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Protective effect of drug delivery systems against the enzymatic degradation of dermally applied DNAzyme

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ABSTRACT

DNAzymes are a group of RNA-cleaving DNA oligonucleotides that contain a catalytic domain and represent a novel class of antisense molecules. Although single-stranded DNAzymes may represent the most effective nucleic acid drug to date, the sensitivity to nuclease degradation is challenging. Therefore, it is important to develop a drug delivery system, which protects the molecule against degradation during dermal application. In the present study, the potential protective effect, regarding the dermal application of DNAzyme, of multiple (W/O/W) emulsions, W/O emulsions, submicron emulsion and microemulsions were investigated using a HPLC method. The HPLC method enables the quantitative analysis of DNAzyme as well as the detection of degradation products. The differences between the activity of DNase I and the activity of nucleases located in the porcine skin were compared. It was found that the degradation of an aqueous solution of DNAzyme is depending on the DNase I activity as well as on the incubation time. Furthermore, the activity of neutral and acid nucleases in skin tissue was determined to be 5.2 and 14.8 U per 1 g of porcine skin tissue, respectively. Investigation of the protective character of different delivery systems revealed that formulations containing DNAzyme in the outer water phase (submicron emulsion and microemulsion) did not exhibit any form of protective effect, whereas formulations containing DNAzyme in the inner water phase (multiple emulsion and W/O emulsion) were able to prevent the DNAzyme degradation to a considerable degree. Consequently, these formulations are promising candidates for the dermal drug delivery of oligonucleotides.

1. Introduction

Chronic inflammatory skin diseases like atopic dermatitis and psoriasis representing complex skin disorders and indicate increased occurrence worldwide, mainly in industrialised countries. Current therapies are based on the treatment of the symptoms, using anti-inflammatory drugs, without directly interfering with the disease-causing mechanisms. Consequently, there is still a significant medical need for novel therapeutic approaches for the treatment of inflammatory skin diseases. Novel concepts for such approaches include the use of antisense-based molecules such as antisense DNA, DNAzymes, small interfering (si)RNA and ribozymes that interfere with the mRNA of proteins that are involved in the pathogenesis of the respective disease.

DNAzymes represent a novel class of antisense molecules that are not yet established in the therapy of any human diseases. The 10–23 DNAzymes are a group of RNA-cleaving DNA molecules that contain a catalytic domain (Silverman, 2005; Cieslak et al., 2003; 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 an 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. Therefore, 10–23 DNAzyme was chosen as a promising active pharmaceutical ingredient candidate for the treatment of atopic dermatitis.

The dermal application of such molecules is still challenging owing to their limited bioavailability due to several reasons (Akhtar et al., 2000; Shoji and Nakashima, 2004; White et al., 2009). For example, penetration into the affected skin compartments is often restricted due to the effective barrier function of the skin (Cork, 1997; Elias, 1988; Roberts et al., 2002) and the rather large size of these molecules, which often exceeds 10 kDa.

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Moreover, oligonucleotide-degrading enzymes such as RNases and DNases, which might be located on the skin and in the skin tissue, may destroy the integrity of such molecules before they come into contact with the target structure. The deoxynucleases on the skin are produced by several micro-organisms such as *Staphylococcus* (Langlois et al., 1989; Wierup, 1978). DNases in the human body are considered to be of lysosomal or pancreas origin and are able to be classified into two groups: DNase I and DNase II. Both types of enzymes are endonucleases, acting at the centre of the polymer chain (Laskowski, 1959) and preferentially hydrolyse the bonds between purine and pyrimidine nucleotides. However, while the products of hydrolysis by DNase I are oligonucleotides carrying the monoesterified phosphoryl group on carbon 5′, DNase II yields fragments carrying the phosphoryl group on carbon 3′. It has been shown that human epidermis mostly contains type II deoxyribonuclease with an optimum pH of 5 (Santoianni and Rothman, 1961). Furthermore, other enzymes capable of participating in nucleic degradation such as phosphodiesterases, phosphatases and others have been identified in the epidermis (King et al., 1975; Tabachnick and Perlish, 1967).

In order to protect DNAzymes from DNA-cleaving enzymes located on the skin and in the skin tissue, the protection efficiency of promising dermal drug delivery systems has been investigated. Systems with an inner water phase (W/O, W/O/W) have been evaluated in respect of their encapsulation properties regarding to hydrophilic drugs such as the oligonucleotide DNAzyme. Due to their distinct structure and properties, multiple emulsions are in particular of interest for several drug delivery approaches, including carriers for the dermal application of encapsulated drugs in pharmaceutical products (Fukushima et al., 1987; Khopade and Jain, 1999; Lindenstruth and Muller, 2004; Schmidts et al., 2010), cosmetics (Tadros, 1992; Vasudevan and Naser, 2002) and the encapsulation of flavours in food (Garti and Benichou, 2004). The oil-in-water (O/W) systems microemulsions and submicron emulsions with the hydrophilic drug located in the outer water phase are well known for excellent dermal drug transport properties. These carrier systems below 1 μm have been of particular interest for the last two decades and showed improved drug transport into and through 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; Yuan et al., 2010).

