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Development of drug delivery systems for the dermal application of therapeutic DNAzymes

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ABSTRACT

DNAzymes are potent novel drugs for the treatment of inflammatory diseases such as atopic dermatitis. DNAzymes represent a novel class of pharmaceuticals that fulfil a causal therapy by interruption of the inflammation cascade at its origin. There are two challenges regarding the dermal application of DNAzymes: the large molecular weight and the sensitivity to DNases as part of the natural skin flora. To overcome these limitations suitable carrier systems have to be considered. Nano-sized drug carrier systems (submicron emulsions, microemulsions) are known to improve the skin uptake of drugs due to their ability to interact with the skin's lipids. To protect the drug against degradation, the hydrophilic drug may be incorporated into the inner aqueous phase of carrier systems, such as water-in-oil-in-water multiple emulsions. In the present study various emulsions of pharmaceutical grade were produced. Their physicochemical properties were determined and the influence of preservation systems on stability was tested. Drug release and skin uptake studies using various skin conditions and experimental set-ups were conducted. Furthermore, cellular uptake was determined by flow cytometric analysis. The investigations revealed that the developed multiple emulsion is a suitable and promising drug carrier system for the topical application of DNAzyme.

1. Introduction

Chronic inflammatory diseases of the skin such as atopic dermatitis are increasingly prevalent worldwide mainly in industrialised countries. They represent complex skin disorders that develop on the basis of a genetic predisposition under the influence of certain environmental factors. The current understanding is that immunological mechanisms are mainly involved in the pathology of these diseases with activation of different T cell subpopulations selectively contributing to the development of either disease or a disease state (Leung, 2000; Leung and Soter, 2001). In atopic dermatitis, the immune hypothesis invokes an imbalance in the T lymphocytes, with Th2 cells predominating in the acute phase; this results, among other things, in the cytokine production of various interleukins such as IL-4, IL-5, IL-12 and IL-13 (Garlisi et al., 1995; Grünig et al., 1998; Maggi, 1998). Therefore, most current therapies are still based on general anti-inflammatory drugs that may reduce symptoms without directly interfering with the disease-causing mechanisms. Novel concepts for approaches include the use of antisense-based molecules such as antisense DNA, DNAzymes, small interfering RNA (siRNA) and ribozymes that interfere with the mRNA of proteins that are involved in the pathogenesis of the respective disease (Kim et al., 2009). DNAzymes represent a novel class of antisense molecules that have not yet been established for the treatment of any human disease. 10–23 DNAzymes are a group of RNA-cleaving DNA molecules that contain a catalytic domain (Cieslak et al., 2003; Silverman, 2005; Tritz et al., 2005) and cleave the RNA sequence at a phosphodiester bond between an unpaired purine and a paired pyrimidine residue (Santoro and Joyce, 1998). 10–23 DNAzymes targeting GATA-3 mRNA have recently been developed, and their anti-asthmatic effect in mouse models has been successfully demonstrated (Sel et al., 2008). As GATA-3 plays a central role in Th2 cell differentiation (Barnes, 2008) and in promoting Th2 responses (Zhu et al., 2006), similar results are expected for the treatment of inflammatory skin diseases with the mRNA-cleaving GATA-3 10–23 DNAzyme. Therefore, this oligonucleotide model was chosen as a promising API candidate for the treatment of

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atopic dermatitis. For the effective treatment of atopic dermatitis, it is necessary to transport the oligonucleotide into the epidermis and to the inflammatory infiltrate. However, the topical application of such molecules is still challenging owing to their limited bioavailability, which is due to several reasons (Akhtar et al., 2000). For example, penetration into the skin compartments is often restricted due to the rather large size of these molecules, which often exceeds 10 kDa. In addition, as a result of size and chemical composition, oligonucleotide molecules are generally not easily taken up by cells; thus the transfection efficiency of target cells in the skin is often low (Bally et al., 1999). Moreover, oligonucleotide-degrading enzymes such as RNases and DNases may destroy the integrity of such molecules before they come into contact with their target structures. DNA-cleaving enzymes might be located on both the skin and in skin tissue. The deoxynucleases on the skin are mainly produced by several microorganisms, which belong to the natural skin flora such as Staphylococcus (Langlois et al., 1989; Wierup, 1978). Drug carrier systems for topical application of such sensitive and large DNA molecules pursue improvement of skin uptake and protection against degradation. Therefore, different systems were evaluated. With regard to the encapsulation properties of DNAzymes, systems with an inner water phase (water-in-oil (W/O) emulsion and water-in-oil-in-water (W/O/W) emulsion) were chosen as appropriate candidates. With respect to their excellent transport properties, a microemulsion and a submicron emulsion with hydrophilic DNAzymes located in the outer water phase were chosen. Due to their distinct structure and properties, multiple emulsions are of particular interest for several drug delivery approaches, including the dermal application of encapsulated drugs in pharmaceutical products (Fukushima et al., 1987; Khopade and Jain, 1999; Lindenstruth and Müller, 2004; Schmidts et al., 2010). Microemulsion (ME) and submicron emulsion (SME) represent carrier systems below one micrometre in size and allow improved drug penetration and permeation into the skin compared to conventional formulations (Bouchemal et al., 2004; Delgado-Charro et al., 1997; Djekic and Primorac, 2008; Kanikkannan and Singh, 2002; Schmalfuß et al., 1997).

In the present study, a W/O/W emulsion, a W/O emulsion, an SME and an ME were produced, their physicochemical properties were determined over time and the formulations were tested for sufficient preservation. Furthermore, drug release and skin uptake studies using various skin conditions and experimental set-ups were conducted and were analysed by HPLC, hybridisation-ELISA and fluorescence microscopy. Finally, a W/O/W emulsion and a SME were compared regarding celluar uptake of the drug by flow cytometric analysis.

