



Development of a skin-friendly microemulsion for dermal allergen-specific immunotherapy

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ABSTRACT

Due to their role in immune responses, the skin dendritic cells (i.e. epidermal Langerhans cells and dermal dendritic cells) have become of great interest to researchers in the past decades. A dermal administration of allergens could target these professional antigen-presenting cells directly and build up immunotolerance. Additionally, many of the adverse side effects, which are seen in the current state of the art specific immunotherapy routes, could be avoided.

Therefore, in this study a penetration enhancing microemulsion was developed and its physicochemical properties were determined under several storage conditions. The influence of different preservatives on the microemulsion stability was observed. We examined epidermal penetration of Alexa Fluor-647 labelled bee-venom phospholipase A2 (Api m 1) using the Franz diffusion cell set up and confocal laser-scanning microscopy. First results of an *in-vivo* Api m 1-allergic mouse model indicated the protective efficacy of dermal AIT with our newly developed microemulsion.

Summarily, the developed microemulsion is a suitable, stable drug delivery system for the topical administration of proteogenic allergens into the epidermis and is able to reach dendritic cells in the skin.

1. Introduction

The prevalence for IgE-mediated allergic reactions to Hymenoptera stings varies, depending on several factors, such as region, occupation or individual risk. The current guidelines of the European Academy of Allergy and Clinical Immunology (EAACI) number the risk of systemic reactions with up to 7.5% for adults and up to 3.4% for children (Sturm et al., 2018). Only the allergen-specific immunotherapy (AIT) is fighting the cause of the allergy – with evidence based success (Bødtger et al., 2002; Bonifazi et al., 2005; Walker et al., 1995). While subcutaneous AIT with Hymenoptera venom is associated with potential severe adverse effects, the non-invasive dermal administration of allergens has become focus of researchers (Di Meglio et al., 2011; Prausnitz and Langer, 2008). One approach to enable dermal drug delivery is the transdermal patch system Viaskin® (DBV Technologies). This mode of application is currently undergoing development for the

treatment of several allergies (peanut-, cow milk- and egg allergy) and has reached different stages of clinical trials. Studies showed, that the Viaskin® system could deliver the allergens to the target cells and help to build up immunotolerance (Jones et al., 2017; Mondoulet et al., 2010). However, the major disadvantage of dermal patch systems is the risk of developing a contact dermatitis, which is characterised by itching, erythema and urticaria (Ale et al., 2009). Our research focuses primarily on using microemulsions as drug delivery devices, to benefit from the positive effects of dermal immunotherapy and increase patient's compliance by avoiding local and systemic side effects. Microemulsions are showing great potential for the formulation of a protein containing drug delivery system, due to their characteristics such as natural penetration enhancement, thermodynamical stability and the solubilisation of a vast variety of hydrophilic and lipophilic molecules (Kogan and Garti, 2006; Kreilgaard, 2002; Lawrence and Rees, 2012). For our research we focussed on bee-venom phospholipase A2 (Api m

Abbreviations: AIT, Allergen-specific immunotherapy; CLSM, Confocal laser-scanning microscopy; CFU/g, colony forming units/g; DLS, Dynamic light scattering; LOD, Limit of detection; LOQ, Limit of quantification; ME, Microemulsion; OD₆₀₀, Optical density at 600 nm; OP, Oil phase; PBS, Phosphate Buffered Saline; PDMPD, Phase Diagram by Microplate Dilution; SCoS, Surfactant/Co-Surfactant; WP, Water phase

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1), the main allergen of the European honeybee (*Apis mellifera*) as the model allergen. The aim of this work was to create a microemulsion with a low surfactant content, which enables the model allergen to penetrate into epidermal skin layers or beyond to reach the Langerhans cells and dermal dendritic cells for inducing allergen-specific immunotolerance. The long-term stability at different temperatures and humidity was analysed. The recovery of the allergen was examined using acetone precipitation followed up by HPLC-analysis. Furthermore, the penetration enhancing abilities of the established microemulsion were studied via Franz diffusion cell penetration tests, pursued by confocal laser-scanning microscopy.

2. Material and methods

2.1. Materials

Caproylocaproylmacrogol-8 glycerides EP (Labrasol®) and Polyglyceryl-6 dioleate (Plurol Oleique®) were provided by Gattefossé (Germany). Isopropyl palmitate, (+)-1,3-Butanediol (butylene glycol), 1,2-Propanediol (propylene glycol), 1,5-Pentanediol (pentylene glycol) were purchased from Sigma-Aldrich (Germany).

2-Phenoxyethanol and isopentane (AnalaR Normapur) was acquired from VWR (Germany).

TissueTek O.C.T. Compound was purchased from Fa.Sakura (Germany).

Cetearyl ethylhexanoate (Tegosoft liquid ®) was generously provided by Evonik Industries (Germany).