The degradation of DNAzymes by DNase I and deoxynucleases located in porcine skin tissue as well as the protective function of different drug delivery systems was investigated. The qualitative and quantitative analysis of DNAzyme and its degradation products were performed using an anion-exchange HPLC (AE-HPLC) method.

2. Materials and methods

2.1. Materials

All ingredients were obtained in pharmaceutical grade. The following excipients were used to prepare the multiple emulsions: the oils used were light and heavy paraffin oil (Fagron, Germany). Soy lecithin (Caelo, Germany) was added as a penetration enhancer. The lipophilic surfactant was Span 80 (Crill4; Croda, Germany) and the hydrophilic surfactant Steareth-20 (Brij 78 P; Uniqema, Germany). Either NaCl solution or $MgSO_4$ solution (both 0.065 M) was used as inner water phase.

The oils used for preparation of the W/O emulsion were isopropyl palmitate (Fagron, Germany) and cetostearyl isononanoate (Cetiol SN; Cognis, Germany). Triglycerol diisostearate (Caelo, Germany) was used as emulsifier. The oil phase also consists of soy lecithin (Caelo, Germany) and a hydrophobic basis gel (Fagron, Germany). The water phase contained glycerol, $MgSO_4 \cdot 7H_2O$, potassium sorbate and citric acid purchased from Fagron (Germany).

To prepare the microemulsion, the following excipients were used: labrasol (caprylocaproyl macrogol-8 glycerides) and plurol oleique (polyglyceryl-6-dioleate) from Gattefossé (Bally et al., 1999) as emulsifiers and isopropyl palmitate and cetostearyl isononanoate as oil phase. The water phase consisted of propylene glycol, glycerol and $MgSO_4 \cdot 7H_2O$ purchased from Fagron (Germany).

The following ingredients were used in order to prepare the submicron emulsion: Oleth-5 and Oleth-10 (Croda, Germany) as emulsifiers and a blend of cetostearyl isononanoate, coco caprylate/caprate (Cetiol LC) and ethyl oleate as oil phase, kindly provided by Cognis (Germany).

A 10–23 DNAzyme was generated and kindly provided by Sterna Biologicals GmbH & Co. KG (Germany), representing the Na-salt of a single-stranded DNA molecule composed of 34 deoxynucleotide bases with a molecular weight of 10.6 kDa. DNase I and proteinase K were purchased from Applichem (Germany). HPLC-grade methanol and NaCl from Merck (Germany) and Tris–HCL UltraPure (pH 7.5) from Invitrogen (Germany) were used as solvent components for HPLC analysis.

2.2. Preparation of the drug delivery systems

2.2.1. W/O/W emulsion

Multiple emulsions were prepared using a 2-step procedure, as reported by Matsumoto et al. (1976). First, the primary W/O emulsion was produced and then, the primary emulsion (40 wt%) was dispersed in the aqueous solution of the hydrophilic emulsifier. In detail, the primary W/O emulsion was obtained by adding the aqueous phase, NaCl or $MgSO_4$ solution plus the DNAzyme Na-salt (0.4%), to the oil phase. The electrolytes NaCl and $MgSO_4$ are necessary for obtaining stable formulations (Kent and Saunders, 2001). Therefore, both phases were heated to approx. 70–75 °C and stirred using a magnetic stirrer. Then, the inner water phase was added to the oil phase and the emulsion was homogenised for 2 min using a rotor/stator homogeniser (Diax 600, Heidolph Germany) at 9500 rpm. In the second step, the chilled primary emulsion was slowly added to the outer water phase under stirring at 1200 rpm using the EUROSTAR digital stirrer (IKA, Germany), until a homogeneous emulsion was obtained. Table 1 shows the composition of the multiple emulsion.

2.2.2. W/O emulsion

W/O emulsion was prepared at room temperature. The DNAzyme Na-salt was added to the inner water phase at a total quantity of 0.4%. The soy lecithin was dissolved in oil phase and then mixed with the surfactant and the hydrophobic basis gel. Finally, the water phase was added slowly to the oil phase and mixed with a mortar and pestle until a homogeneous emulsion was obtained. Table 2 shows the composition of the W/O emulsion.

Table 1
Composition of the multiple emulsion.

Ingredient		[wt%]
0.065 M NaCl or $MgSO_4$ solution	Inner water phase	19.6
DNAzyme Na-salt		0.4
Heavy paraffin oil		15.8
Span 80	Oil phase	4.0
Soy lecithin		0.2
Distilled water		59.0
Steareth-20	Outer water phase	1.0

Table 2
Composition of W/O emulsion.

Ingredient	[wt%]
Triglycerol diisostearate	3.0
Isopropyl palmitate	1.2
Cetostearyl isononanoate	1.2
Soya lecithin	0.2
Hydrophobic basis gel	24.6
Potassium sorbate	0.14
Distilled water	63.9
Glycerol 85%	5.0
Na-salt of DNAzyme	0.4
$MgSO_4 \cdot 7H_2O$	0.3
Citric acid	0.07

Table 3
Composition of the microemulsion.