2. Materials and methods

2.1. Materials

All ingredients were obtained in pharmaceutical grade. Light and heavy paraffin oil, isopropyl palmitate, sodium chloride (NaCl), magnesium sulphate heptahydrate ($MgSO_4$), propylene glycol, glycerol 85%, potassium sorbate, citric acid, butylene glycol, propyl-4-hydroxybenzoate and hydrophobic basis gel (DAC) were supplied by Fagron (Germany). Cetostearyl isononanoate and Coco-Caprylate/Caprate were provided by Cognis (Germany). Ethyl Oleate, Sorbitan Oleate (Span®80), Oleth-5, Oleth-10 and Steareth-20 were provided by Croda (Germany). Soy lecithin, methyl-4-hydroxybenzoate and triglycerol diisostearate were purchased from Caelo (Germany). Caprylocaproyl macrogol-8-glycerides (Labrasol®) and Polyglyceryl-6-dioleate (Plurol Oleique®) were obtained from Gattefossé (France). Acrylamide/Sodium acryloyldimethyl Taurate Copolymer/Isohexadecane/Polysorbate®80 (Sepineo®P600) was obtained from Seppic (Germany). Methanol (gradient grade), water (gradient grade), sulphuric acid, ethylenediamine tetra-acetic acid (EDTA) and sodium hydroxide (NaOH) were obtained from VWR (Germany). Benzoic acid was obtained from Euro OTC Pharma (Germany), and the Phenoxyethanol was from Th. Geyer (Germany). An unlabelled and a 6-carboxyfluorescein (FAM) labelled 10–23 DNAzyme was provided by Sterna Biologicals (Germany) representing the sodium salt of DNAzyme, a single-stranded DNA molecule composed of 34 deoxynucleotide bases with a molecular weight of 10.6 kDa (unlabelled). UltraPure™ Tris Hydrochloride (Tris–HCl) was supplied by Invitrogen (Germany). NaCl and magnesium chloride ($MgCl_2$), both nuclease-free, were obtained from Applied Biosystems (Germany). Polysorbate 20 (Tween®20) and Sodium dodecyl sulphate ultra-pure (SDS) were purchased from Carl Roth (Germany). Trizma® hydrochloride and Trizma® base were supplied by Sigma–Aldrich (Germany). Oligonucleotide probes labelled with either digoxigenin or biotin were synthesised by Operon (Germany). Anti-digoxigenin-peroxidase and BM Blue POD substrate were supplied by Roche Diagnostics (Germany). Phosphate buffered saline (PBS) without Mg^{2+} and Ca^{2+} was supplied by Biochrom AG (Germany), and proteinase K was purchased from Qiagen (Germany). Dispase II was obtained from Roche Molecular Biochemicals (Germany). Trypsin/EDTA and Trypsin Inhibitor were both supplied by Pan (Germany). Collagenase was purchased from Worthington Biochemical Corporation (USA) and Hyaluronidase from Sigma–Aldrich.

2.2. Preparation of delivery systems

DNAzyme was added to the water phase of the formulations. Regarding the multiple emulsion, DNAzyme was added to the inner water phase at a total quantity of 0.4 wt%. For skin penetration studies, 10% of the DNAzyme was replaced by *the fluorescent labelled* FAM-DNAzyme.

W/O emulsions were prepared at room temperature. The water phase (5 wt% glycerol, 0.3 wt% $MgSO_4$, 0.14 wt% potassium sorbate, 0.07 wt% citric acid and 63.9 wt% distilled water) was added slowly to the oil phase (3.0 wt% triglycerol diisostearate, 1.2 wt% isopropyl palmitate, 1.2 wt% Cetostearyl Isononanoate, 0.2 wt% soy lecithin and 24.6 wt% hydrophobic basis gel) and stirred with a mortar and pestle until a homogeneous emulsion was obtained.

W/O/W emulsions were prepared using a 2-step procedure previously reported by Matsumoto et al. (Matsumoto et al., 1976). First, the primary W/O emulsion was produced. Second, the obtained primary emulsion (40.0 wt%) was dispersed in the aqueous phase containing the hydrophilic emulsifier. In detail, the simple W/O emulsions were produced by adding the aqueous phase (0.065 M $MgSO_4$ solution containing the DNAzyme) to the oil phase (15.8 wt% heavy paraffin oil, 4.0 wt% Sorbitan oleate and 0.2 wt% soy lecithin) followed by a homogenisation step using a rotor-stator homogeniser (Diax 600, Heidolph, Germany) for 2 min at 9500 rpm. Next the chilled primary emulsion was slowly added to the outer water phase (1.0 wt% Steareth-20 and 58.7 wt% preserved water) under stirring at 1200 rpm using a EUROSTAR digital stirrer (IKA, Germany) at room temperature. Finally, 0.3 wt% Sepineo®P600 was added as thickening agent under gentle stirring.

An ME was obtained by gently mixing the appropriate quantities of the components plus the DNAzyme at room temperature. The oil phase consisted of 7.20 wt% Labrasol®, 4.80 wt% Plurol Oleique®, 2.50 wt% cetostearyl isononanoate and 2.50 wt% isopropyl palmitate. The water phase consisted of 2.49 wt% glycerol, 16.60 wt% propylene glycol, 0.30 wt% $MgSO_4$ and 63.21 wt% distilled water.

SMEs were prepared as followed. The water phase (a blend of Oleth-3 and Oleth-10 in total 6.0 wt%, 3.0 wt% glycerol, 0.3 wt%

$MgSO_4$ and 75.3 wt% preserved water) and oil phase (5.0 wt% Coco-Caprylate/Caprate, 5.0 wt% cetearyl isononanoate, 5.0 wt% Ethyl oleate) were heated separately to 70 °C. The two phases were then combined and homogenised for 1 min using a rotor/stator homogeniser at 24,000 rpm. Three different SMEs were produced with HLB values of 10, 11 and 12. Oleth-3 and Oleth-10 were used to adjust the HLB value.

2.3. Characterisation of the drug delivery systems

Droplet size analysis was performed with respect to the requirements of the samples. The mean droplet sizes (*z*-Average) of W/O emulsion and ME were determined by dynamic light scattering (High Performance Particle Sizer, Malvern Instruments, UK). W/O emulsions were diluted 1:1000 with light paraffin oil (viscosity: 33 mPa s) prior to measurement, while the MEs were measured undiluted. The droplet sizes of the multiple W/O/W emulsions and the SMEs were determined by laser diffraction (Mastersizer S, Malvern Instruments, UK). The results are presented as the mean diameter $D(v,0.5)$ based on the volume distribution. Determination of the viscosity was performed at 25 °C using the RheoStress 300 Rheometer (ThermoHaake, Germany) with a cone and plate geometry of 20 mm in diameter and a 2° angle. The apparent viscosity was measured over a shear rate of 0.1–100 s^{-1} and presented as the mean value ($n = 3$). All of the above mentioned parameters were recorded over a period of 3 months.

