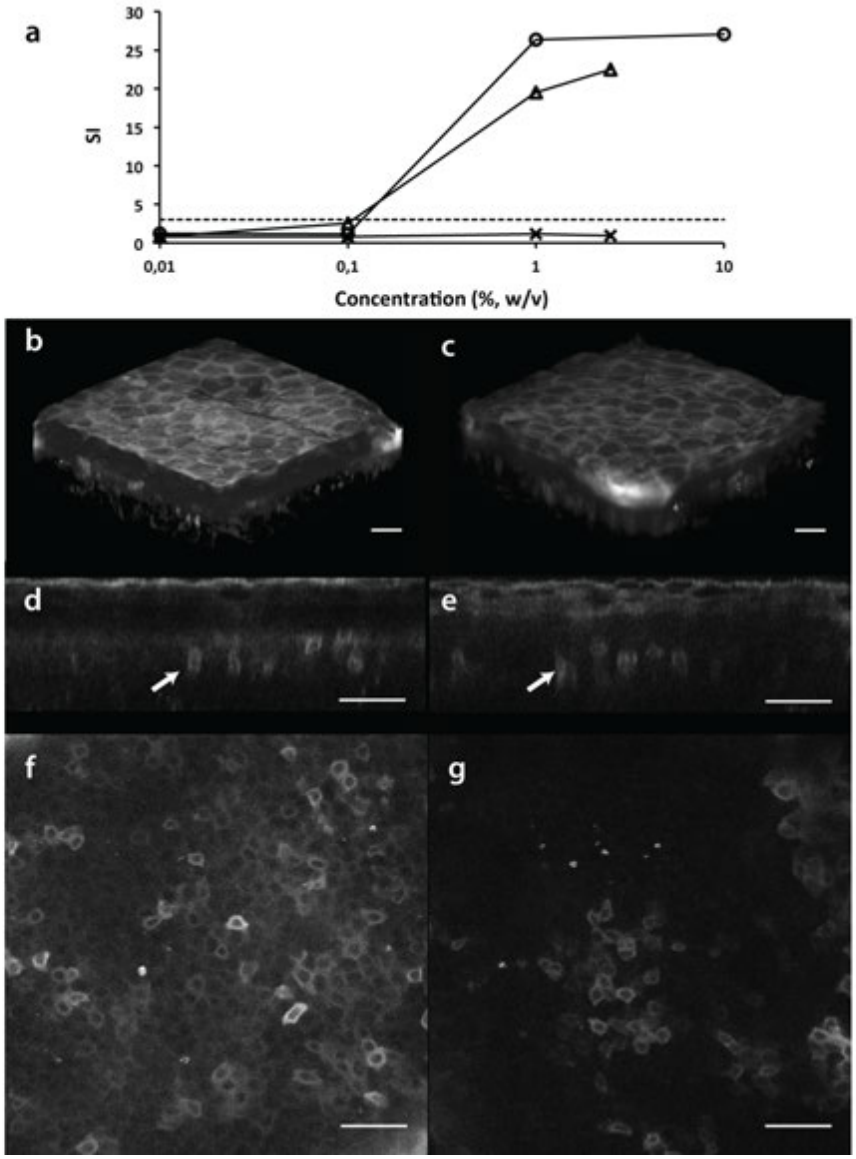

Figure 7.5. LLNA dose response curves for mBBr (EC3 = 0.12%, **Δ**), dBBr (EC3 = 0.17%, **O**) and syn(methyl,methyl)bimane (**×**). Three-dimensional visualization of the epidermal distribution of mBBr (**b**) and dBBR (**c**) protein targets. Optical z-cross section of a mBBr exposed sample (**d**) and a dBBr exposed sample (**e**). Lateral distribution of mBBr (**f**, z = 52 µm) and dBBr (**g**, z = 62 µm) in or adjacent to the basal cell layer.

Initially, it was suspected that the fluorescent cells were epidermal Langerhans cells. However an immunohistochemical analysis of cryosectioned skin samples using CD1a, K5 and K14 antibodies, excluded the Langerhans cells and indicated that the bimane labelled cells corresponded to basal cell keratinocytes. This was later supported by both western blot and mass spectrometry analysis. Another observation made in both the TPM and confocal images, was that the bromobimanes did not label all cells in the basal cell layer.