Ingredient	[wt%]
Labrasol	7.2
Plurol	4.8
Cetostearyl isononanoate	2.5
Isopropyl palmitate	2.5
Glycerol 85%	2.49
Propylene glycol	16.6
DNAzyme Na-salt	0.4
$MgSO_4 \cdot 7H_2O$	0.3
Distilled water	63.21

Table 4
Composition of the submicron emulsion.

Ingredient	[wt%]
Coco-caprylate/caprate	5.0
Cetearyl isononanoate	5.0
Ethyl oleate	5.0
Oleth-5	5.118
Oleth-10	0.882
Glycerol 85%	3.0
DNAzyme Na-salt	0.4
$MgSO_4 \cdot 7H_2O$	0.3
Distilled water	75.3

2.2.3. Microemulsion

A microemulsion was spontaneously formed by gently mixing the appropriate quantities of the components and the drug at room temperature. The concentration of DNAzyme in the formulation was 0.4%. Table 3 shows the composition of the microemulsion.

2.2.4. Submicron emulsion

The water phase and the oil phase of the submicron emulsion were heated separately to 70 °C. The two phases were then combined and homogenised using a rotor/stator homogeniser (Diax 600, Heidolph Germany) for 1 min at 24,000 rpm. Table 4 shows the composition of the submicron emulsion.

2.3. Determination of the physicochemical properties of the delivery systems

The mean droplet size (z-average, mean ± S.D., $n = 3$) in the W/O emulsion, submicron emulsion and microemulsion was determined by dynamic light scattering (High Performance Particle Sizer (HPPS), Malvern Instruments, UK). Prior to measurement, W/O samples were diluted 1:1000 using light paraffin oil (viscosity: 33 mPa s) and the submicron emulsion was diluted 1:100 using distilled water. The droplet size of the microemulsion was determined without dilution. Oil droplet size (mean ± S.D., $n = 3$) in the multiple W/O/W emulsions was determined using a laser diffraction particle size analyser (Mastersizer S, Malvern Instruments, UK). The results are presented as the mean diameter $D(\nu, 0.5)$ based on the volume distribution.

Rheological analysis was performed at 25 °C using a RheoStress 300 Rheometer (Thermo Haake, France) with a cone and plate geometry 20 mm in diameter and 2° angle. The apparent viscosity was measured over a shear rate of 0.1–100.0 s^{-1}. The results are presented as mean values (mean ± S.D., $n = 3$).

2.4. HPLC analysis

A DNAPac PA-100 4 mm × 250 mm anion-exchange column and guard column (Dionex, USA) were used. The resin consisted of 100 nm quaternary-ammonium functionalised MicroBeads bound to a 13 μm diameter solvent compatible nonporous substrate. The HPLC system (VWR-HITACHI, Germany) consisted of a L2130 pump equipped with an L2200 autosampler, column heater set and a L2455 photodiode array detector. The detection wavelength was set at 260 nm and EZChrome EliteClient/Server Version 3.1.7 was used for peak integration. The equilibration buffer (A) was composed of 20 mM Tris–HCl, 20% methanol, 260 mM NaCl, pH 7 and the elution buffer (B) of 20 mM Tris–HCl, 20% methanol and 1 M NaCl, pH 7. The flow rate was 0.8 mL/min and the injection volume 20 μL. Gradient run is shown in Table 5. Quantification was done using a calibration curve over the investigated concentration range.

2.5. Degradation of DNAzymes by DNase I in water solution

DNAzyme degradation in the presence of DNase I was investigated. The DNase I was solubilised in Tris buffer (pH 7.5) containing 10 mM $MgSO_4$. DNase I belongs to the group of metallonucleases and therefore, it is only active in the presence of magnesium ions (Baranovskii et al., 2004). The following concentrations of DNase I were tested: 105 U, 10.5 U, 5 U and 1 U. For this assay, 0.5 mL of DNAzyme solution (0.1625 mg/mL) was mixed with 1 mL DNase I solution and incubated at 25 °C. The quantitative analysis of a non-degraded DNAzyme strain was carried out by HPLC analysis. The degradation products were only analysed qualitatively.

2.6. Degradation of DNAzymes by deoxyribonucleases from the skin

Nuclease activity from domestic porcine skin tissue (ear) obtained from a local slaughterhouse was investigated in three different circumstances. Therefore, 60 mg of porcine skin (dermis and epidermis) of fresh and washed tissue was finely hashed and incubated with 3 mL of reaction medium. Acid nuclease activity was measured in a reaction mixture containing 10 mM sodium acetate buffer, pH 5.0. Neutral nucleases were determined in a reaction mixture containing 200 mM Tris–HCI buffer, pH 7.5 and 10 mM $MgSO_4$. Additionally, degradation in distilled water was investigated. Afterwards, 1 mL of DNAzyme solution (0.1625 mg/mL) was added to the tissue solution in order to determine the degradation of DNAzyme. Subsequently, the samples were filtered using a 0.45 μm filter and HPLC measurement was performed.