2.4. Preservative efficacy test

Preservative efficacy tests were performed according to the European Pharmacopoeia (2008) using the microbial strains: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger*. The microbial impurities were determined after storage at 25 °C for one week. Preservative efficiency test was considered a success when the count of bacteria is reduced within one week by more than 99.9% and fungus by more than 99.0%.

2.5. Drug release studies

The DNAzyme release from the drug delivery systems was studied using the Franz diffusion cell set-up (surface area 1.76 cm^2 and 12 mL acceptor volume) (Gauer Glas, Germany). A pre-soaked mixed cellulose ester membrane (pore size 0.2 μm, Ø 25 mm, Whatman, Germany) was placed between the donor and receptor compartment. The acceptor medium (distilled water) was continuously stirred (500 rpm) and the temperature was maintained at 32.5 °C. After equilibration (30 min), 500 μL of the investigated formulation was applied on the top side of the membrane and the released DNAzyme was monitored by collecting samples (300 μL) of the acceptor fluid at different time points. Sampling time was adjusted for every formulation. The samples were analysed by hybridization-Enzyme Linked Immunosorbent Assay (ELISA). All experiments were repeated three times and are presented as the mean value ± SD.

2.6. Skin penetration studies

DNAzyme skin uptake studies were performed in vitro using the Franz diffusion cell set-up (OECD, 2004a,b). Porcine skin was chosen due to its similarity to human skin in terms of its morphology and permeability characteristics (Diembeck et al., 1999) and due to its availability. Therefore, untreated porcine ears (domestic pig) were obtained directly from a local slaughterhouse, and immediately transferred to the lab under cool conditions. Porcine ears were washed by rinsing with mildly warm water and wiped with paper towels, and the bristles were carefully shortened by trimming. Full thickness porcine skin was either used immediately (fresh skin) or stored at −20 °C (frozen skin; up to 6 months) and thawed prior to use in experiments. Untreated skin (intact skin) and skin with an impaired barrier were used to conduct skin uptake studies. To simulate the impaired barrier of atopic dermatitis skin, the porcine skin underwent a tape stripping procedure following Simonsen and Fullerton (2007), which was controlled by transepidermal water loss (TEWL) measurements. On the day of the experiment, the prepared skin samples were mounted into the Franz diffusion cell with PBS as the acceptor fluid and equilibrated for 30 min at 32.5 °C. Then, the investigated formulations were evenly applied as either an infinite dose (500 μL) or a finite dose (20 μL) onto the skin surface for 24 h. To mimic potential human exposure, the finite dose was applied and gently massaged for 1 min. At the end of the experiment, any remaining formulation was removed by a wash-off procedure. Skin samples were analysed by hybridisation-ELISA and fluorescence microscopy. Skin sections for the fluorescence microscope analysis were stamped out, shock frozen and stored at −20 °C. The remaining parts were reduced to small pieces and covered with lysis buffer (50 mM Tris–HCl, 25 mM EDTA, 100 mM NaCl, 0.5% SDS) plus proteinase K overnight. The samples were then centrifuged, and the supernatant was used to conduct hybridisation-ELISA. All experiments were repeated three times and are presented as the mean value ± SD.

2.7. Cellular uptake of DNAzymes

Fluorescence activated cell sorter (FACS, Calibur, BD Bioscience, Germany) was used to determine the cellular uptake of the FAM-DNAzyme. Skin penetration studies (finite dose experimental set up, chapter 2.6) were conducted and the total amount of DNAzyme was replaced by FAM-DNAzyme. At the end of the experiment, cells were isolated out of the epidermis and measured by FACS. Therefore, punch biopsies (4 mm) were taken, and incubated over night at 4 °C in 1 mL Dispase II (2.4 U/mL). Subsequently, the epidermis was taken-off the dermis and incubated for 5 min at 37 °C in 1 mL Trypsin (0.05%)/EDTA (0.02%). The enzymatic digestion was stopped by adding the same amount of Trypsin Inhibitor to the epidermis. To obtain a single cell suspension, cells were separated by using a cell strainer (40 μm). After two additional wash steps with PBS cells were measured by FACS and analysed using Cell QuestPro software (BD Bioscience). Results are presented as geometric mean value (GeoMean), representing the mean value of the total fluorescence of the cell population, and percentage of FAM-DNAzyme positive cells (%pos). Therefore, the threshold values were adjusted in order to obtain low values of skin cells treated with the placebo formulations.

2.8. Fluorescence microscopy analysis

For fluorescence microscopy analysis, 10-μm-thick cryosections were prepared with the Leica CM 1850UV (Leica, Germany). Slices were analysed using the fluorescence microscope DMI 6000B (Leica, Germany) with the L5 filter cube. The fluorescent pictures analysis is based on summing the pixel intensity values (total intensity) and counting the number of the summed pixels. Then the total intensity is normalised to the number of the included pixels representing the intensity per pixel for the given image; at least 15 images were analysed for each sample, and values are presented as the mean ± SD.

2.9. DNase activity assay

To determine the DNase activity on the skin surface, the first three tape strips obtained by skin tape stripping were analysed. The

single tape strips were incubated for 20 min with 1.5 ml of 10 mM sodium acetate buffer (pH 5.0) and 0.5 mL of an aqueous DNAzyme solution (0.1625 mg/mL). The samples were filtered (0.2 μm pore size), and the remaining DNAzyme amount was analysed by HPLC (Schmidts et al., 2011).

2.10. Quantification of the DNAzyme

DNAzyme recovery and stability testing in the developed drug delivery systems as well as testing of DNase activity on the skin surface was performed by a slightly modified anion-exchange HPLC method (DNAPac PA-100 4 mm × 250 mm anion-exchange column and guard column (Dionex, USA)) previously described by Schmidts et al. (2011). Briefly, equilibration buffer (A) consists of 20 mM Tris–HCl, 20% methanol and 263 mM NaCl, and elution buffer (B) consists of 20 mM Tris–HCl, 20% methanol and 650 mM NaCl. Both buffers were adjusted to pH 7. The following gradient was run with a flow of 1.0 mL/min: 0–11 min (100% A), 11–50 min (from 100% A to 100% B), 50–56 min (100% B), 56–60 min (from 100% B to 100% A) and 60–70 min (100% A). If necessary, sample preparation was done by sonication at 50 °C for 30 min prior to HPLC analysis.