Instead clusters of bimane-labelled cells were detected. The clustered pattern led to a hypothesis that the labelled cells might be epidermal stem cells, which have been shown to reside in stem cell niches, i.e. clusters in the basal cell layer [177, 178]. The exact reason for patchy distribution of the bimane labelled cells was not further investigated, thus the reason for the irregular lateral distribution of the bimane labelled cells remains speculative.

To identify the protein targets, protein extracts from skin samples incubated with bimane formulations were prepared and separated by SDS-PAGE. The strongest fluorescent band from the mBBr treated sample had a molecular weight of approx. 60 kDa and the strongest fluorescent band in the dBBr treated sample had a molecular weight of approx. 110 kDa. In combination with the results from the TPM and the Immunohistochemical analysis, demonstrating that basal keratinocytes were hapten targets, it was speculated that the protein band around 60 kDa corresponded to K5 (58 kDa) and that the band at around 100 kDa could be cross-linked K5 or K14 monomers (100 or 116 kDa), alternatively cross-linked K5/K14 heterodimers (108 kDa). A Western Blot confirmed this hypothesis.

The most intense protein bands in the SDS-PAGE gels were excised and analyzed by tandem mass spectrometry. A Mascot search corroborated the previous identification of K5 and K14 as hapten targets in the skin. K5 was positively identified in the 58 kDa band from the analysis of the mBBr treated sample and both K5 and K14 were present in the excised band from the dBBr treated sample. In addition, manual interpretation of the tandem mass spectrometry data revealed a tryptic fragment (V48 to R62, Figure 7.6) with a haptenated cysteine residue (C54). Subsequent, analysis of the fragmentation pattern of the MS/MS spectra confirmed the modification on C54.

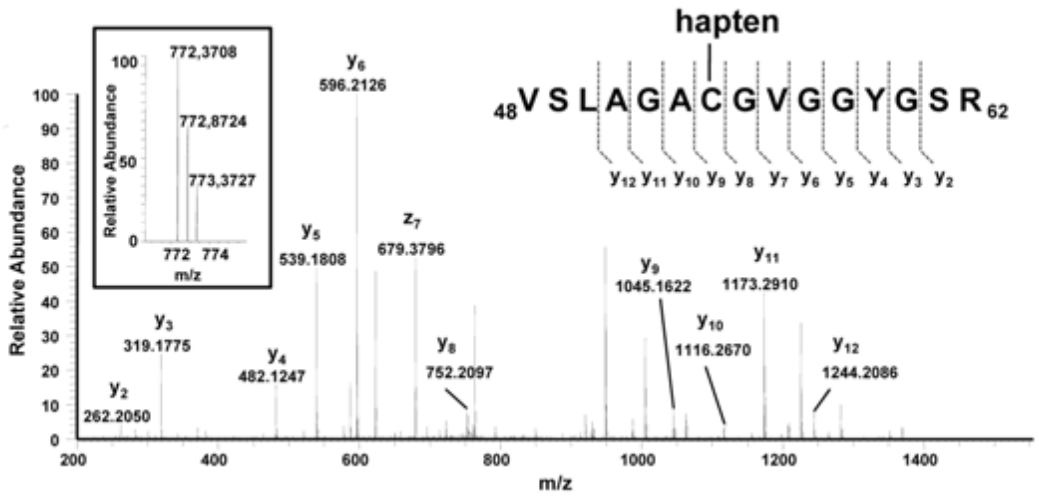

Figure 7.6. Tandem mass spectra of the tryptic fragment (V48 to R62) of keratin 5 with a mBBr modified cysteine (C54).

In conclusion, a combination of caged fluorescent haptens, fluorescent microscopy, immunohistochemistry and proteomics led to the identification basal keratinocytes and specifically the keratins K5 and K14 as potential hapten targets in human skin. A hapten modification of the residue C54 on K5 was also identified. The result highlights the role of the keratinocytes and the keratins in ACD.

7.5 The pilosebaceous unit – a phthalate-induced highway to skin sensitization (Paper V)

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8 General Discussion

The development of efficient epicutaneous drug delivery systems rely on a fundamental understanding of the skin barrier and its interaction with topically applied compounds and formulations. This is equally important when evaluating biologic effects following topical exposure to harmful xenobiotics, e.g. skin sensitizers. A central component in all research and development are tools to measure and analyze specific processes and effects. TPM offers unique possibilities to study the relative uptake, penetration pathways, skin targets and depot effects of topically applied fluorescent compounds relatively deep into intact skin samples, giving valuable information about the interactions between the skin and xenobiotics. The application of TPM within the work of this thesis has provided new insights in drug delivery as well as mechanisms involved in contact allergy, as will be discussed below. In addition, a discussion of the methods chosen will be provided, along with a final conclusion and some future perspectives.