Bee-venom phospholipase A2 (Api m 1) was purchased from Latoxan (France). Alexa Flour-647 protein labelling kit was purchased from ThermoFisher Scientific (Germany).

Phosphate buffered saline buffer (PBS) was manufactured and consisted of sodium chloride 0.8 wt%, potassium chloride 0.2 wt%, Na₂HPO₄ 0.144 wt%, KH₂PO₄ 0.024 wt%, purified water ad 100 wt%.

2.2. Development of microemulsion using a modified PDMPD method

Pseudoternary diagrams were obtained, combining Phase Diagram by Micro Plate Dilution (PDMPD)-method (Schmidts et al., 2009) and a measurement of the optical density at 600 nm (Synergy HTX, BioTek) at room temperature. Therefore, mixtures of the oil phase (OP; cetearyl ethylhexanoate and isopropyl palmitate 7:1 wt%), surfactant/co-surfactant phase (SCoS; caproylocaproylmacrogol-8 glycerides EP and polyglyceryl-6 dioleate NF 11:8 wt%) and water phase (WP; PBS, glycerol, butylene glycol 83:3:14 wt%) had been prepared. Mixtures of OP and SCoS ranging from 10 wt% up to 90 wt% SCoS were assembled. Microtiter plates (96-wells, 350 µl filling volume) were filled with the OP/SCoS mixture and WP as described by Schmidts et al. (2009) and afterwards gently shaken on a temperature controlled thermomixer at 25 °C. Microemulsions were identified via visual evaluation of the isotropy and measurement of the OD₆₀₀ after a 30 min equilibration-time.

2.3. Characterisation of the developed microemulsion

The developed microemulsion was characterised with regard to physicochemical properties and sufficient preservation. Rheological characterisation of microemulsion was performed using an Anton Paar MCR 102. Cone-and-plate set-up (diameter 40 mm, cone angle 1°) was used for rotational viscosimetry performed at 25 °C. Microemulsion was placed between the plate and cone with a gap of 80 µm, rotation measurement was carried out at increasing shear rate from 500 to 1000 1/s.

The microstructure of undiluted microemulsion was analysed using dynamic light scattering measurement (DLS, Zetasizer Nano ZS90, Malvern, 25 °C, 90° measurement angle). The aggregate size and aggregate distribution were presented as the z-average size (z-average) and polydispersity index (PDI).

To detect signs of incompatibility, turbidity measurements were carried out via determination of the OD₆₀₀ (Synergy HTX, BioTek) at 25 °C.

In order to find the most suitable preservative, placebo microemulsion was tested according to the Pharmacopoeia Europaea 2017 (Topical formulations a) with a preservative efficacy test. The following preservatives were tested: butylene glycol (10 wt%), propylene glycol (20 wt%), pentylene glycol (5 wt%), as well as combinations of butylene glycol/ethylhexylglycerol (10 wt% + 0.5 wt%) and butylene glycol/phenoxy ethanol (10 wt% + 1 wt%) had been prepared. The testing methodology is given in brief: to 10 ml of the formulation 0.1 ml inoculum suspension (bacterial load between 10⁵ and 5 × 10⁶ cfu/g and 10⁴–10⁵ for mould fungus, respectively) was added. They were incubated at 25 ± 1 °C. Microbial growth was controlled via serial dilution method, followed by control records after two days, one week, two weeks and four weeks. The microbial strains used, were: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231 and *Aspergillus brasiliensis* ATCC 16404. The preservative efficacy test was considered as a success, if the amount of bacteria was reduced to over 99.0% within two days and over 99.9% within one week followed up by a non-increase of bacterial impurities after that time. The amount of fungi had to be reduced by 99.0% within two weeks, again followed-up by a non-increase to pass the test.

2.4. HPLC-analysis

The quality and quantity of the model allergen Api m 1 were examined via reversed-phase high performance liquid chromatography (RP-HPLC). Therefore stock solutions of 1 mg/mL, 0.625 mg/mL and 0.25 mg/mL (Api m 1 in microemulsion) were prepared. After acetone precipitation, the protein pellet was resuspended in PBS-buffer to a concentration of 0.04 mg/mL. We performed the gradient elution method, as shown in Table 1. The HPLC-system used was a LaChrom Elite with a diode array detector (VWR-Hitachi, Germany), with a Jupiter C18 column (Phenomenex, USA). The detection wavelength of the diode array detector was set to 220 nm. Calibration curves were determined with a range from 0.02 to 0.06 mg/mL. The limit of detection (LOD) was determined at 5.1 µg/mL and the limit of quantification (LOQ) at 15.5 µg/mL. Eluent A consisted of a mixture of acetonitrile + 0.1% trifluoroacetic acid, eluent B a mixture of ultrapure water + 0.1% trifluoroacetic acid. The injection volume was 30 µl. Column temperature was set to 25 °C and the analysis was performed at a flow rate of 0.75 (ml/min).

