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Development of a protective dermal drug delivery system for therapeutic DNAzymes

Kay Marquardt [a,*], Anna-Carola Eicher [a], Dorota Dobler [a], Ulf Mäder [b], Thomas Schmidts [a], Harald Renz [c], Frank Runkel [a]

[a] Institute of Bioprocess Engineering and Pharmaceutical Technology, University of Applied Sciences Mittelhessen, Wiesenstrasse 14, 35390 Giessen, Germany
[b] Institute of Medical Physics and Radiation Protection, University of Applied Sciences Mittelhessen, Wiesenstrasse 14, 35390 Giessen, Germany
[c] Institute of Laboratory Medicine and Pathobiochemistry, Molecular Diagnostic, Baldinger Strasse, 35043 Marburg, Philipps-University Marburg, Germany

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ABSTRACT

RNA-cleaving DNAzymes are a potential novel class of nucleic acid-based active pharmaceutical ingredients (API). However, developing an appropriate drug delivery system (DDS) that achieves high bioavailability is challenging. Especially in a dermal application, DNAzymes have to overcome physiological barriers composed of penetration barriers and degrading enzymes. The focus of the present study was the development of a protective and penetration-enhanced dermal DDS that was tailor made for DNAzymes. DNAzyme Dz13 was used as a potential API for topical therapy against actinic keratosis. In the progress of development and selection, different preservatives, submicron emulsions (SMEs) and the physiological pH range were validated with respect to the API's integrity. A physicochemical stable SME of a pharmaceutical grade along with a high API integrity was achieved. Additionally, two developed protective systems, consisting of a liposomal formulation or chitosan-polyplexes, reduced the degradation of Dz13 in vitro. A combination of SME and polyplexes was finally validated at the skin and cellular level by in vitro model systems. Properties of penetration, degradation and distribution were determined. The result was enhanced skin penetration efficiency and increased cellular uptake with a high protective efficiency for DNAzymes due to the developed protective DDS.

1. Introduction

DNAzymes represent a novel class of nucleic acid-based potentially active pharmaceutical ingredients (API). The synthetic molecules can be grouped into different subclasses. The molecules of the 10–23 DNAzyme subclass consist of a single DNA strand that can be divided into a conserved catalytic domain and two flanking binding domains (Breaker and Joyce, 1994; Santoro and Joyce, 1997). The catalytic domain is able to cleave the transcripts of gene expressions (post-transcriptional silencing). Variations of the binding domain can specifically address different pathogen gene expression patterns, resulting in a high number of potential therapeutic approaches (Cairns et al., 1999; Tan et al., 2009). Promising approaches have been made towards developing therapies for different skin diseases, for instance, atopic dermatitis or basal cell and squamous cell carcinomas (Cai et al., 2012; Schmidts et al., 2012).

A new dermally applied API could be DNAzyme Dz13. Dz13 is a typical representative of the 10–23 DNAzymes with hydrophilic properties, a high molecular weight (MW) of 10.6 kDa and a negative charge (Khachigian et al., 2002; Santoro and Joyce, 1997). Studies revealed that Dz13 can inhibit the translation of the overexpressed protein c-Jun consequently suppressing the growth of squamous and basal cell carcinomas (SCC and BCC) (Cai et al., 2012; Cho et al., 2013; Zhang et al., 2004). Similar results are expected for the treatment of actinic keratosis, a precursor form of

* Corresponding author. Tel.: +49 641 309 2630; fax: +49 641 309 2553.
E-mail address: Kay.Marquardt@kmub.thm.de (K. Marquardt).

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SCC and BCC (Criscione et al., 2009). In this context, Dz13 was chosen as a promising API candidate for topical treatment of actinic keratosis.