Samples obtained by skin penetration studies and drug release studies were analysed by a hybridisation-ELISA. The hybridisation–ELISA developed for the specific detection of DNAzyme (Dicke, 2009) was adjusted for the present requirements and performed as follows. Samples were diluted within a concentration range between 1 ng/mL and 78 ng/mL (dilution buffer: 20 mM Tris–HCl, 2.6% SDS, 0.5 mM $MgCl_2$; pH 7.4). Then 100 μL of the hybridisation buffer (20 mM Tris–HCl, 52.5 mM NaCl, 0.5 mM $MgCl_2$; pH 7.4), 80 μL dilution buffer and 0.22 μL of a mixture of oligonucleotide probes labelled with digoxigenin or biotin (each with 0.5 mg/mL) were added to 30 μL of the sample. This solution was incubated for 5 min at 75 °C and then immediately chilled to room temperature. Next, 100 μL of this solution was incubated in a 96-well streptavidin-coated microtiter plate (StreptaWell, High Bind, Roche) for 2 h at 37 °C and washed ten times with a buffer (20 mM Tris Base, 150 mM NaCl, 0.1% Tween®20; pH 10.0). Each well was filled with 100 μL of an antibody mixture (100 μL hybridisation buffer plus 0.5 μL anti-digoxigenin-peroxidase (anti-digoxigenin-POD, 250 mU/mL)), incubated by gently shaking for 1.5 h at 37 °C and washed again as previously described. Subsequently, 100 μL of BM Blue POD substrate was transferred into each well and incubated for 5 min in darkness. The reaction was stopped with 50 μL 2 M H_2SO_4, and the absorbance was measured with a Multiskan FC photometer (Thermo Fisher Scientific, Germany) at 450 nm and 650 nm as the sample and reference wavelengths, respectively. The calculation was done by a logarithmic standard curve.

3. Results

3.1. Selection of preservatives for the delivery systems

To identify the applicable preservative agent for the investigated drug delivery systems, various commonly used preservative systems were examined by visual observation regarding microbiological and physical stability (Table 1).

Propylene glycol as one component of the ME was sufficient to preserve this formulation. With regard to the W/O/W emulsion, preservatives showed a bearing influence on the physical stability leading to coalescence and phase separation. Only the application of 0.05% of benzoic acid showed sufficient microbiological and physical stability. Based on the preservative efficacy test and the stability data, the formulations were preserved as follows: the W/O emulsion contained 0.14% potassium sorbate, the ME contained 20% propylene glycol in the water phase, the SME contained 0.20% potassium sorbate and the W/O/W emulsion contained 0.05% benzoic acid.

Table 1
Influence of different preservative systems on the microbiological and physical stability of various drug delivery systems; (–) failed, (+) passed, (○) no data. Concentrations are given as total amount of the formulation.

Preservative	Preservative efficacy test	Stability (3 months)
W/O emulsion		
0.14% potassium sorbate	+	+
ME		
16.60% propylene glycol	+	+
SME		
0.10% Parabens[a]	–	+
20.00% propylene glycol	+	–
10.00% propylene glycol/0.10% Parabens[a]	–	+
5.00% butylene glycol/0.10% Parabens[a]	–	+
0.20% potassium sorbate	+	+
W/O/W emulsion		
0.10% Parabens[a]	–	+
0.20% Parabens[a]	–	–
0.40% Parabens[a]	–	–
20.00% propylene glycol	○	–
10.00% propylene glycol/0.10% Parabens[a]	–	–
5.00% butylene glycol/0.10% Parabens[a]	–	–
5.00% butylene glycol/0.10% Parabens[a]/0.50% Phenoxyethanol	○	–
8.00% butylene glycol/0.10% Parabens[a]	–	–
8.00% butylene glycol	–	–
0.14% potassium sorbate	○	–
0.20% benzoic acid	+	–
0.05% benzoic acid	+	+

[a] Parabens (75% methyl-4-hydroxybenzoate and 25% propyl-4-hydroxybenzoate).

3.2. Stability of delivery systems

To compare different drug delivery systems, the physicochemical properties were determined. First, stable placebo formulations were developed. The placebo formulations ($n = 3$) were considered as stable if the physicochemical parameters did not change within 3 months (stored at 25 °C and 60% RH). Next, these formulations were produced with DNAzyme, and the physicochemical parameters of the formulations were observed over 3 months (Table 2).

Regarding the droplet size and viscosity in the long-term stability analysis, the addition of DNAzyme did not appreciably influence these parameters in the case of the W/O emulsion, the ME and the SMEs. Only in the case of multiple emulsions was an increase of droplet sizes and a slight decrease in viscosity observed over three months. DNAzyme stability in the different formulations ranged in the order of W/O/W ≫ W/O ≫ ME ≥ SMEs. ME and SMEs showed a considerable decrease of DNAzyme content of about 35% after three months. Only with the W/O/W emulsion was a sufficient DNAzyme stability observed over three months. Additionally, regarding the different SMEs, the droplet size depended on the HLB of the surfactant blend.

3.3. Drug release studies

Drug release of the different formulations and an aqueous solution of DNAzyme were studied. First, the three SMEs, varying in droplet size, were compared (Fig. 1A). DNAzyme released from the three SMEs is very similar, and an influence of the droplet size could not be detected. In general, SMEs showed an immediate release; about 22.3 ± 1.3% was released within the first hour increasing up to 30.1 ± 3.2% after five hours.