2.5. Enzymatic degradation test

To examine the impact of dermal proteases on the stability of Api m 1, fresh full porcine ear skin samples of 350 mg had been cut into small pieces and incubated at room temperature using 500 µl PBS buffer containing Api m 1 [1 mg/ml]. The control group consisted of samples, which were incubated without allergen. The samples were centrifuged

Table 1

Gradient elution HPLC-method for the determination of Api m 1, acetonitrile + 0.1% trifluoroacetic acid (eluent A), ultrapure water + 0.1% trifluoroacetic acid (eluent B).

Time [min]	Eluent A [%]	Eluent B [%]
0.0	5.0	95.0
3.0	5.0	95.0
10.5	52.0	48.0
12.0	95.0	5.0
19.0	95.0	5.0
22.0	5.0	95.0
28.0	5.0	95.0

after 15 min, 1 h, 2 h, 3 h and 24 h incubation time, respectively. The amount of intact Api m 1 in the supernatant was analysed by RP-HPLC (2.4) after preparing the samples by acetone precipitation.

2.6. Skin penetration studies

The dermal uptake of Alexa Fluor-647 labelled Api m 1 was studied using the Franz diffusion cell set-up (OECD, 2004a,b).

Due to its similarity to human skin regarding the morphology and the permeability characteristics, porcine ear skin was used (Diembeck et al., 1999).

The porcine ears were obtained from a local slaughterhouse and transported to the laboratory under cold storage conditions on the day the pigs were slaughtered.

They were hand rinsed with mildly warm water and dried with paper towels and the bristles were removed with an electric hair trimmer. The acceptor chamber of the Franz diffusion cell was filled with PBS and full-thickness skin samples were cut out and mounted into the Franz diffusion cell. The system was equilibrated at 32.5 °C for 30 min and the Franz diffusion cells were then either loaded with 500 µl microemulsion (with/without labelled Api m 1) [0.08 µg/µl] or 500 µl PBS (with/without allergen) [0.08 µg/µl]. Allergen was labelled using the Alexa Fluor-647 protein labelling kit (ThermoFisher Scientific, Germany) and following the manufacturer's instructions. After 24 h, the skin was dismantled from the experimental set up and hand-rinsed with mildly warm water, to remove any remaining formulation.

2.7. Sample preparation for fluorescence microscopy

Isopentane was filled into a metal box and cooled down with liquid nitrogen. Small rectangular probes were cut out from the penetrated skin tissue, embedded in cryomedia (TissueTek O.C.T. Compound, Sakura) and shock frosted in the previously cooled-down isopentane.

Cross-sections of the frozen skin samples with a thickness of 12 µm were cut out using a Leica CM 1850UV Cryostat (Leica, Germany). For further analysis, the cross-sectioned samples were mounted on microscope slides and stored at minus 21 °C.

2.8. Fluorescence microscopy analysis

Confocal laser-scanning microscopy (CLSM) using the TCS SP5CLSM (Leica, Germany) was performed to analyse the amount of penetrated Alexa Fluor-647 labelled allergen into the skin. Excitation wavelength was set to 633 nm, emission wavelength to 645–700 nm. The objective used was a HCX PL APO CS 10.0 × 0.40 DRY UV. Polygonal areas of the epidermis were marked and the fluorescence intensity/pixel ratio of Alexa Fluor-647 labelled Api m 1 was calculated.

2.9. Statistics

Statistical analysis was performed with GraphPad Prism 5. To determine significant differences, the one-way Analysis of Variance (ANOVA) was performed. As a post hoc test the Tukey analysis was used. Significance was assumed for $p < 0.05$.

3. Results and discussion

3.1. Developing a skin-friendly microemulsion

Microemulsions are thermodynamically stable, optical clear systems with a good potential to act as drug delivery systems for a broad variety of substances (Kogan and Garti, 2006).

They appear translucent, because their particle size is smaller than the wavelength of visible light (400–800 nm). Macroemulsions in contrast, consist of particles, mostly larger than 1 µm. They absorb some of

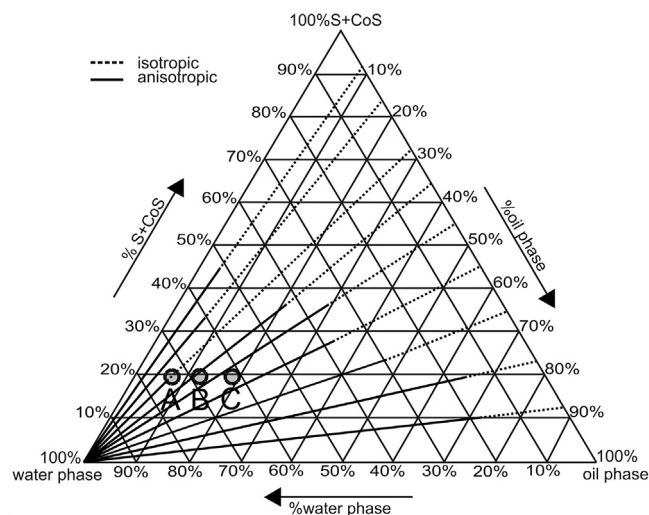


Fig. 1. Pseudoternary diagram of a caproylocaproylmacrogol-8 glycerides EP/polyglyceryl-6 dioleate surfactant blend. The diagram was obtained at room temperature. S + CoS: mixture of caproylocaproylmacrogol-8 glycerides EP/polyglyceryl-6 dioleate ratio 11:8 (wt%). Water phase: butylene glycol, glycerol, PBS. Oil phase: cetaryl ethylhexanoate, isopropyl palmitate.