The target of Dz13 is the messenger RNA of c-Jun in the cytoplasm of keratinocytes located within the outer skin layers. Dermally applied Dz13 is at the site of action but is separated by multiple physiological barriers compromising therapeutic outcomes. The stratum corneum is the outermost skin layer and forms a first lipid barrier (Jepps et al., 2013), restraining DNAzymes from penetration. The second main barrier of the skin is enzymatically active. Enzymes of exogene and endogene origin on the surface and in the skin can irreversibly degrade DNA (reviewed by Eckhart et al., 2012). If DNAzyme overcomes the two barriers, a high bioavailability in the top skin layers will be helpful to sufficiently internalize Dz13 into cells. A variety of approaches may be possible to pass through the crucial barriers, despite the challenging properties of DNAzymes, including the following strategies. (i) Enhancing the permeability of the stratum corneum by hydration or disruption. In this setting, using penetration enhancers, for instance propylene glycol, oleic acid or even whole galenic formulations, such as submicron emulsions (SME), could be possible (Mohammed et al., 2014; Ongpipattanakul et al., 1991). SMEs have excellent penetration enhancement properties due to their compositions and nano-scaled droplet size, which have already shown an ability to transport and accumulate a DNAzyme into the first skin layers (Amselem and Friedman, 1998; Schmidts et al., 2012). (ii) Maintaining the integrity of DNAzymes by using protective systems. For example, the systems could consist of liposomal formulations or chitosan-polyplexes that could counteract the degradation of DNAzymes by encapsulation or complexation, respectively (Richardson et al., 1999; Semple et al., 2001). Furthermore, an advantage of both systems could be that they enhance cellular uptake. It is expected that a combination of the approaches in one protective drug delivery system (DDS) will increase the topical bioavailability of DNAzymes, resulting in an increase in therapeutic efficiency (Elsabahy et al., 2011; Zhu and Mahato, 2010).

The present study focused on an advancement towards a protective DDS for the dermally applied Dz13. In preliminary examinations, the compatibility and suitability of different preservatives and pH range with Dz13 was investigated. Subsequently, a SME with an appropriate preservation was developed to enhance the penetration of the API. The SME was investigated in terms of physicochemical stability and the integrity of Dz13. To protect the DNAzyme against degradation, a liposomal formulation and chitosan-polyplexes were developed and validated. Finally, a combination of the SME and polyplexes generated a novel protective DDS. Aspects of penetration, degradation and distribution with and without the protective DDS were determined on the skin and cellular level with in vitro model systems.

2. Materials and methods

2.1. Materials

Triethylammonium acetate and phenol–chloroform–isoamyl alcohol were provided by Applichem Lifescience (Germany). Phosphatidylcholin and phosphatidylglycerol were thankfully provided by Lipoid (Germany). Deoxyribonuclease I from bovine pancreas, 10x TAE buffer, and agarose were purchased from Sigma–Aldrich (USA). DNAzyme Dz13 sodium salt was synthesized by Integrated DNA Technologies (Belgium). Dz13 consists of 34 deoxyribonucleotides including an inverted thymidine with a MW of 10.6 kDa (Khachigian et al., 2002). Especially for fluorescence analytics, a 5'FAM-labelled DNAzyme (FAM-DNAzyme) with similar characteristics was synthesized by Biospring (Germany). The fluorescence-labelled FAM-DNAzyme (MW: 10.6 kDa) consists of 34 deoxyribonucleotides and an inverted thymidine (Turowska et al., 2013). In addition, SYBR Gold nucleic acid gel stain was bought from Life Technologies (Germany). PBS buffer with and without Mg^{2+} and Ca^{2+}, Dulbecco's modified eagle's medium, fetal bovine serum, and trypsin/EDTA solution were purchased from Biochrom AG (Germany). The human keratinocyte cell line HaCaT was kindly provided by Prof. Dr. Weindl of the Institute of Pharmacy (Pharmacology and Toxicology), Freie Universität Berlin, Germany. The following ingredients were obtained at pharmaceutical grade. Phenoxy ethanol and benzoic acid were purchased from FrankenChemie (Germany) and OTC Pharma (Germany), respectively. Glycerol, magnesium sulphate were purchased from Fagron (Germany). Glyceryl stearate, stearyl alcohol and ceteareth-20 were supplied by Evonik (Germany). Benzyl alcohol, methylparaben, propylparaben, propylene glycol, laureth-12 and caprylic/capric triglyceride were bought from Caelo (Germany). Cetearyl isononanoate, oleyl oleate and chitosan (Chitopharm S) were provided by Cognis (Germany). The chitosan has a MW range of 50–1000 kDa and a degree of deacetylation of 70%. The following ingredients were obtained at HPLC grade. Acetonitrile, ethanol, sodium perchlorate, hydrochloric acid, sodium hydroxide, potassium dihydrogen phosphate, disodium hydrogen phosphate and sodium acetate were supplied by VWR BDH Prolabo (Germany).