Table 2
Physicochemical parameters of DNAzyme containing formulations.

	pH	Viscosity [mPa s]	$D(v,0.5)$ [μm]	z-Average [nm]	Recovery [%]
W/O emulsion					
Day 1	–	4954	–	816	97.3 ± 1.1
Month 1	–	4703	–	800	88.9 ± 1.5
Month 3	–	4764	–	900	88.8 ± 0.3
ME					
Day 1	6.9	9.1	–	95	99.3 ± 1.4
Month 1	6.6	9.6	–	105	99.8 ± 2.9
Month 3	6.4	11.8	–	114	65.6 ± 0.8
SME HLB = 10					
Day 1	4.6	2.4	0.23	–	104.1 ± 0.9
Month 1	4.6	2.6	0.23	–	100.6 ± 1.3
Month 3	4.5	2.7	0.22	–	62.6 ± 0.3
SME HLB = 11					
Day 1	4.6	2.6	0.40	–	102.3 ± 2.0
Month 1	4.6	2.6	0.40	–	101.4 ± 0.5
Month 3	4.6	2.6	0.37	–	62.9 ± 0.6
SME HLB = 12					
Day 1	4.6	2.8	0.77	–	100.8 ± 1.7
Month 1	4.6	2.9	0.77	–	100.2 ± 1.2
Month 3	4.5	3.1	0.75	–	66.3 ± 0.8
W/O/W emulsion					
Day 1	5.8	935.0	14.65	–	98.3 ± 0.5
Month 1	5.7	903.8	17.99	–	100.5 ± 0.5
Month 3	5.2	803.8	29.27	–	103.5 ± 0.3

Fig. 1B shows the comparison among the different formulations and an aqueous DNAzyme solution. The amount of DNAzyme released was in the following order: aqueous solution > SME (HLB 12) ≥ ME > W/O/W emulsion > W/O emulsion. Drug release was strongly dependent on the drug carrier system. An undisturbed release was recognised with the aqueous solution (81.4 ± 4.3% after two hours). While DNAzyme from SME (HLB 12) was released immediately and yielded a plateau phase after two hours, ME showed a delayed release and a lag time of 90 min and was continuously increasing over time. DNAzyme released from W/O/W emulsion and W/O emulsion was either close to the quantification limit or not detectable at all. Less than 0.1% of the DNAzyme was released after 24 h.

3.4. DNase activity assay

To study the influence of DNase activity of the skin on the penetration studies, the degradation of DNAzyme was determined. Fig. 2 represents the data of the DNase activity experiment of fresh and frozen skin on top of the *stratum corneum* (tape 1) up to tape 3. The results exhibited a reduction of DNase activity from the top of the skin (tape 1) to the deeper skin layers. The frozen-thawed skin showed a similar decreasing profile but an overall lower amount of DNase activity.

3.5. Skin penetration studies

DNAzyme transport into the skin was compared with respect to the different drug carrier systems and different experimental designs. The comparison of the three SMEs, differing in droplet size, showed no significant difference (Fig. 3).

To compare the influence of the different drug carrier systems, the DNAzyme transport into skin was studied (Fig. 4A + B). The penetration of DNAzyme into impaired and intact skin ranged in the order SME (HLB 12) > ME ≫ aqueous solution (aq. s.) > W/O/W and W/O emulsion after 24 h. In general, the DNAzyme uptake from impaired skin was slightly increased compared to the intact skin except with the SME (HLB 12). Examination of the fluorescence assay showed similar results (Fig. 4B), thus are in agreement with the results by hybridisation-ELISA.

Fig. 5 depicts representative examples of fluorescence microscopy images of vertically cross-sectioned skin following topical application of DNAzyme in various formulations prepared for the fluorescence assay (Fig. 4B). The permeation of FAM-DNAzyme was restricted to the epidermal layer, especially the stratum corneum.

The most promising drug carrier systems regarding drug transport into the skin, SME (HLB 12), and DNAzyme stability, W/O/W emulsion, were subjected to further penetration studies using fresh

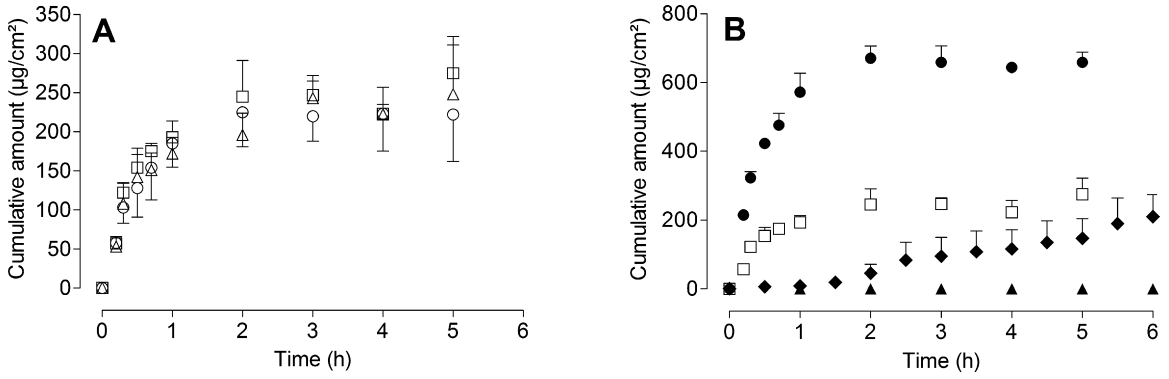

Fig. 1. Cumulative drug release (HPLC analysis) of DNAzyme from various drug carrier systems. (A) SME with different HLB values: (○) HLB 10, (Δ) HLB 11, (□) HLB 12. (B) Various drug carrier systems (●) aqueous solution, (□) SME (HLB 12), (♦) ME, (▲) W/O/W emulsion, W/O emulsion below limit of detection; (mean ± SD, $n = 3$).

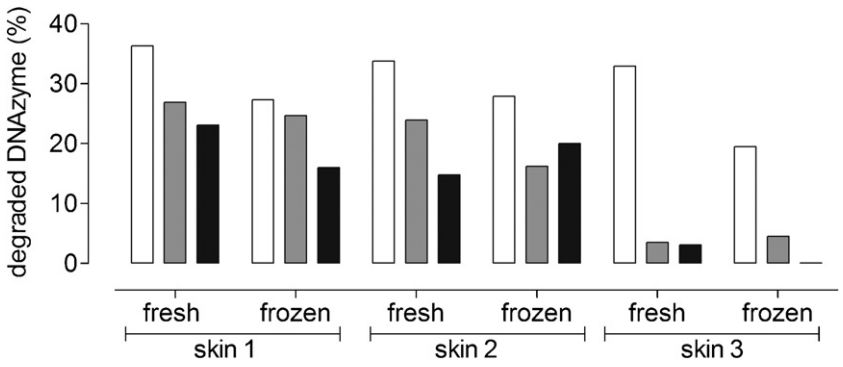

Fig. 2. Results of the DNase activity assay of fresh and frozen skin samples by tape stripping; analysed by HPLC.