the wavelengths of the visible light spectrum and therefore appear “cloudy” or “milky”. The used components (2.1) did not absorb the light at 600 nm.

Consequently, only an increase in particle size can lead to increasing turbidity and therefore higher OD₆₀₀ values.

By combining PDMPD and OD₆₀₀ measurement, we were able to develop the pseudoternary diagram as presented in Fig. 1.

The emulsions A, B and C (Fig. 2) were chosen as an example to set apart the visually detectable differences of mikro- and macroemulsions and to correlate this with their differing OD₆₀₀-values. OD₆₀₀ of the macroemulsions B and C is much higher than the OD₆₀₀-value of the microemulsion A, corresponding to their visual appearance.

The corners of the pseudoternary diagram represented 100% of the single phases (water-, oil- and surfactant/co-surfactant-phase). Every point within the diagram showed a defined mixture of oil-, water- and surfactant-phase.

Microemulsions were identified as translucent formulations without streaks visualized as dashed lines (OD₆₀₀ < 0.1). Areas with anisotropic, turbid mixtures, represented with solid lines, were macroemulsions (OD₆₀₀ > 0.1). Microemulsions were formed in big areas, but mostly the surfactant-/co-surfactant amount was very high (over 40 wt %). A surfactant content this high, would increase the risk of adverse dermal side effects and therefore was not suitable for our aims. Some microemulsions were created with lower surfactant content. However, in most cases the OP:WP ratio was not optimal for our model allergen (Api m 1). Due to the good water solubility of Api m 1, the microemulsion formed at point A was of particular interest.

The high water content (73.0 wt%) enabled the successful solubilisation of Api m 1, whereas the oily components (8.0 wt%) acted as emollient, moisturizer and penetration enhancer. The amount of surfactant mixture (19 wt%) was high enough to lower the interfacial tension between OP and WP and should be low enough to minimise the risk of skin irritancy. The final formulation is shown in Table 2.

Since it has been shown that major factors interfering with patient compliance to AIT include side effects, treatment duration and ease of use, the choice of a suitable, skin-compatible surfactant and co-surfactant is crucial. Preliminary studies carried out by Djekic et al. showed the potential of the surfactants caproylocaproylmacrogol 8 glycerides EP and polyglyceryl-6 dioleate NF to form stable microemulsions with different oils in a vast variety of blends (Djekic and Primorac, 2008).

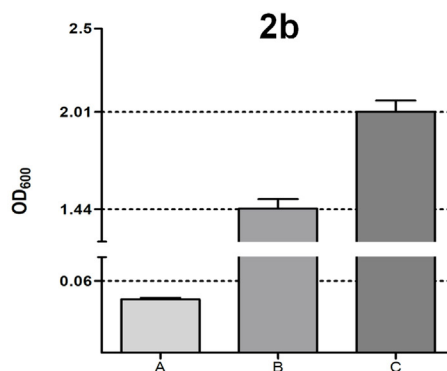
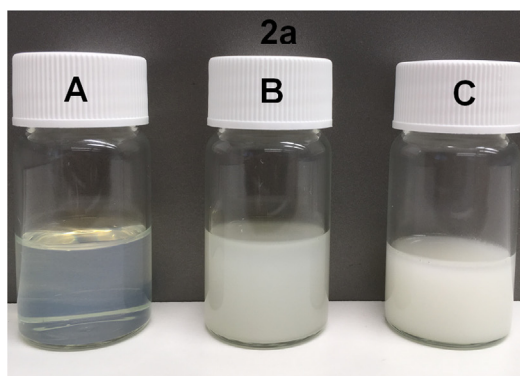


Fig. 2. Image of three selected mixtures, marked in the pseudoternary diagram (Fig. 1). 2a: Mixture A consists of 8.0 wt% OP, 73.0 wt% WP and 19.0 wt% SCoS; mixture B is 12.0 wt% OP, 69.0 wt% WP, 19.0 wt% SCoS; mixture C is 20.0 wt% OP, 61.0 wt% WP and 19.0 wt% SCoS; 2b: OD₆₀₀-values of the emulsions of the three marked areas in the pseudoternary diagram (A, B, C), mean value ± SD (n = 4) at 25 °C.