2.2. DNAzyme analytics

For the present examinations, Dz13 and the alternative 5'FAM-labelled DNAzyme (FAM-DNAzyme) were analysed by the following four different methods.

(i) The quality and quantity of Dz13 and the FAM-DNAzyme were analysed by using anionic exchange high pressure liquid chromatography (AEX-HPLC). Prior to analysis, samples were purified using phenol-chloroform extraction and an ethanol-sodium acetate precipitation. The purified samples were analysed with a DNAPac PA200 column (Thermo Fisher Scientific, Germany). The HPLC system was a LaChrom Elite system with a fluorescence detector and UV–vis (VWR-HITACHI, Germany). The UV–vis detector wavelength was set to 260 nm. Calibration detector was performed between 15 and 200 μg/mL ($r^2 = 0.999$) with a limit of quantification (LOQ) of 3.7 μg/mL and a limit of detection (LOD) of 11.4 μg/mL. The fluorescence signal was measured using a 490 nm excitation wavelength and 520 nm emission wavelength. The calibration curve was established in a range from 4 to 40 μg/mL ($r^2 = 0.999$). The LOQ and LOD can be specified with 1.0 μg/mL and 0.3 μg/mL, respectively. The equilibration buffer consisted of 20% acetonitrile and 4% triethylammonium acetate (pH 7.0), while the elution buffer also included 0.2 M acetate sodium perchlorate. The AEX-11;HPLC system was performed in gradient mode, starting with 45% elution buffer and, after 18 min, ending with 100% elution buffer. The flow rate was 1 mL/min at a temperature of 60 °C. The EZChrome Elite Software was used for peak integration. The quantification was performed using a standard calibration curve at the investigated concentration range and above the quantification limit.

(ii) The quantities of intact Dz13 and FAM-DNAzyme were measured by hybridization-ELISA as previously described (Schmidts et al., 2012). Hybridization probes were adjusted to Dz13 or FAM-DNAzyme, and the substrate was exchanged to ABTS solution.

(iii) FAM-DNAzyme was detected using confocal laser-scanning microscopy (CLSM) using the TCS SP5CLSM (Leica, Germany). To distinguish between the specific fluorescence of the dye and

autofluorescence of skin specimens or cell extracts, a spectral analysis of each pixel was performed according to Maeder et al. (2012). The method was adapted to the FAM dye. Therefore, the excitation wavelength and emission wavelength spectrum were set to 488 nm and 500–600 nm, respectively. The method-specific *p*-value was set to 0.85. After identifying the dye, the specific intensity was summed and is presented as a mean intensity per summed pixel ± SD.

(iv) The fluorescences of FAM-DNAzyme in the HaCaT keratinocytes were detected using flow cytometry (guava easyCyte 5HT, EMD Millipore, Germany) and analysed using corresponding software (GuavaSoft 2.5). After 5000 events per sample, the cell population was defined in the forward-scattering and sideward-scattering diagram. The fluorescence emission of 488 nm excitation wavelength of the gated cell population was used to identify FAM-DNAzyme positive cells. The threshold was adjusted to obtain the lowest signal of the medium control.

2.3. Preparation of drug delivery systems

2.3.1. Submicron emulsion

The SMEs were produced by high-energy emulsification. The blend of the emulsifiers of the SME without preservatives was previously optimized regarding most thermo-stable droplet size. The SMEs were manufactured in triplicate with and without Dz13 according to Table 1 and each was conserved using different preservatives analogous to Table 2. In the case of the parabens, a blend of methylparaben and propylparaben was used. The aqueous phase and oil phase of the SME were separately heated to 65 °C until all ingredients were dissolved. The aqueous phase was added to the oil phase and homogenized for 2 min at 24,000 rpm by a rotor/stator homogenizer (D-15 homogenizer with DS-20/PF-EMR, MICCRA, Germany).