Table 5
Gradient elution method for HPLC.

t [min]	A [%]	B [%]
0–2	100	0
5	85	15
35	45	55
40	0	100
45	100	0
45–50	100	0

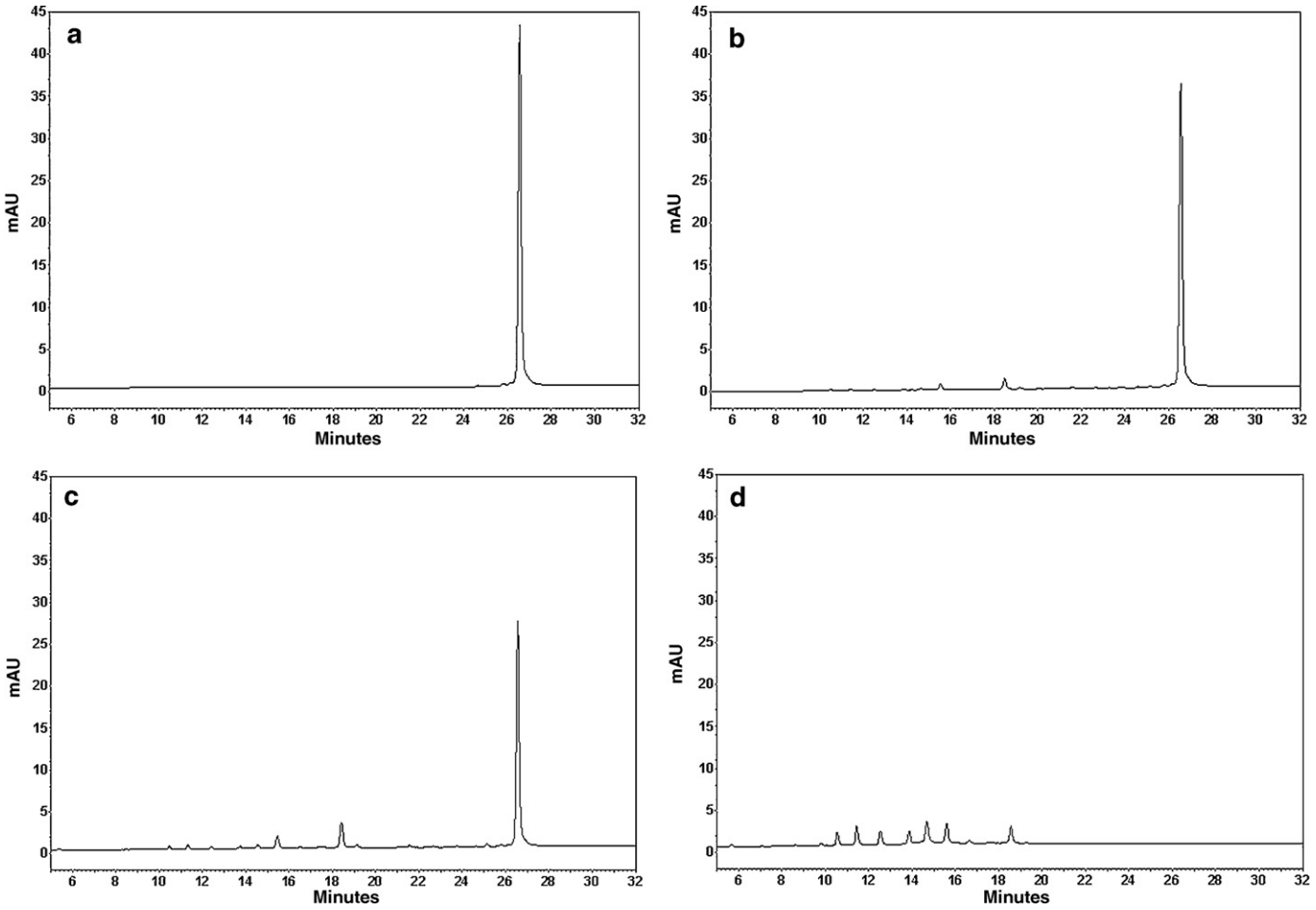

Fig. 1. Degradation of 10–23 DNAzyme in the presence of various concentrations of DNase I. HPLC analysis was performed after 2 min of incubation time. (a) Without DNase I, (b) 5.25 U, (c) 10.5 U, and (d) 105 U

2.7. DNAzyme protection assays against DNase I in emulsions

The protective effect of different drug delivery systems containing DNAzyme against DNase I was investigated. Therefore, 20 mg of the different emulsions were transferred to 1 mL of digestion buffer containing 105 U of DNase I and incubated at 25 °C for 1 min. In order to interrupt the DNase I activity, the samples were heated to 99 °C for 10 min. Thereafter, the samples were treated for 10 min at 50 °C in an ultrasonic bath and filtered using 0.45 μm filters. The degradation of DNAzyme was examined using a HPLC method.