8.1 New insights in drug delivery

The improved cutaneous uptake from lipid cubic phases applied to human skin offers clinical applications due to the penetration enhancing effect of these formulations. In this work, TPM provided supplementary data regarding the cutaneous distribution and skin penetration pathways. A putative mechanistic explanation for the observed penetration enhancing effect of the cubic phases could be proposed based on the penetration pathways observed. In a broader perspective, better insight into drug uptake and distribution using TPM could improve interpretation of pharmacokinetic effects and support in future development and improvements of topical formulations. It could also assist in the choice of vehicle depending on target tissue. Specifically, cosmetic products are generally intended to target, either the skin surface or the *stratum corneum*. Penetration to the deeper skin layer is often undesirable and vehicle induced penetration enhancing effects might increase the risk of adverse health effects.

8.2 New insights in contact allergy

8.2.1 Vehicle effects on the sensitization potency of haptens

The LLNA revealed significant vehicle induced variations in sensitization potency of haptens. Specifically, the results showed that isothiocyanates are extreme sensitizers when applied in combination with phthalate (DBP) but non- or weak sensitizers when applied in e.g. DMSO.

There is a general correlation between bioavailability and biological effects. However, FITC did not seem to penetrate the skin when applied in combination with DBP (Paper II), at least not via the *stratum corneum.* An alternative penetration pathway via the hair follicles was hypothesized based on confocal microscopy of cryosectioned human skin samples exposed to FITC. This pathway was later clearly identified as major contributor to the cutaneous and percutaneous absorption of both RBITC and FITC when applied in combination with DBP *in vivo* in mouse (Paper V). Compared to the absorption of RBITC in acetone and DMSO, the target specific delivery via the PSU appears as a likely explanation for the increased sensitization potency of isothiocyanates in the LLNA.

In Paper III it was found that the increased sensitization potency of RBITC correlated with an improved general cutaneous absorption in excised human skin, but no specific uptake via the hair follicles was detected. A preliminary experiment in connection with the study presented in Paper V, indicated that ethosomal formulations also improves the uptake of RBITC into the pilosebaceous units compared to the control vehicle, *in vivo* in mouse (data not shown). Thus there seems to be a connection between follicular absorption and sensitization potency also in this case.

The PSUs have traditionally not been considered to play any significant role in the uptake of topically applied compounds. However, new studies indicate that follicular transport has been underestimated and hair follicles have become attractive targets in epicutaneous drug delivery [24, 28, 182, 183]. The PSU have so far not been widely discussed as a mediator of CHS reactions, although it could be regarded as an ideal route for hapten to penetrate the viable compartments of the skin, bypassing the *stratum corneum*. In addition, the surrounding tissue has a relatively high abundance of immune cells, i.e. dendritic cells, T-cells and macrophages [25, 26]. The PSU is also the skins largest reservoir of stem cells [9] and it has a closely connecting extensive network of perifollicular capillaries, enabling

effective systemic resorption of compounds absorbed via the PSU [28]. Moreover, compounds stuck in the follicle orifice will likely reside in the skin for longer time periods compared to compounds trapped in the *stratum corneum* [24]. The reservoir capacity of the PSU could have a role in e.g. primary ACD reactions [184]. It is also likely that repeated contact with non-sensitizing doses of hapten accumulating in the PSUs eventually leads to sensitizing concentrations in the skin. Repeated exposure is an important predisposing factor in ACD [185].

Being a well-known penetration enhancer, DMSO was chosen as one of the control vehicles in Paper V. Considering its capacity to improve the uptake of topically applied compounds, it was initially surprising to find that RBITC did not induce any proliferative response in the LLNA in DMSO. It was even more surprising that RBITC did not penetrate the skin more when applied in this vehicle. A reactivity assay suggested that DMSO provokes a rapid conjugation of RBITC to proteins in the upper *stratum corneum*, which limits the uptake of RBITC into the skin. Thus, vehicle induced variations in reactivity towards skin surface proteins should be considered as a regulating factor when exploring the uptake of reactive compounds like sensitizers.