2.4. Preparation of protective systems

2.4.1. Liposomes

Liposomes were manufactured using a combination of the lipid film method and high pressure homogenization. A mixture of 4 wt % phosphatidylcholine and 1 wt% phosphatidylglycerol were dissolved in chloroform in a round bottom flask. The organic solvent was completely removed using rotary evaporation (BUCHI, Germany). Dz13 was encapsulated using the passive loading technique. For that purpose, the remaining thin lipid film was hydrated with a 0.01 M Sorensen's buffer and 4 mg/mL Dz13 at 40 °C resulting in large multilamellar vesicles (LMV). The LMVs were homogenized in a high pressure homogenizer (EmulsiFlex-C5, AVESTIN, Germany). The homogenization process was performed for 30 cycles at 40 °C and 1000 bar. A control group (spiked liposomes) was produced without Dz13. Therefore, Dz13 was spiked to the formulation to a final concentration of 4 mg/mL after liposome manufacturing process.

Table 1
Composition of the submicron emulsion.

Ingredient	wt%
Aqua	ad 100
DNAzyme	0.40
Caprylic/capric triglyceride	10.0
Cetearyl isononanoate	6.00
Ceteareth-20	3.85
Glycerol 85%	3.00
Glyceryl stearate	0.65
Laureth-12	1.00
Magnesium sulphate	0.30
Oleyl oleate	4.00
Preservatives	[a]
Stearyl alcohol	1.00

[a] For preservatives' concentration, see Table 2.

Table 2
Concentration of the preservatives.

Preservatives	wt%
Benzoic acid	0.10
Benzyl alcohol	1.00
Phenoxy ethanol	1.00
Propylene glycol	20.0
Parabens	
Methylparaben	0.15
Propylparaben	0.05

2.4.2. Chitosan-DNAzyme polyplexes

Chitosan-DNAzyme polyplexes were prepared in the following manner. Chitosan was dissolved in 1 M sodium acetate buffer with a pH value of 5.0. A concentrated, aqueous DNAzyme solution was added to the chitosan solution to a final DNAzyme concentration of 4 mg/mL. The final chitosan concentrations were in a range between 0.1 and 2 mg/mL equal to a N/P ratio of 0.035 and 0.7. A stepwise mixing of both solutions at 25 °C and 1300 rpm in a Thermomixer (Eppendorf, Germany) formed coarsely dispersed polyplexes.

2.5. Preparation of a protective drug delivery system

To generate a protective DDS, the developed SMEs and polyplexes were combined. Placebo SMEs with preservatives were manufactured according to Section 2.3.1. The polyplexes with DNAzymes were produced as described in Section 2.4.2 and dried overnight. The dried polyplexes were resuspended in the SMEs. Final concentrations of the DNAzymes and chitosan in the protective drug delivery system were 4 mg/mL and 2 mg/mL, respectively.

2.6. Stability of DNAzymes

The influence of different matrices on the integrity of DNAzymes was investigated. Therefore, Dz13 was incubated at a final concentration of 4 mg/mL with the following matrices: (i) Aqueous solutions were in a range of the physiologic pH values. The pH values from 5 to 7 in 0.5 steps were maintained by a 67 mM Sorensen's buffer. (ii) Aqueous solutions with different preservatives were used. The preservatives and their concentrations were used according to Table 2. (iii) SMEs were used with different preservatives. SMEs were produced as described in Section 2.3.1. All samples were selectively incubated at 8 °C, 25 °C at a 60% relative humidity (RH) or 40 °C with 75% RH. Each condition was performed in triplicate and analysed at fixed time intervals over a period of 3 months. Dz13 was qualified and quantified by AEX-HPLC. The data are presented as the mean with SD relative to the mean after 1 day.

2.7. Characterization of drug delivery systems and protective systems

The characterization of the DDSs and protective systems was selectively performed with regard to physicochemical properties, sufficient preservation and complexation efficiency. The physicochemical properties were determined as described in the following steps. The mean droplet size and droplet size distribution of diluted SMEs or liposomes were analysed using dynamic light