3. Results and discussion

3.1. Degradation of DNAzyme by DNase I in the water solution

The degradation of 10–23 DNAzymes was investigated using the nuclease DNase I. DNase I belongs to the class of Mg^{2+} and Ca^{2+} dependent nucleases (Melgar and Goldthwait, 1968; Santoianni and Rothman, 1961) and therefore, the experiments were performed in a reaction medium containing 10 mM $MgSO_4$. As expected, no degradation was observed in the absence of Mg^{2+} ions.

Fig. 1 shows the HPLC chromatograms of DNAzyme incubated with various concentrations of DNase I and an incubation time of 2 min. Using the HPLC method described above, the retention time of intact 10–23 DNAzyme in water solution was 26.6 min. Treatment of DNAzyme with DNase I results in a decrease of the DNAzyme peak and simultaneous appearance of additional peaks with shorter retention times. These peaks can be correlated with degradation products of DNAzyme. The separation of DNA molecules by AE-HPLC is predicated on the interaction between negatively charged phosphate groups on the DNA backbone and positively charged groups on the resin stationary phase. The strength of this interaction is based not only on net charge, but also on local charge density. Generally, the more phosphate groups the stronger the affinity for the stationary phase. With increasing salt concentration, DNA molecules should be eluted in the order of chain length (number of charged phosphates) (Prazeres et al., 1998). The first degradation products of 10–23 DNAzyme appeared at about 18.5 min. Thus, the DNA chain must be considerably shorter in this case than the original chain. However, the exact length and composition of the degradation product could not be determined due to a lack of references. The next clearly visible degradation product could be detected at 14.7 min. As degradation progressed, the fragments appeared at increasingly shorter retention times. This result can be related to the DNase I degradation mechanism. Since DNase I is an endonuclease, the phosphodiester bond is cleaved within a polynucleotide chain. In contrast, exonucleases cleave phosphodiester bonds at the end of a polynucleotide chain (Baranovskii et al., 2004).

Fig. 2 presents the results of the quantitative analysis of DNAzyme degradation subject to DNase I activity. The higher the DNase I activity, the more DNAzyme is degraded. DNA hydrolysis is found to be roughly dependent on the enzyme concentration. At activities of 105 U after 1 min of incubation, no intact DNAzyme was able to be found in the HPLC chromatogram.

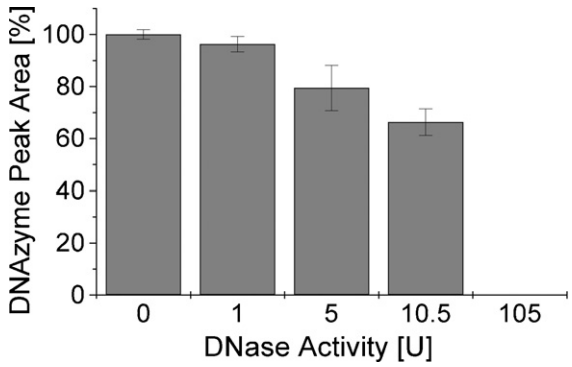

Fig. 2. Quantitative analysis of degradation of 10–23 DNAzyme in the presence of various concentrations of DNase I.

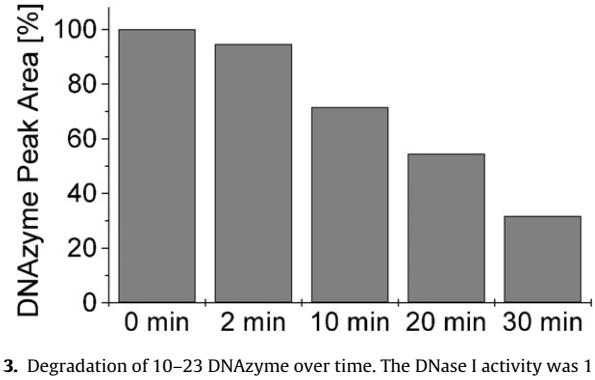

Fig. 3. Degradation of 10–23 DNAzyme over time. The DNase I activity was 1 U.

In addition, DNAzyme degradation was seen to be time-dependent. For this purpose, DNAzyme was incubated with DNase I (activity 1 U) for 2 min up to 30 min (Fig. 3). The degradation is dependent on the effective treatment time. After 30 min, approximately 70% of DNAzyme was decomposed.

3.2. Degradation of DNAzymes by deoxyribonucleases from the skin

To gain more knowledge about the degradation process of dermally applied DNAzyme, the degradation of 10–23 DNAzyme by deoxyribonucleases from native porcine skin tissue was determined. In general, it is known that the human body produces several other deoxyribonucleases apart from DNase I. The list of studied human DNase includes non-specific DNase I, DNase II, phosphodiesterase I, DNase DFF40, endonuclease G and lactoferrin (Baranovskii et al., 2004). The deoxyribonucleases differ in terms of their structure, mechanism of hydrolysis, catalytic properties and appearance in the various regions of the human body. Deoxyribonucleases have been found in the epidermis (Santoianni and Rothman, 1961; Tabachnick and Perlish, 1967) and are responsible for the disappearance of DNA in corneocytes during keratinisation. The enzymes involved in DNA degradation in the epidermis are endonucleases, exonucleases and phosphatases. The existence of epidermal acid DNA cleaving exonucleases and exonucleases was confirmed using guinea-pig skin (Miyagawa et al., 1975). Epidermal acid phosphatase was characterised as a non-specific phosphatase (Miyagawa et al., 1977). These acid phosphohydrolases are most active around pH 5.0 and presumably of lysosomal origin.