Taken together, the results indicate that isothiocyanates, due to their reactivity, do not readily penetrate the skin directly via the *stratum corneum*, except perhaps if applied in very high concentrations. Thus these compounds need an alternative route of entry into the skin to induce an allergic response; e.g. via shunts or via damaged skin. Uptake via the PSUs seems to be facilitated in combination with DBP. The results also reveal that vehicle dependent hapten reactivity towards *stratum corneum* proteins regulates the uptake and thus the potency of the hapten.

It is important to point out that modifications in the chemical composition of the vehicle can have other consequences, which might influence the potency of the haptens. For example phthalate or POPC induced adjuvant effects, i.e. induction of inflammatory mediators, have not been investigated here, and can therefore not be ruled out.

Regardless of the mechanism behind the vehicle-induced variations, the results emphasise the importance to consider formulation effects when evaluating the potency of skin sensitizer. Variation in the sensitizing capacity due to formulation effects should be

considered when performing risk evaluation based on the LLNA EC3 value. Similarly, vehicles effects should also be considered when performing patch tests of ACD patients.

8.2.2 Identification of hapten targets

One of the great challenges in the research related to contact allergy is to identify immunogenic hapten-protein complexes and the nature of the modification of the protein targets. Considering the amount of protein targets in the skin, this might be like looking for a needle in a haystack. In Paper IV, a combination of caged fluorescent haptens, TPM, proteomics and mass spectrometry led to the first published identification of a haptenation site in human skin tissue. The thiol reactive bromobimanes specifically reacted with basal keratinocytes and the keratins K5 and K14. Furthermore C54 on K5 was identified as a hapten target.

Structural and chemical variations in the differentiating epidermal keratinocytes could be one reason for the specific reactivity of the bromobimanes with the basal keratinocytes. A reactivity assay revealed that bromobimanes readily reacts with thiols but not with disulfides. Thus, hapten targets in the skin should be expected to be free thiols. The relatively high abundance of available free thiols in the basal compared to the suprabasal keratinocytes, where the sulfhydryles are concealed due to extensive bundling of the keratins and cross linking of the cysteines, could lead to a more intense labelling of the basal cell layer. A similar localisation have previously been observed in studies on the epidermal distribution of DNCB, another thiol reactive skin sensitizer [186].

Based on the results in Paper IV, further investigations has been conducted *in vitro* using cultured human keratinocytes [187]. Incubation with the bromobimanes led to a rapid internalization of the haptens and labelling of structures resembling the keratin intermediate filaments. In addition, the keratinocytes responded by forming fluorescent membrane vesicles (blebs). It could be shown that the vesicles contained both haptenated K5 and K14. The specific labelling of C54 in K5 could also be confirmed. As C54 is part of the head-region of K5, which binds to desmoplakin and the desmosomes, an interesting speculation would be if haptenation could lead to malfunction of the desmosome complex and subsequently the keratinocyte. The outcome of this malfunction could perhaps result in

the release of non-self immunogenic keratin-epitopes in membrane vesicles; however, this has yet to be demonstrated *in vivo*.

The new restrictions of the use of animal based toxicity assays, e.g. using the LLNA for predicting the sensitization potency of haptens, has led to an urgent need for new *in vitro* based assays, as discussed in chapter 3. Thus, the results obtained within this project have stimulated to further investigation whether the keratinocyte response, i.e. the blebbing, could be used as an alternative *in vitro* assay to test the sensitization potency of skin sensitizers [188].

8.3 The choice of skin model

In four out of five papers included in this thesis, skin penetration experiments were performed *in vitro* using excised human skin, obtained from breast reduction surgery. The skin was stored at -70 °C up to 3 month before it was used. Penetration studies using TPM were carried out using full thickness skin samples and permeation experiments were conducted using heat separate epidermal sheets. In either case, viability was not maintained in these assays. This is generally not an issue, assuming uptake is a passive diffusive process and *stratum corneum* being the rate limiting membrane. However, some artefacts e.g. due to heat-separation cannot be ruled out. Evaluating the integrity of the isolated epidermal sheets, e.g. measuring electrical resistance, might be appropriate, but has not been performed in these studies. Reconstructed skin is an interesting viable alternative; however cases of deviations in permeability compared to isolated human skin has been reported. Moreover, reconstructed skin models do not include shunts pathways and potential effects of the circulatory system. Although mouse models (Paper V), provides *in vivo* setting, differences in the architecture of mouse and human skin, e.g thickness and follicle density, must be considered.