scattering (DLS) with the Zetasizer Nano Z (Malvern, UK). The droplet size and droplet distribution were presented as the z-average size (z-average) and polydispersity index (PDI), respectively. The pH value was monitored with a pH probe (FE20, Mettler-Toledo, Germany) or pH indicator strips (pH-Fix 2.0–9.0, MACHEREY-NAGEL, Germany), depending on the sample volume. Viscosity measurements were conducted at 25 °C with a cone and plate geometry of 2.2 cm in diameter and 2° cone angle (RheoStress 300, ThermoHaake, Germany). The share rate ramp was 0.1–100/s. The test of sufficient preservation was restricted to placebo SMEs. The preservation efficiency tests were conducted according to the European Pharmacopoeia using the microbial strains *Staphylococcus aureus*,*Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* (Council of Europe, 2013). The test of efficient complexation was restricted to polyplexes. A gel retardation assay was performed to visualize the degree of complexation. A 1.5% agarose gel was run in a Tris-acetate-EDTA (TAE) buffer (pH 8.3) for 1 h at 100 V (EV 200, Consort bvba, Belgium), and Dz13 was stained with 1x SYBR Gold. Each sample in the gel contained approximately 10 ng of Dz13 premixed with loading buffer. Furthermore, the degree of complexation was quantified by AEX-HPLC. The samples were centrifuged for 5 s at 13,000 rpm, and the recovery of Dz13 in the supernatant was analysed. To analyse Dz13, the polyplexes were decomplexed by adding sodium hydroxide to the samples until obtaining a final molarity of 1 M. All experiments were performed in triplicates and are presented as the mean with SD.

2.8. DNAzyme degradation assay

The degradation of Dz13 was investigated with deoxyribonuclease I (DNase I, EC 3.1.21.1). A 0.45 M Sorensen's buffer (pH 7.4) with a 10 mM magnesium sulphate was produced. The DNase I was diluted with Sorensen's buffer to a concentration of 400 units/mL and stored on ice until use. The different samples including 4 mg/mL Dz13 were incubated at a ratio 1:2 with the DNase I solution. The incubation parameters were 25 °C at 300 rpm for 0.5, 1 or 2 h in a Thermomixer (Eppendorf, Germany). The degrading reaction was immediately stopped by adding phenol-chloroform solution following an extraction and ethanol-sodium acetate precipitation. Negative control groups of each sample were equally treated without adding DNase I. The purified samples were analysed by AEX-2011;HPLC, allowing a qualitative and quantitative measurement of the degradation of Dz13. Each sample was tested in triplicate, and the data are presented as the mean with SD.

2.9. In vitro studies

2.9.1. Skin penetration study

The in vitro penetration study was performed in a Franz diffusion cell with fresh and intact porcine skin (*sus scrofa domestica*) in accordance with OECD guidelines and as previously described (OECD, 2004a,b,b; Schmidts et al., 2012). Infinite doses (0.5 mL) of 4 mg/mL Dz13 and FAM-DNAzyme at a 9:1 ratio were applied. After 24 h, the penetration study was stopped and analysed by multiple methods. The remaining formulation in the donor chamber was completely washed off the skin and was analysed using AEX-HPLC. Dz13 and the FAM-DNAzyme were detected simultaneously. A skin area of 0.5 cm^2 was snap frozen and vertically sliced in 15 specimens using the Leica CM 1850UV cryostat (Leica, Germany) and analysed by CLSM. The remaining skin (1.26 cm^2) was analysed using hybridization-ELISA. For this purpose, the skin was lysed by proteinase K, and the amount of intact Dz13 was quantified using specific probes. Each sample was tested in 5 Franz diffusion cells. The data are presented as the means with SDs.

2.9.2. Cell uptake study

HaCaT cells were cultivated in 24-well plates (2 cm^2/well) in DMEM medium with 10% FBS at 37 °C, 5% CO_2. At a confluence level of more than 75%, the cell monolayer was washed with PBS. The cells were treated with different media (0.5 mL). For this purpose, the total amount of Dz13 was replaced by the FAM-DNAzyme at a final concentration of 1 μg/cm^2. The FAM-DNAzyme was complexed with chitosan to polyplexes (N/P ratio: 0.7) and diluted with DMEM ($n = 18$). Control groups consisted of FAM-DNAzyme without chitosan ($n = 18$), chitosan itself ($n = 6$) or medium only ($n = 6$). After 24 h, the cell monolayer was washed twice with PBS, and the distribution of the FAM-DNAzyme was monitored by CLSM. Therefore, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) and Alexa Fluor 555 Phalloidin. Additionally, trypsinized cell suspension was analysed by flow cytometry. The cell extract of 9 wells were pooled to 3 samples, lysed with Proteinase K, and analysed by hybridization-ELISA.