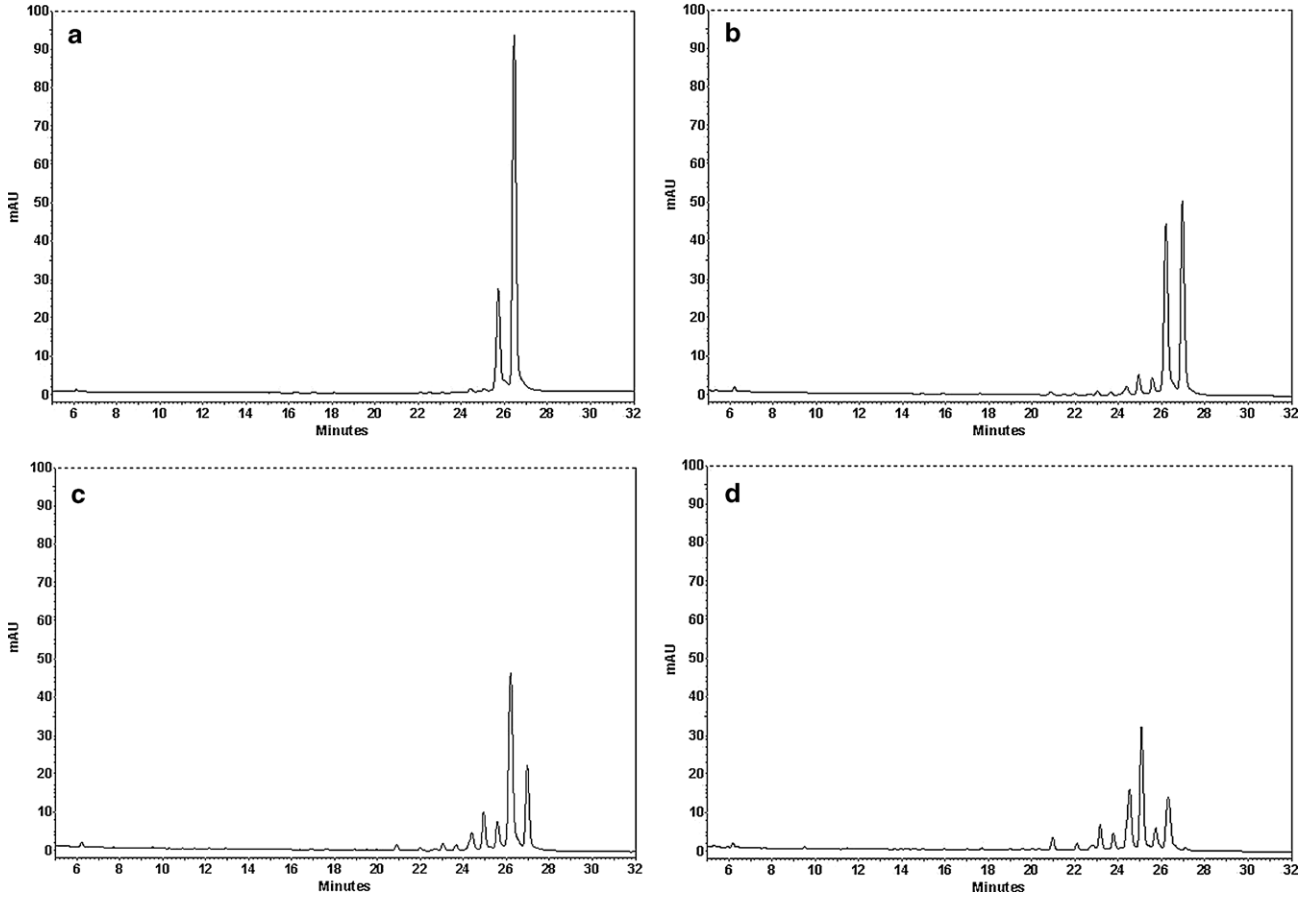

Fig. 4. HPLC chromatogram of degradation products of 10–23 DNAzyme in the porcine skin extract at different incubation times. (a) 1 min, (b) 10 min, (c) 30 min, and (d) 60 min.

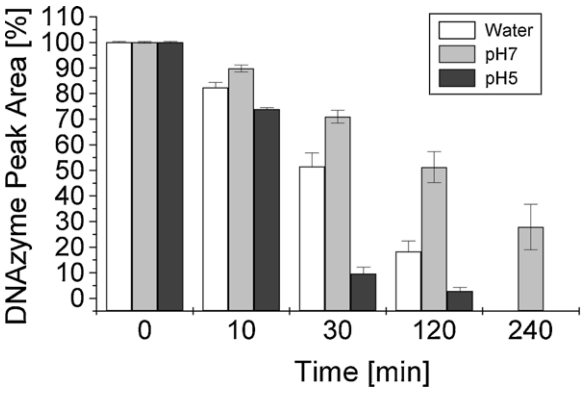

Fig. 5. Enzymatic hydrolysis of DNAzyme by porcine skin extract as a function of time. Skin weight was 60 mg and a total volume of 4 mL reaction mixture. Final DNAzyme concentration was 0.041 mg/mL.