Table 2

Formulation of the selected microemulsion (A).

Compound	wt%
Caprylocaproylmacrogol-8 glycerides EP	11.0
Polyglyceryl-6 dioleate	8.0
Cetearyl ethylhexanoate	7.0
Isopropyl palmitate	1.0
Butylene glycol	10.0
Glycerol	2.6
PBS	60.4

Both surfactants belong to the class of non-ionic tensides, which generally have lower potential to cause skin irritancy than ionic ones (Effendy and Maibach, 1995), which is important for the adherence to patient therapy. Cetearyl ethylhexanoate, the main component of the oil phase, is well-known for its skin moisturising abilities, oxidation stability, and good spreading properties.

Isopropyl palmitate, the second component of the oil phase, is an emollient and works as penetration enhancer (Kogan and Garti 2006). Butylene glycol, glycerol, and PBS are the components of the water phase. Polyols, such as butylene glycol, are well-known for their mild penetration enhancing and preserving properties. Glycerol was chosen as skin moisturiser and could avoid skin dryness. Phosphate buffered saline was used for stability reasons of our chosen allergen.

3.2. Selection of the most suitable preservative

To guarantee a successful protection against bacterial and fungal contamination without causing contact dermatitis, the identification of a suitable preservative is crucial. In the present work polyols and mixtures of polyols with other preserving agencies were tested (Table 4). Propylene glycol and pentylene glycol both destabilised the microemulsion and led to turbidity and in case of pentylene glycol even to phase separation. Only butylene glycol and the butylene glycol mixtures led to stable, optical-clear microemulsions. Butylene glycol alone was not able to sufficiently reduce the microbial burden of *Staphylococcus aureus* (Gram positive), *Pseudomonas aeruginosa* (Gram negative) and *Aspergillus brasiliensis* (mould fungus). Because Kinnunen and Koskella reported, that butylene glycol showed antibacterial and antifungal properties (Kinnunen and Koskela, 1991), we conclude, that the used concentration of 10 wt% is too low, compared to the concentration used in their work (30 wt%). We decided to combine the antimicrobial activity of butylene glycol with ethylhexylglycerol and phenoxy ethanol. The butylene glycol/ethylhexylglycerol combination was not able to fulfill all the requirements of the European Pharmacopoeia, too. The phenoxy ethanol/butylene glycol mixture solely reduced the microbial burden of all used strains as required by the European Pharmacopoeia and passed the preservative efficacy test (Table 3). Phenoxy ethanol is a synergist for butylene glycol, due to its efficacy against Gram positive and Gram negative bacteria, as well as

Table 3

Influence of the preservatives on microemulsion- and microbiological stability of the developed microemulsion: (+) passed, (–) failed, (o) no examination. Concentrations are the final concentration in the formulation [wt%].

Preservative	wt%	Microemulsion stability	Preservative efficacy test
Butylene glycol/Phenoxy ethanol mixture (10.0:1.0)	11.0	+	+
Butylene glycol/Ethylhexylglycerol mixture (10.0:0.5)	10.5	+	–
Butylene glycol	10.0	+	–
Propylene glycol	20.0	–	o
Pentylene glycol	5.0	–	o

yeasts. Additionally, it shows a very low risk of causing contact dermatitis – a prominent side effect of many preservatives (Bohn and Bircher, 2001; Lovell et al., 1984).

3.3. Determination of physicochemical properties

The Pharmacopoea Europaea prescribes various testing techniques and conditions for determining long-term stability of novel therapeutic devices. The mostly used characterisation method for microemulsions is visual examination. For better objective characteristic, we additionally used OD₆₀₀ measurements, while changes in microstructure were determined via DLS-measurement.

The developed microemulsion remained a homogenous, optical clear system over a period of three months. Neither storage at 25 °C, 60%rH, nor 40 °C, 75%rH led to phase separation or increased turbidity (Table 4). Storage in a refrigerator (6 °C) led to phase separation and an increase in OD₆₀₀. However, after allowing the phase-separated microemulsion to achieve room temperature and initial gentle shaking, we were able to observe spontaneous re-microemulsification.

DLS-measurement was performed, to observe the microemulsion microstructure while storage under given conditions. We observed that storage at 25 °C, 60%rH and 40 °C, 75%rH both led to an increase in z-average (Table 4). The viscosity of fresh microemulsion was 29.63 ± 0.56 mPa·s. After 3 months the slight increase of viscosity up to 32.11 ± 0.45 mPa·s was observed.

However, the interpretation of the data is not that trivial. In general, for spontaneous microemulsification the free energy of mixing or inner energy (ΔG_f) has to have a negative value, as described by previous studies (Kahlweit et al., 1992; Lawrence and Rees, 2012; Paul and Moulik, 1997; Ruckenstein and Chi, 1975). The inner energy can be described as shown in Eq. (1):

$$\Delta G_f = \gamma \cdot \Delta A - T \cdot \Delta S \quad (1)$$

where ΔG_f is the Gibbs free energy of microemulsion formation, γ the surface tension of the oil/water interface, ΔA the change of the

Table 4

Physicochemical properties of the microemulsion during storage at different conditions; z-average (d.nm), polydispersity index (PDI) measured by DLS, mean value $\pm$ SD (n = 9); OD₆₀₀ (n = 9).