2.9.3. Statistics

Statistical analysis was performed with OriginPro 8. To determine significant differences in the mean of families the one-way Analysis of Variance (ANOVA) was performed. The homogeneity of variance was tested with the Levene's test. As a post hoc test the Bonferroni analysis was used. $p < 0.05$ was considered as significant.

3. Results and discussion

3.1. Stability of DNAzymes

In preliminary investigations, the influence of different matrices on the Dz13's integrity was investigated. Matrices or ingredients could interact with the API or trigger degradation, resulting in decreased therapeutic activity. Dz13 was tested over a pH range similar to the physiologic gradient of the skin (Wagner et al., 2003) and conventional for a final DDS. No different alteration in comparison with each pH value could be observed (data not shown). Especially, no degradation of the DNA's phosphodiester bonds by acid hydrolysis was monitored, which would be one degradation pathway. In a further analysis, appropriate preservatives and their concentrations were selected according to the German Pharmaceutical Codex. The integrity of Dz13 was unaffected by the tested preservatives with the exception of benzoic acid (Table 3). Samples with benzoic acid continually lost Dz13 recovery. An interaction between DNAzyme and benzoic acid is conceivable because Zhang and Ma, (2013) discovered that the sodium salt of benzoic acid is able to interact with DNA by hydrophobic and hydrogen binding. This interaction can change the retention time of Dz13 in the AEX-HPLC analysis, which led to a decreased detection of the DNAzyme. As a result, all tested preservatives, except benzoic acid, can be used for further formulations containing Dz13.

Table 3
Dz13 recovery from aqueous solutions with different preservatives after 3 months. The means of triplicate samples with SDs relative to the corresponding means after 1 day. O, not performed.

	DNAzyme recovery (%)		
Preservatives	8 °C	25 °C	40 °C
Without	98 ± 1	103 ± 3	101 ± 4
Benzoic acid	O	58 ± 1	O
Parabens	O	101 ± 3	O
Benzyl alcohol	O	100 ± 3	O
Phenoxy ethanol	100 ± 1	100 ± 3	107 ± 4
Propylene glycol	97 ± 1	101 ± 5	101 ± 2

Table 4
Physicochemical properties of SMEs with different preservatives with or without Dz13 measured by DLS, pH indicators or a pH probe (mean ± SD, $n = 3$). PE test, preservative efficacy test; O, not performed.

		SME without DNAzyme				SME with DNAzyme		
Preservative		pH	z-average (d.nm)	PDI	PE test	pH	z-average (d.nm)	PDI
Without	1 day	6.7	303 ± 29	0.3 ± 0.1	O	O	O	O
	1 month	7.0	223 ± 2	0.1 ± 0.0				
	3 months	7.0	223 ± 1	0.1 ± 0.0				
Parabens	1 day	5.7	243 ± 8	0.1 ± 0.0	Fail	6.2	258 ± 38	0.1 ± 0.1
	1 month	6.2	315 ± 19	0.2 ± 0.0		6.2	305 ± 82	0.2 ± 0.1
	3 months	6.0	300 ± 13	0.1 ± 0.1		6.3	286 ± 70	0.2 ± 0.0
Benzyl alcohol	1 day	6.6	243 ± 12	0.1 ± 0.1	Fail	7.0	268 ± 35	0.1 ± 0.1
	1 moth	7.0	326 ± 7	0.2 ± 0.0		7.0	267 ± 30	0.1 ± 0.1
	3 months	7.0	288 ± 14	0.2 ± 0.0		7.0	259 ± 30	0.1 ± 0.0
Phenoxy ethanol	1 day	6.8	261 ± 5	0.1 ± 0.0	Fail	6.8	330 ± 13	0.3 ± 0.1
	1 month	7.0	334 ± 8	0.3 ± 0.1		6.8	347 ± 15	0.2 ± 0.1
	3 months	7.0	319 ± 21	0.1 ± 0.1		6.8	324 ± 2	0.2 ± 0.1
Propylene glycol	1 day	6.7	225 ± 1	0.1 ± 0.1	Pass	7.0	223 ± 10	0.1 ± 0.0
	1 month	7.0	255 ± 0	0.1 ± 0.0		7.0	235 ± 28	0.1 ± 0.1
	3 months	7.0	249 ± 3	0.2 ± 0.1		7.0	211 ± 35	0.1 ± 0.1

The SME formulations of Section 2.3.1 (except benzoic acid) were chosen as the DDS. The integrity of Dz13 in all SMEs was over a range of 100 ± 12% after a period of 3 months. Thus, the ingredients of the particular SME did not affect the stability of Dz13.