Before the degradation process was investigated, the HPLC chromatogram of lysed porcine skin tissue was analysed. No peaks of lysed porcine skin tissue could be detected at the retention time of 10–23 DNAzyme and the degradation products. To determine the mechanism of action of the skin's own deoxyribonucleases, the degradation process was performed using different reaction media. Acid deoxyribonuclease activity was measured in a reaction mixture containing 10 mM sodium acetate buffer, pH 5.0. Neutral deoxyribonucleases were determined in a reaction mixture containing 200 mM Tris–HCl buffer, pH 7.5 and 10 mM $MgSO_4$. Distilled water was used as control.

Fig. 4 presents the HPLC chromatograms of the degradation products of 10–23 DNAzyme incubated with homogenised skin in distilled water over time. Similar chromatograms were obtained using the other two reaction mixtures. A significant difference compared to the degradation using DNase I was observed. First, degradation products appeared close to the intact 10–23 DNAzyme peak, already 1 min in front. This indicates that only a few bases were cut from the compact strain. Such a degradation pattern is more likely a result of exonuclease activity than to endonuclease activity. Miyagawa et al. (1975) presented a model where the degradation of the DNA chain in the epidermis was initiated by an endonuclease, followed by an exonuclease hydrolyses resulting in mononucleotides. Finally, phosphatase and other enzymes converted these mononucleotides to nucleosides and further metabolites. In the present study, no endonuclease activity was detected as an initial degradation step in respect of DNAzyme. Similar results were obtained by Tidd and Warenius (1989) who implicated a predominantly exonucleolytic attack on c-myc 15-mer oligodeoxynucleotides by serum nucleases. However, due to a low level of sensitivity of the HPLC method, endonuclease activity cannot completely be ruled out.

Fig. 5 presents the degradation of DNAzyme by deoxyribonucleases from the skin subject to the reaction medium and reaction time. Degradation was seen to be greatly dependent on the reaction medium. The highest deoxyribonuclease activity was observed at pH 5. Similar observations have been made by other authors (Beck et al., 1972; Santoianni and Rothman, 1961). Santoianni and Rothman (1961) found that, in contrast to guinea pig epidermis, human and rat epidermis mainly contains type II deoxyribonuclease with an optimum pH of 5. However, no deoxyribonuclease II activity was found in the dermis extract. Studies of other authors (Beck et al., 1972; Reimer et al., 1978) have shown the existence of several deoxyribonucleases at different pH optima in the human dermis, epidermis and horny layer.

On the basis of DNAzyme degradation in buffer solutions after 10 and 30 min, the activity of neutral and acid deoxyribonucleases in native porcine skin tissue was calculated. Owing to the involvement of all types of deoxyribonucleases, the results obtained for distilled water were not able to be evaluated in this instance. The results are presented in Table 6. Activity was described in units, with 1 U representing the transformation of 1 μmol of the substrate DNAzyme per minute at 25 °C.

Table 6
Activity of neutral and acid deoxyribonucleases in pig skin for DNAzyme (mean ± S.D, $n = 3$).

	Activity [U/g of skin tissue]
Neutral deoxyribonucleases (pH 7.5)	5.2 ± 0.1
Acid deoxyribonucleases (pH 5)	14.8 ± 1.5

These data are similar compared with results obtained by other authors (Santoianni and Rothman, 1961). However, a direct comparison is often difficult due to the different species used.

3.3. Degradation of DNAzymes incorporated in the emulsions by DNase I

3.3.1. Physicochemical characterisation of delivery systems

During topical application, DNAzymes are exposed to nucleases located on the skin as well as in the skin cells. In the last few years, several systems such as liposomes (Ali et al., 2008), lipoplexes (Henriques et al., 2009), cationic amphiphiles and dendrimers (Bielinska et al., 1996; Delong et al., 1997) have been developed for delivery and protection of nucleic acid. In the present work, the dermal formulations W/O/W emulsion, W/O emulsion, submicron emulsion and microemulsion were used as drug delivery systems. The properties of the different formulations vary in terms of lipophilicity, droplet size, viscosity and DNAzyme localization (inner or outer phase). With regard to a potential successful topical application, all formulations present advantages as well as disadvantages. Due to the high water solubility of DNAzyme, it is situated in the water phase of the formulations. In case of W/O and W/O/W emulsions, the DNAzyme is encapsulated in the inner water phase. In contrast, the DNAzyme is located in the outer phase with the microemulsion and submicron emulsion. The encapsulation of drugs is of great importance for the protection against nucleases. Thus, W/O and W/O/W emulsions appear to be advantageous. However, the formulations are characterised by large droplet sizes of 800 nm and a few micrometers, respectively, which might have a negative influence on drug permeation and penetration. Several studies have shown that the droplet size of delivery systems can play an important role in skin penetration of drugs. A decrease in the droplet diameter below 200 nm showed a significantly increase in skin penetration of several drugs (Friedman et al., 1995; Kreilgaard, 2002). Therefore, systems with small droplets such as microemulsions and submicron emulsions could be a more powerful tool for topical application and thus were not excluded from this study. The physicochemical properties of tested formulations are presented in Table 7.