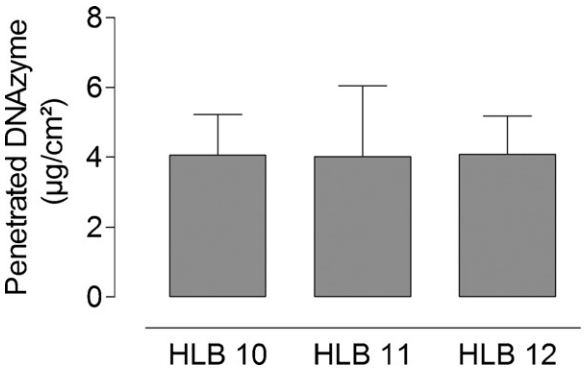

Fig. 3. DNAzyme skin uptake of SMEs varying in HLB values and droplet sizes. Infinite dose approach and frozen skin with intact skin barrier (mean ± SD, $n = 6$); 24 h, analysed by hybridization ELISA.

skin (Fig. 6) and the finite dose approach to mimic a more realistic experimental design (Fig. 7).

DNAzyme uptake from W/O/W emulsion into skin was not significantly affected by the skin conditions, but it exhibited a slightly higher DNAzyme uptake with the frozen skin (Fig. 6). Regarding the SME, a significant decreased amount of DNAzyme uptake was seen with fresh intact skin compared to the frozen intact skin, while no difference was seen with the impaired skin.

Finally, the influence of finite dose application under a low pressure massage on the skin uptake of DNAzyme was investigated to mimic the common conditions. Then, a formulation was applied on the skin. This experiment showed a nearly 4-fold higher DNAzyme uptake from the W/O/W emulsion (0.26 ± 0.15 µg/cm^2) compared to the SME (0.07 ± 0.03 µg/cm^2; HLB 12) (Fig. 7).

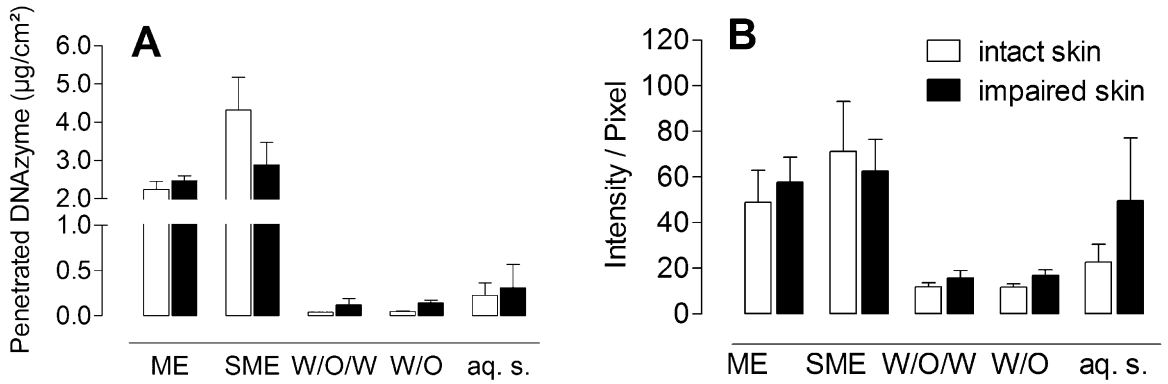

Fig. 4. DNAzyme skin uptake of various drug carrier systems; results of (A) hybridisation ELISA and (B) fluorescence assay. Infinite dose approach, frozen skin for 24 h (mean ± SD, $n \geq 3$).

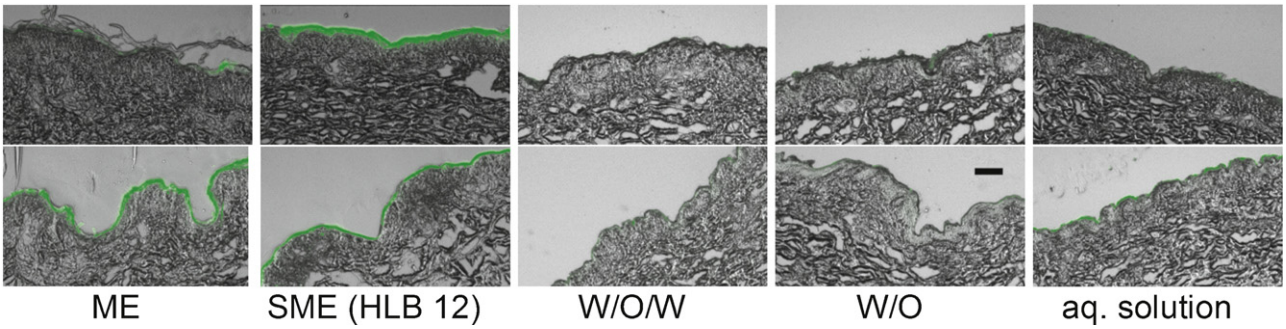

Fig. 5. Fluorescence microscopy images of vertically cross-sectioned skin following topical application of FAM-DNAzyme (green) in various formulations. Scale bar represents 100 µm. Pictures in the upper row represent the intact skin, and those in the lower row represent the impaired skin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

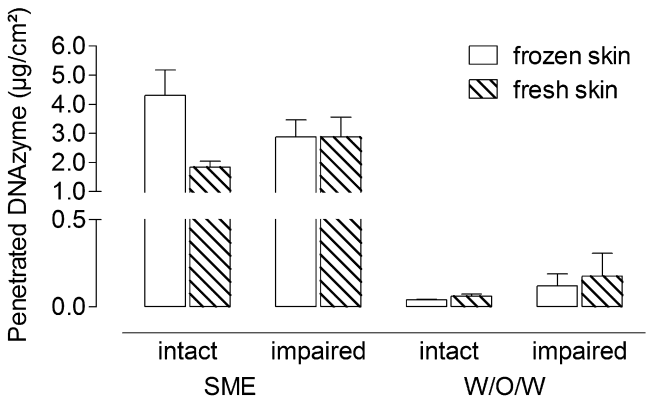

Fig. 6. DNAzyme skin uptake analysed by hybridisation-ELISA; Infinite dose and 24 h analysed by hybridisation-ELISA (mean ± SD, $n \geq 3$).