3.2. Development of a drug delivery system

The SME as a promising DDS was further characterized. Therefore, a selection of compatible preservatives was added to the basic SME formulation according to Section 2.3.1. The stability of the DDSs were monitored with regard to physicochemical properties (Table 4). The droplet size of the formulation without preservatives and Dz13 was approximately 300 nm and decreased slightly within the first weeks of storage. The addition of preservatives and Dz13 did not significantly influence the droplet size and droplet size distribution ($p < 0.05$). Furthermore, the parameters of all SMEs did not change significantly with $p < 0.05$ over the period of time. The viscosity of most SMEs was in a range of 21 ± 7.6 mPa s. SMEs containing propylene glycol had the highest viscosity of 46.5 ± 8.7 mPa s. The preservative efficacy test revealed sufficient preservation only by the SMEs that contained propylene glycol. Overall, the SME represents an appropriate DDS for DNAzymes due to the physicochemical stability along with the compatibility with Dz13.

3.3. Development and validation of the protective systems

3.3.1. Liposomes

Liposomes are able to encapsulate hydrophilic APIs into the aqueous inner phase. A lipid bilayer separated the inner phase from the external phase, resulting in a protection against degrading enzymes located in the external phase. Therefore, a liposomal formulation with DNAzymes was produced. The incorporation of Dz13 into the liposomal formulation increased the liposome size (z-average) from 36 ± 2 nm to 52 ± 12 nm. However, the droplet size distribution of both formulations remained similar with a PDI of 0.4. Encapsulating DNAzymes into liposomes is challenging using the passive loading technique, especially at high API concentrations and small liposome sizes (Xu et al., 2012a). Purifying the liposomes by diafiltration determined a Dz13 encapsulation efficiency of less than 6% (data not shown). A theoretical encapsulation efficiency of 9.2% based on the calculation by Xu et al. (2012b) affirmed the result. Therefore, the majority of the DNAzymes were present in the external aqueous phase and unprotected towards DNase activity. In the DNAzyme degradation assay with DNase I, the liposomal formulation was validated to be suitable as a potential protective system. The liposomal formulation with encapsulated Dz13 was compared to a liposomal formulation with spiked Dz13 after the liposome formation (Fig. 1). The formulation with encapsulated Dz13 was significantly more protective than the liposomal formulation with spiked Dz13 and an aqueous solution ($p < 0.05$). After 2 h, Dz13 in the aqueous solution was completely degraded, while 8.7 ± 0.8% and 5.0 ± 0.5% of Dz13 in the encapsulated and spiked formulation, respectively, was intact. Although the DNAzyme of the spiked formulation was not encapsulated, protective properties were monitored. This might be explainable by the observations by Gregoriadis et al. (1996). The group described that DNA was able to adsorb onto similar liposomal lipids and thus protected against degradation to a certain extent (Gregoriadis et al., 1996). Nevertheless, the recovery of Dz13 from the liposomal formulations was reduced over the time, indicating that the majority of the DNAzyme was insufficiently protected.

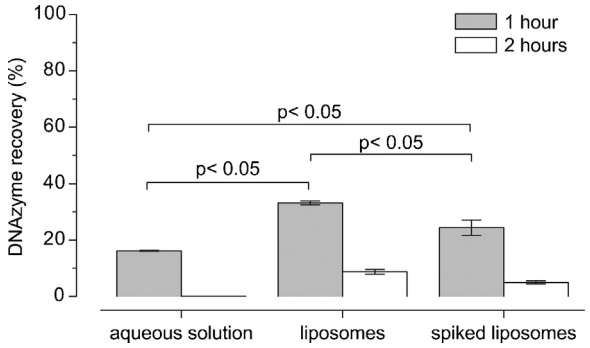

Fig. 1. DNAzyme degradation assay with liposomal formulations. Comparison of liposomes directly manufactured with Dz13 versus liposomes spiked with Dz13 after liposome formation. The Dz13 was analysed by AEX-HPLC (mean ± SD, $n = 3$). Significant differences were calculated by one-way ANOVA following a Bonfferroni test ($p < 0.05$).