3.3.2. Investigation of the protective character of different delivery systems

With respect to the formulations, the protection of DNAzyme against the attack by degrading enzymes was examined. All formulations containing DNAzyme were treated with DNase I solution (105 U) and incubated for 1 min. The high activity of DNase I was chosen for a better comparison of the formulations. Degradation of DNAzyme was evaluated using HPLC analysis. The degradation of DNAzyme depending on the delivery system is shown in Table 8.

As expected, DNAzyme located in the outer water phase of the microemulsion and the submicron emulsion was completely degraded immediately. After 1 min, as with the water solution, no

Table 7
Physicochemical properties of delivery systems containing DNAzyme Na-salt (mean ± S.D., $n = 3$).

Formulation	Droplet size D (v, 0.5) [μm]	Droplet size z-average [nm]	Viscosity [Pa s]
W/O/W with NaCl	10.8 ± 0.5	–	1.2565 ± 0.3
W/O/W with $MgSO_4$	14.6 ± 0.5	–	0.9797 ± 0.1
W/O emulsion	–	816 ± 20	4.9596 ± 0.4
Microemulsion	–	96 ± 5	0.0088 ± 0.0005
Submicron emulsion	–	68 ± 4	0.0025 ± 0.0003

intact DNAzyme chains were detected. In contrast, the degradation of DNAzyme encapsulated in the O/W and W/O/W emulsion was delayed significantly. DNAzyme enclosed in the W/O emulsion completely survived the treatment with DNase I. It could be assumed that the high hydrophobicity of the formulation and a potent encapsulation is responsible for this result. With respect to the DNAzyme encapsulated in the inner water phase of the W/O/W emulsion, 50–60% was well protected against degradation. This protection is the result of efficient DNAzyme encapsulation in the inner water phase, and consequently, the DNase I has no direct access to the DNAzyme. This observation was verified by treating a W/O/W emulsion containing DNAzyme in the outer water phase with DNase I. In this instance, DNAzymes were completely degraded. The differences between both W/O/W formulations are probably associated with the DNase activity in the presence of different salts. Melgar and Goldthwait (1968) showed that monovalent Na^+ ions have an inhibitory effect on DNase I activity, whereas additional Mg^{2+} ions could increase the activity. However, Mg^{2+} ions are an important co-factor for DNAzymes and therefore are preferred additives for formulations (Feldman et al., 2006). Furthermore, Mg^{2+} ions decrease DNase II activity, which primarily takes place in the epidermis (Murai et al., 1980) and are known for their stabilising effect on eczematous skin (Denda et al., 1999). Regarding the influence of native porcine skin tissue on the degradation of DNAzyme, a similar protective effect is expected due to a much lower neutral and acid deoxyribonuclease activity compared to the DNase I activity.

However, it must be kept in mind that the prevalent conditions during this experiment obviously differ from those that are prevalent during real circumstances where the formulation is applied on the skin. During dermal application, the behaviour of the individual delivery system can also affect the degradation of the DNAzyme.

Table 8
Degradation of DNAzyme incorporated into different delivery systems (mean ± S.D., $n = 3$).

Formulation	Degraded DNAzyme [%]
W/O/W with NaCl	39.5 ± 5.2
W/O/W with $MgSO_4$	49.1 ± 9.8
W/O/W with $MgSO_4$ (DNAzyme in the outer water phase)	100.0
W/O emulsion	2.3 ± 1.1
Microemulsion	100.0
Submicron emulsion	100.0

4. Conclusion

In the present work, the degradation of 10–23 DNAzymes using an AE-HPLC method was investigated. It was demonstrated that the DNAzyme was degraded by endonucleases as well as by exonucleases. The degree of degradation depends on the activity as well as on the period of application of the nucleases. However, using porcine skin tissue nucleases, a predominant exonucleotic mechanism of degradation was discovered. The activity of acid skin nucleases was three times higher compared to neutral nucleases.

The present investigation was primarily designed to determine the protection of DNAzyme by different emulsion types against the breakdown by nucleases from skin tissue. Using DNase I as an equivalent for the skin's own DNases, the protective character of W/O and W/O/W emulsions compared to a submicron emulsion and a microemulsion was demonstrated. It was shown, that despite of a high level of nucleases activity, encapsulation of DNAzyme into the inner aqueous phase could warrant the DNAzyme stability and the resistance DNase. Thus, W/O and W/O/W emulsions were found to be the most promising emulsion systems for the dermal application of DNAzyme and other oligodeoxynucleotides. Nevertheless, skin permeation and penetration tests of topically applied DNAzyme have to be done to determinate the most appropriate drug delivery system for DNAzymes.

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