	25 °C, 60%rH			40 °C, 75%rH		
	z-Ave (d.nm)	PDI	OD ₆₀₀	z-Ave (d.nm)	PDI	OD ₆₀₀
Day 1	50.43 $\pm$ 3.52	0.71 $\pm$ 0.13	0.044 $\pm$ 0.00	50.43 $\pm$ 3.52	0.71 $\pm$ 0.13	0.044 $\pm$ 0.00
Month 1	71.65 $\pm$ 2.79	0.53 $\pm$ 0.01	0.045 $\pm$ 0.00	94.90 $\pm$ 2.86	0.48 $\pm$ 0.11	0.045 $\pm$ 0.00
Month 2	84.90 $\pm$ 3.45	0.49 $\pm$ 0.02	0.043 $\pm$ 0.00	130.34 $\pm$ 10.48	0.37 $\pm$ 0.04	0.044 $\pm$ 0.00
Month 3	98.52 $\pm$ 8.39	0.48 $\pm$ 0.02	0.041 $\pm$ 0.00	194.43 $\pm$ 15.58	0.29 $\pm$ 0.02	0.040 $\pm$ 0.00

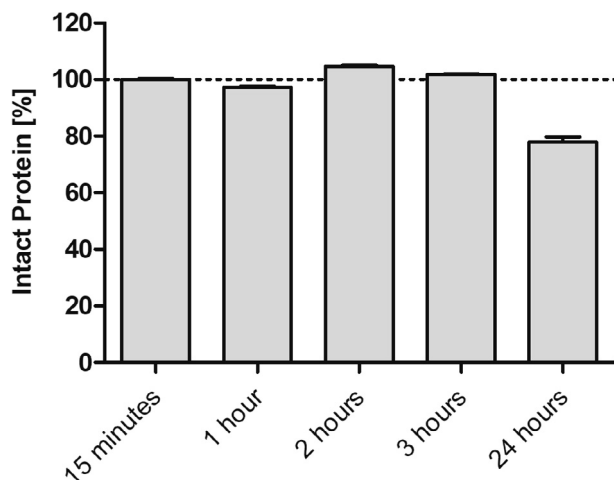


Fig. 3. Amount of intact Api m 1 over a 24 h time period, mean value $\pm$ SD (n = 3), 25 °C, dashed line represents 100% Api m 1.

interfacial area during the emulsification process, T the temperature during the emulsification process and ΔS the entropy change. To remain a homogenous, thermodynamically stable system ΔG must show a minimum value. Temperature changes or water loss could influence the inner structure of the microemulsion and even modify it (Wennerström et al., 2006).

As result, the DLS-measurement would be directly influenced, resulting in altering of the calculated z-average values. The calculation of the particle size is based on the assumption, that there are spherical particles existing. However, previous research showed, that from spherical droplets, over cubic structures, lamellar phases, bicontinuous phases to ellipsoid droplets many structural differences, regarding the structure of microemulsions can occur. Even mixed forms are possible (Lawrence, 1994; Lawrence and Rees, 2012; Nagarajan and Ruckenstein, 2000; Scriven, 1976). In our research, the inner structure of our ME is not estimated. Therefore the calculation of z-average is only used as a tool to determine possible structure changes and not for real particle size measurement. All in all, the composition remained a homogenous and clear system without streaks or cloudiness and therefore still is considered a microemulsion. This can also be underlined by having a closer look at the OD₆₀₀ values, which stay nearly constant in a narrow corridor. Therefore we conclude that the observed changes in z-average show a change in the microemulsion inner structure caused by minimal water loss during storage. The increase in viscosity and the fact, that the observed changes are bigger at higher temperatures, support this thesis. Therefore, we recommend storage of the microemulsion at room temperature (22–25 °C).

3.4. HPLC analysis and enzymatic degradation test

The primary function of the intact human skin is to defend the body against physical, microbial and chemical assaults. To fulfil this task, the integrity of the skin itself is decisive. These dermal integrity is a result

of a complex interaction between endogenous proteases and protease-inhibitors (de Veer et al., 2014). Additionally, the outermost skin surface, the epidermis, is colonised by a gathering of various micro-organisms, the so-called skin microbiome. However, some of these proteases may cut proteogenic substances onto the top of the skin and those that have penetrated into the skin. Therefore we investigated the stability of Api m 1 against the dermal degradation mechanisms. Prior to the degradation essay the HPLC-analysis (2.4) had been developed.