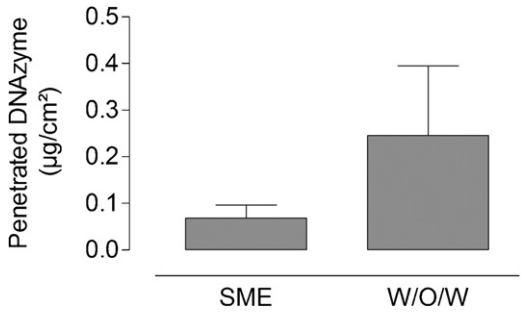

Fig. 7. DNAzyme skin uptake analysed by hybridisation-ELISA. Finite dose approach with low pressure application on fresh intact skin for 24 h analysed by hybridisation-ELISA (mean ± SD, $n = 6$).

3.6. Cellular uptake of DNAzymes

To determine the cellular uptake of the FAM-DNAzyme, FACS analysis of cells obtained from skin penetration studies with W/O/W and SME were done. As depicted in Fig. 8, it is shown that the penetrated FAM-DNAzyme was taken up by viable epidermal cells, mainly keratinocytes. Cellular uptake of the FAM-DNAzyme is improved by the W/O/W emulsion as compared to the SME resulting in an obvious higher increase of the respective uptake parameters as compared to the SME.

4. Discussion

The dermal therapy of skin diseases is often limited due to insufficient therapeutic drug levels at the target site. Therefore, the identification and application of suitable drug carrier systems are essential for a successful therapy. On the one hand, 10–23 DNAzyme is a relatively large molecule (10.6 kDa); therefore, appropriate inactive ingredients have to be chosen to enhance or even enable skin uptake. On the other hand, the DNAzyme is extremely sensitive towards DNases naturally occurring on the skin surface and in the skin tissue, therefore an encapsulation of the drug, in multiple emulsions, would be preferable.

The aim of this study was to identify formulations that best meet these requirements. Therefore, two drug carrier systems, SME and ME, with excellent penetration-enhancing properties due to their excellent drug solubility characteristics, alternation of skin lipids and small droplet size (Friedman et al., 1995; Kreilgaard, 2002) were developed. Furthermore, two formulations with the DNAzyme encapsulated in the inner aqueous phase were produced: a conventional W/O emulsion and, as innovative approach, a W/O/W multiple emulsion. Particular attention was paid to the preservation of the developed formulations due to the expected influence on the formulation stability. The preservatives tested and concentrations used were chosen according to the German Pharmaceutical Codex (DAC). No incompatibility occurred when the preservative potassium sorbate (0.14%) for the W/O emulsion and propylene glycol (16.60%) as formulation constituent of the ME was used and the preservative efficacy test was successful. The selection of an appropriate preservative for the W/O/W emulsion and the SME was more difficult. One possible explanation could be the influence of the inactive ingredients, e.g., soy lecithin, on the efficiency of the preservative (Darwish and Bloomfield, 1995; Kohn et al., 1963) and the distribution of the preservative between the aqueous and lipophilic phase of the emulsions (Kurup et al., 1991). Furthermore, W/O/W emulsions are extremely sensitive towards variation of components and additives (Schmidts et al., 2010). However, 0.05% benzoic acid and 0.20% potassium sorbate showed an appropriate preservation for W/O/W emulsion and W/O emulsion, respectively.

Regarding the stability of the formulations, the SME, ME and W/O emulsion did not meet the requirements for the recovery of the DNAzyme over 3 months. Degradation of the DNAzyme over time might be attributed to the non-encapsulated drug in the outer water phase in the case of ME and SME (Schmidts et al., 2011), DNase contamination during non-aseptic sampling for the stability testing or unspecific interactions with the formulation

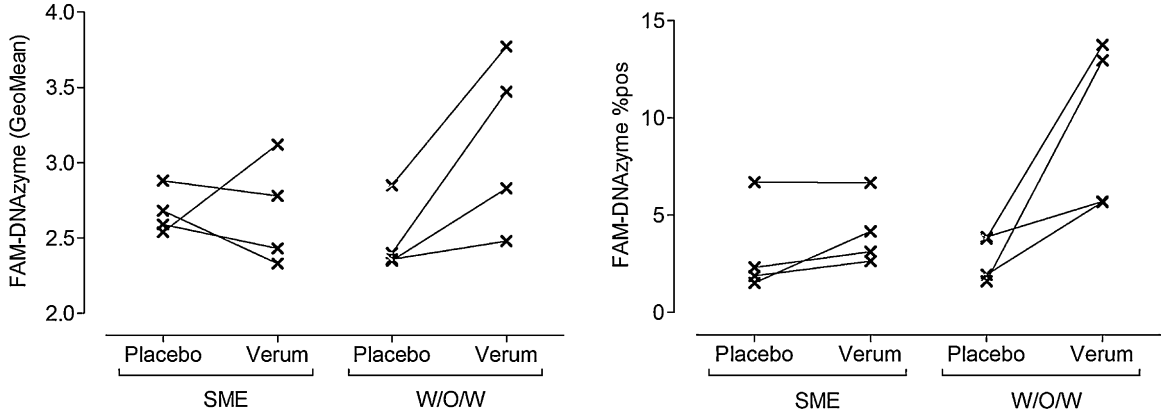

Fig. 8. Results of the FACS analysis of the cellular uptake of FAM-DNAzyme after skin penetration studies ($n = 4$). Left: geometric mean value (GeoMean). Right: percentage of FAM-DNAzyme positive cells (%pos).

components (Langlois et al., 1989). In contrast, only the W/O/W emulsion revealed sufficient DNAzyme recovery and protection during the period of observation that can be attributed to an appropriate encapsulation.

Next, the pharmacokinetic data of the developed DNAzyme containing formulations by drug release and skin penetration studies were collected. A comparison of the three different SMEs showed no significant influence of the droplet size on the drug release and skin uptake of DNAzyme. In contrast, Verma et al. (2003) showed a size-dependent influence of liposomes on drug penetration. One explanation might be the width of the droplet size distribution and therefore an overlapping droplet size of the developed SMEs. Furthermore, it must be considered that the hydrophilic DNAzyme was solubilised in the outer water phase.