8.4 Two-photon microscopy of the skin, benefits and limitations

TPM offers a unique possibility to explore the interactions between the skin and xenobiotics, e.g. skin sensitizers, and cutaneous immunological reactions in or close to *in vivo* conditions. It is also an interesting method to study topical drug delivery. TPM provides information, which would be difficult to extract from more traditional techniques, e.g.

regarding local absorption and targeted delivery. Compared to confocal microscopy, TPM provides an improved imaging depth and a more straightforward approach to multicolor imaging. Multiple fluorophores, both endogenous and exogenous, and single harmonic generation can be visualized using a single laser. This facilitates imaging of the skin morphology when e.g. studying the absorption of topically applied compounds.

However, imaging depth is not unlimited and lack of knowledge regarding signal losses makes absolute quantification more or less impossible. In addition, high concentration of fluorophores at the skin surface, which is a common situation when studying the cutaneous absorption of topically applied compounds, seems to generate 'ghost' signals which can complicate the imaging of the deeper skin layers. Furthermore, the vertical resolution is generally better when imaging cryo-sectioned skin samples, though at the cost of a reduced field of view and an increased risk for artifacts and sample contamination. A drawback using this technique is that only fluorescent model drugs or haptens can be visualized. Antibodies can be used on sectioned skin, but their utility is limited in thick samples.

An important limitation of the TPM technique to analyze the cutaneous absorption of topically applied compounds is the incapacity to determine absolute fluorophore concentrations in tissue. Only relative estimations of the concentration of the same compound at a specific depth can be done. It is difficult to e.g. compare the cutaneous absorption of two different compounds or the relative concentration of a single compound at to different depth in the skin. One possible solution to this problem is the combination of TPM and fluorescence correlation spectroscopy, i.e. a technique referred to as two-photon fluorescence correlation microscopy. By measuring the fluctuations of fluorescent signal in the subfemtoliter two-photon excitation volume it is possible to determine both absolute concentrations and diffusion constants of fluorescent molecules in in the skin [189].

Despite the limitations mentioned above, TPM has opened up a unique opportunity to study biological processes in real time in living animals. *In vivo* and intra vital TPM has consequently attracted a lot of interest in the last years, in particularly within the field of immunology. The skin penetration studies included in thesis were performed either *in vitro* using excised human skin or combining *in vivo* exposure and *ex vivo* imaging (Paper V). While the experimental design used in e.g. Paper V is practical and provide the possibility to

image several samples at the same occasion, it does not provide a dynamic view of what is happening in the skin. Thus for a more detailed and complete picture of the processes related to exposure to skin sensitizers and the faith of a hapten in a living system, *in vivo* and intra vital techniques should be further investigated.

8.5 Conclusions and future outlooks

In this thesis, TPM has been used to analyze the cutaneous absorption and distribution of a model drug and a series of model skin sensitizers. A potential mechanistic explanation for the penetration enhancing effect of lipid cubic phases has been proposed and the first confirmed hapten amino acid target in skin has been identified based on studies using TPM. Moreover, vehicle induced variations in the sensitization potency of haptens has been revealed in the LLNA. Specifically, the increased sensitization potency of isothiocyanates applied in combination with phthalates could be linked to a PSU-targeted delivery of the haptens. It could also be shown that vehicles alters hapten reactivity to *stratum corneum* proteins and that this can lead to significant variations in sensitization potency. Together, these results demonstrate some of the potentials of implementing TPM into investigations of the interactions between the skin and xenobiotics.

In the future *in vivo* and *intra vital* techniques should be further investigated to study processes related to exposure to skin sensitizers and the faith of haptens in a living systems. Specifically, some of the findings presented in this thesis should be interesting to investigate further in an *in vivo* model, e.g. the uptake caged fluorescent compounds. Moreover, improvements in two-photon-fluorescence correlation spectroscopy have the potential to give a new perspective on the interactions between xenobiotics and the skin. The possibility to determine exact concentrations and diffusion constants of model drugs in specific regions of the skin or other organs *in vivo* could be valuable e.g. in drug delivery studies.

In conclusion, the work presented in this thesis contributes to the general understanding of the mechanisms involved in the cutaneous absorption of topically applied drugs. It also puts contact allergy in a new focus, using TPM to image cutaneous uptake and distribution of haptens and their protein targets in the skin.

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