HPLC-analysis of Api m 1 in PBS showed a characteristic minor peak at a retention time of 11.5 min and a larger peak at 11.8 min. When untreated porcine ear skin was incubated with PBS buffer and afterwards analysed with acetone precipitation and HPLC-analysis, the signals of dermal proteins did not interfere with the signals of Api m 1. For quantitative analysis both peak areas of the Api m 1 signal were summed up and the amount of recovered Api m 1 was calculated. The recovery was concentration-dependent and amounts of $100.00 \pm 2.63\%$ for the 1 mg/mL, $98.40 \pm 1.47\%$ for the 0.625 mg/mL and $79.08 \pm 5.5\%$ for the 0.25 mg/mL stock solutions (mean, n = 3, $\pm$ SD), have been calculated respectively.

We assume, that small amounts of the protein adsorbed on the surface of the used vials. Because of that, we observed a bigger decrease in the recovery of Api m 1 for the lower concentration (0.25 mg/mL) compared to the higher concentrations. Due to these results, the concentration of Api m 1 was set to 1 mg/mL for the following enzymatic degradation tests. Fig. 3 depicts the amount of intact Api m 1 over a period of 24 h incubation with porcine skin samples. During the first three hours, nearly 100% of the protein was still intact. Even after 24 h, $77.83 \pm 1.94\%$ of intact protein was recovered. These experiments indicated a good stability of Api m 1 against the dermal defence mechanisms which is important for the interpretation of the following penetration studies.

3.5. Penetration studies

Commonly, the efficacy of AIT is characterised *ex vivo* by immune alterations in both allergen-specific T cell subpopulations and antibody titers in correlation to the severity of symptoms, when provoking an allergic reaction (Shamji and Durham, 2017; van de Veen et al., 2017). In this study we used an *ex vivo* pig ear skin read-out model to investigate the impact of the developed microemulsion on dermal absorption of proteogenic drugs.

The dermal uptake was studied with fluorescence-labelled Api m 1. Fig. 4 depicts representative examples of vertically cross-sectioned ear skin after 24 h treatment and the data of the spectral analysis of the CLSM pictures.

The ear skin samples, treated with PBS without allergen and microemulsion without allergen, showed a very similar fluorescence signal of 5.06 (intensity/pixel) (PBS without allergen) and 5.21 (intensity/pixel) (microemulsion without allergen), respectively. This indicates that the developed microemulsion does not influence the autofluorescence signal of the treated samples. Therefore microemulsion without allergen was chosen as control group for statistical analysis.

A strong fluorescence signal of the Alexa Fluor-647-labelled Api m 1 was able to be detected for the microemulsion-treated ear skin samples