Drug release is influenced both by the formulation and the drug properties. The formulation viscosity, the drug distribution among the phases (partition coefficient) and the thermodynamic activity of the drug define the diffusion coefficient and thus the drug release. An expected fast and unimpeded diffusion of the DNAzyme from aqueous solution was observed. DNAzyme was released from various formulations in the order SME (HLB 12) ≥ ME > W/O/W emulsion > W/O emulsion, which is in agreement with the results of Ferreira et al. (1994), who showed that the hydrophilic glucose was released fastest from an O/W emulsion, but was barely released from a W/O/W emulsion and a W/O emulsion. While the maxima of DNAzyme released from the SME and ME were comparable, the DNAzyme release was delayed with the ME. This can be associated with the higher viscosity of the ME as well as the possible inner structure of the ME, e.g., the formation of a bicontinuous structure (Kreilgaard, 2002) that hampers an immediate release. The extent of DNAzyme released from SME was less compared to the aqueous solution and might be attributed to the SME ingredients that block the pores of the membrane. Finally, the DNAzyme release from W/O/W and W/O emulsion was practically non-exist, which is a result of the DNAzyme encapsulation in the inner water phase of W/O/W and W/O emulsion and the high viscosity of the systems.

However, drug release studies are not sufficient enough to predict skin uptake due to the lack of skin–vehicle interactions. Thus, skin penetration was investigated to elucidate the most suitable drug carrier system regarding protective effect and skin uptake. DNAzyme may be used in the treatment of atopic dermatitis that is characterised by a defective skin barrier. Therefore, both skin conditions, intact and impaired skin, were investigated. Skin uptake followed the order SME > ME ≫ aqueous solution > W/O/W and W/O for both impaired and intact skin after infinite dose application for 24 h. The enhanced DNAzyme uptake by the SME and ME compared to the aqueous solution is attributed to the well-known enhancing properties of these carrier systems (Friedman et al., 1995; Schwarz et al., 1995). Furthermore, the low DNAzyme uptake by the W/O/W and W/O emulsions might be a result of a delayed drug release of the encapsulated DNAzyme. As expected, the DNAzyme uptake was slightly enhanced when the skin barrier was impaired. However, this is not true in case of SME. Drug penetration is higher using intact skin compared to impaired skin where the stratum corneum is absent. This phenomenon might be attributed to the oil blend used, especially ethyl oleate, the ethyl ester of oleic acid. Oleic acid is able to facilitate a transport of hydrophilic substances into the skin by increasing the fluidity of lipid portion (Kanikkannan and Singh, 2002) and the generation of water pools within the stratum corneum (Ongpipattanakul et al., 1991). Thus, the enhancing effect of the SME is less expressed in the absence of the stratum corneum layer. Fluorescence microscopic images revealed that the DNAzyme was mainly located in the upper skin. As the target GATA-3 is mainly expressed in the inflammation infiltrate containing T cells but also in eosinophils, basophils, mast cells and epithelial cells (Justice et al., 2002; Masuda et al., 2004; Zon et al., 1993), the observed DNAzyme uptake should be sufficient for an appropriate treatment of atopic dermatitis. Next, the influence of fresh skin on the DNAzyme uptake was investigated using the SME and the W/O/W emulsion. Skin penetration studies revealed the influence of DNase activity on the uptake of DNAzyme. While no significant difference was observed for the encapsulated and thus protected DNAzyme in the W/O/W emulsion, DNAzyme uptake from SME clearly exceeded that of fresh skin with an intact skin barrier. The DNase activity assay showed that DNase activity was highest with fresh skin and consequently degraded the DNAzyme in the outer aqueous phase of the SME. Furthermore, DNase activity showed a decreasing profile following the stratum corneum layers from the outside inwards (Fig. 2). Subsequently, the difference between fresh and frozen skin is less distinctive with the impaired skin.

Florence and Whitehill (1981, 1982) showed that several mechanism of W/O/W multiple emulsions breakdown exist, for example by rupture of the oily membrane due to the expulsion of the internal water droplets or the diffusion of the water through the oil phase owing to osmotic effects. Thus, the entrapped drug can be released (Raynal et al., 1993). As a breakdown of the oily membrane can also be induced by moderate shear stress (Muguet et al., 2001; Olivieri et al., 2003), the influence of the topical application (a shear rate of $1000\,s^{-1}$ is commonly reached in topical application (Olivieri et al., 2003)) was investigated. DNAzyme uptake with finite dose set-up and application of the formulation under a gentle massage led to the superiority of the W/O/W emulsion over the SME. This indicates that the oily membrane of the developed W/O/W multiple emulsion broke down under the applied shear, and the DNAzyme was released and successfully penetrated into the skin. The targets of the DNAzyme are the cells of the viable epidermis. In order to proof an uptake of DNAzyme by the cells of the epidermis, FACS analysis of viable epidermal cells was performed. The FACS analysis revealed that the FAM-DNAzyme uptake by the skin is not limited to the interstitial of the epidermal compartment and the stratum corneum. Importantly, Compared to the SME the W/O/W emulsion mediated a higher drug uptake which confirmed the data obtained by the skin penetration studies.

5. Conclusion

In summary, different drug carrier systems for the topical application of DNAzyme were developed and investigated with regard to the physicochemical stability and skin uptake. The encapsulation of the hydrophilic DNAzyme into the inner aqueous phase of the W/O/W multiple emulsion and the W/O emulsion clearly improved the DNAzyme stability. DNAzyme uptake from SME to the skin indicates that composition components, e.g., ethyl oleate, interact with the *stratum corneum*, leading to enhanced DNAzyme penetration compared to skin with impaired barrier, which is characterised by a reduced *stratum corneum*. The release and consequently skin uptake of DNAzyme from the W/O/W multiple emulsion can be induced by shear commonly applied at topical application. With regard to the experimental set-up, the results emphasise the reduction of skin DNase activity due to deep freeze storage. To conclude, the developed W/O/W multiple emulsion is a suitable and promising drug carrier system for the transport of DNAzyme across the skin and into epidermal cells of the atopic dermatitis affected skin.

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