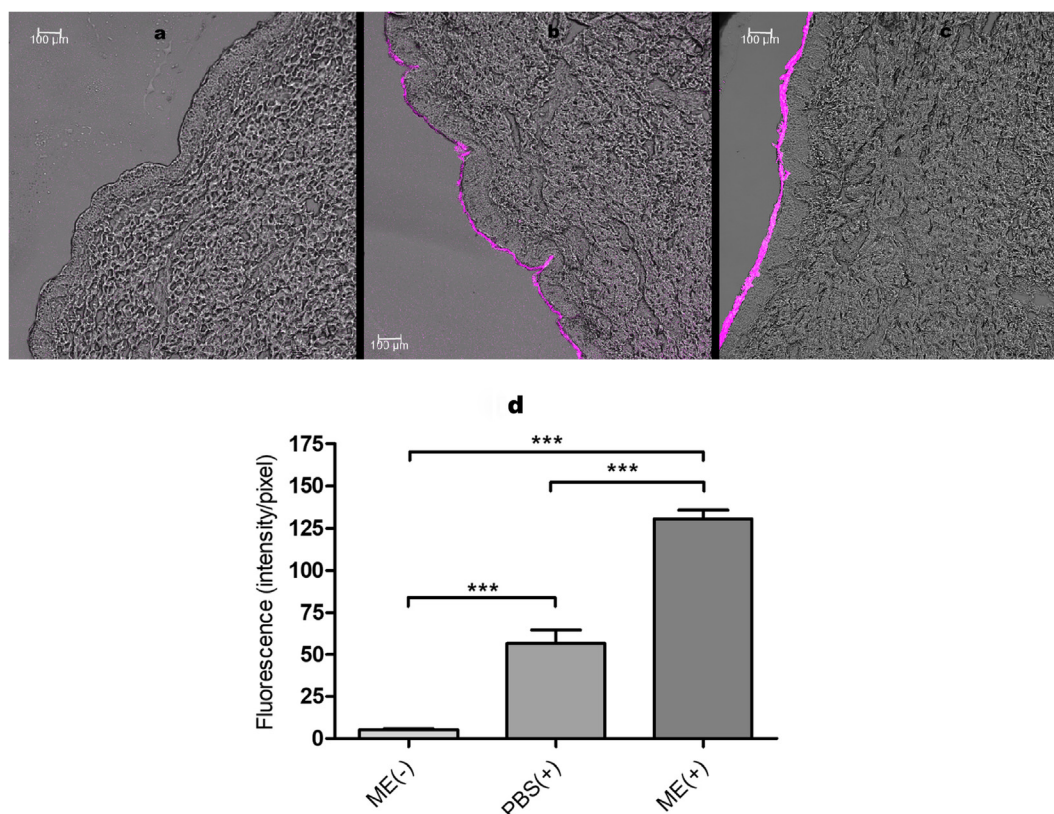


Fig. 4. Confocal laser-scanning microscopy images of vertically cross-sectioned porcine ear skin after 24 h penetration studies with Franz cells. Overlay of fluorescence signal and transmitted-light image; 4a: Microemulsion without allergen incorporated; 4b: Model allergen incorporated into PBS-buffer; 4c: Model allergen incorporated into microemulsion; 4d: Data of the spectral analysis CLSM pictures presented as mean $\pm$ SD ($n = 4$), microemulsion without allergen (ME(-)), PBS with allergen (PBS(+)) and microemulsion with allergen (ME(+)), significance was tested by one-way ANOVA following a Tukey test ($p < 0.05$).

in the upper epidermal area.

For PBS-treated ear skin samples, only a weak fluorescence signal was recorded. In numbers, the fluorescence intensity/pixel ratio analysis of each skin sample shows a mean value of 130.60 (intensity/pixel) for the microemulsion treated ear skin and 56.58 (intensity/pixel) for PBS-buffer treated samples ($n = 4$).

We assume increased tissue hydration of the stratum corneum, reviewed by Barry (2001), is the main reason for the penetration of Api m 1 for the PBS-treated skin samples.

The high fluorescence intensity/pixel ratio for microemulsion treated ear skin samples can be attributed to the microemulsions combination of penetration enhancing effects. Microemulsions, in general may improve the solubility of a drug in the skin, disrupt the lipid barrier of the stratum corneum, increase skin hydration and enable better movement into or across the skin (Benson, 2005; Kreilgaard, 2002; Lopes, 2014; Williams and Barry, 2012). The highest fluorescence signal was recorded in the upper epidermal layer, the stratum corneum. In the literature there are several reports in which different substances were able to form epidermal drug depots. Oleic acid, for example, was able to increase the transport of hydrophilic substances into the skin by increasing the fluidity of the lipid portion of the stratum corneum as reported by Kanikkannan and Singh, while Ongpipattanakul et al. showed its potential to form a separate phase within the stratum corneum (Hadgraft, 1979; Kanikkannan and Singh, 2002; Ongpipattanakul et al., 1991). Hadgraft developed a mathematical model for the building of such epidermal drug depots (Hadgraft, 1979). Plurol oleique®, a diester of oleic acid and polyglycerol showed penetration enhancing effects for many substances.

On the basis of these facts, we postulate the formation of an epidermal drug depot of Api m 1, which could release Api into deeper epidermal layers over time, where it can be processed by the dermal

Langerhans cells and immunotolerance could be built up. To correlate these data with a clinical outcome and immunological changes, Schuppe et al. utilised the above described microemulsion in an Api m 1-allergic mouse model (Schuppe et al., 2018). The first results revealed, that dermal AIT with the newly developed microemulsion prevented anaphylaxis in a dose-dependent manner which was associated with increase in Api m 1-specific IgG antibodies. It has been shown that induction of allergen tolerance is induced by this antibody subclass accounting for inhibition of allergen-IgE complex formation (Möbs et al., 2015), therefore indicating protective efficacy of dermal AIT with our microemulsion.

4. Conclusion

Summarily, we were able to develop a stable microemulsion with a low surfactant content for the topical administration of proteogenic drugs and to characterise the physicochemical properties of the microemulsion under different storage conditions. Due to possible water lost at higher temperatures resulting in change of the microstructure and by long-time storage possible destabilization of the formulation, we recommend storage of the microemulsion at room temperature. Api m 1 showed a very good stability against degradation by dermal proteases. By combining the Franz diffusion cell set-up with confocal laser-scanning microscopy, we were able to record a strong epidermal fluorescence signal with the highest intensity in the stratum corneum for microemulsion treated porcine ear skin. We presume the formation of an epidermal drug depot, which could release Api m 1 over time into deeper skin layers and reach Langerhans-cells or even dendritic cells – the target cell population of dermal AIT. First *in vivo* studies, performed by Schuppe et al., were approving the results of the penetration studies and are indicating immunomodulating and allergen protective

therapeutic effects of our Api m 1/microemulsion drug delivery system.

Subsequent *in vivo* studies have already been planned to continue our investigations and to confirm these promising results. In conclusion, the developed microemulsion is a suitable, stable drug delivery system for the topical administration of proteogenic allergens into the epidermis which enables allergens to reach dendritic cells in the skin.

5. Declaration of interests

None.

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