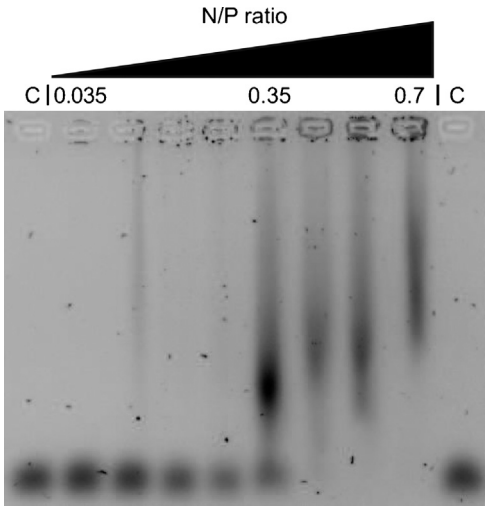

Fig. 2. Gel retardation assay with polyplexes at different N/P ratios. A fixed Dz13 concentration was complexed with different concentrations of chitosan resulting in different N/P ratios. The samples were analysed by gel electrophoresis. Lane C: Dz13 without chitosan.

3.3.2. Chitosan-DNAzyme polyplexes

The complexation between DNAzyme and cationic chitosan to polyplexes spontaneously occurs by electrostatic attraction (Dautzenberg and Jaeger, 2002). The resulting close bond may be able to shield the DNAzyme against enzymatic degradation. Investigation in the present study revealed that the properties of the chitosan-DNAzyme polyplexes depended on the ratio of chitosan's amino groups to DNAzyme phosphate groups (N/P ratio). The stability of the polyplexes increased with higher N/P ratios. Stable polyplexes had the ability to cover the negative charge of Dz13 as visualized by a retarded gel mobility in the electric field (Fig. 2). In contrast, Dz13 without chitosan or decomplexed Dz13 (sample not shown) had an unhindered run length.

The degree of complexation of the polyplexes was measured by AEX-HPLC (Fig. 3A). Using this method, free Dz13 in the supernatant of polyplex samples was detectable, while complexed Dz13 was not detected. Therefore, the degree of complexation was reciprocally proportional to the recovery of the free DNAzyme. An increased amount of chitosan did increase the degree of complexation. At a N/P ratio above 0.61, maximal complexation efficiency was achieved. The protective properties of the polyplexes for DNAzymes were further tested in the DNAzyme degradation assay. The recovery of Dz13 was monitored at multiple time points (Fig. 3B). At a N/P ratio of 0.7, no time-dependent degradation of Dz13 was observed, and a maximal integrity of $81 \pm 2\%$ was maintained. The observed loss might be due to partial exposure of outer complexed Dz13 to DNase activity. A correlation between degradation and degree of complexation after 2 h of DNAzyme degradation assay could be generated (Fig. 3C). The correlation demonstrated that polyplexes with a high N/P ratio represent sufficient protective systems for DNAzymes.

3.4. Protective drug delivery system

Compared to the liposomal formulation, the polyplex samples (N/P ratio: 0.7) provided a significant higher and time-independent protective efficiency. Therefore, the promising polyplexes were incorporated into a SME containing propylene glycol to generate a protective DDS. Propylene glycol was selected as a compatible agent with sufficient preservative activity. The polyplexes maintained the degree of complexation ($97 \pm 2\%$) while also incorporating into the protective DDS. The protective property of the system was finally validated by the DNAzyme degradation assay (Table 5). A high DNase concentration was subjected to the samples to maximise the degrading stress and exceed the activity of human skin (Reimer et al., 1978). At that concentration, unprotected Dz13 was almost fully degraded in every sample after 2 h. In contrast, the protective DDS obtained significant higher protection efficiency ($p < 0.05$). The complexation of Dz13 increased the protective efficiency similar to the previous results of Section 3.3.2. However, unlike the polyplexes in aqueous solution,

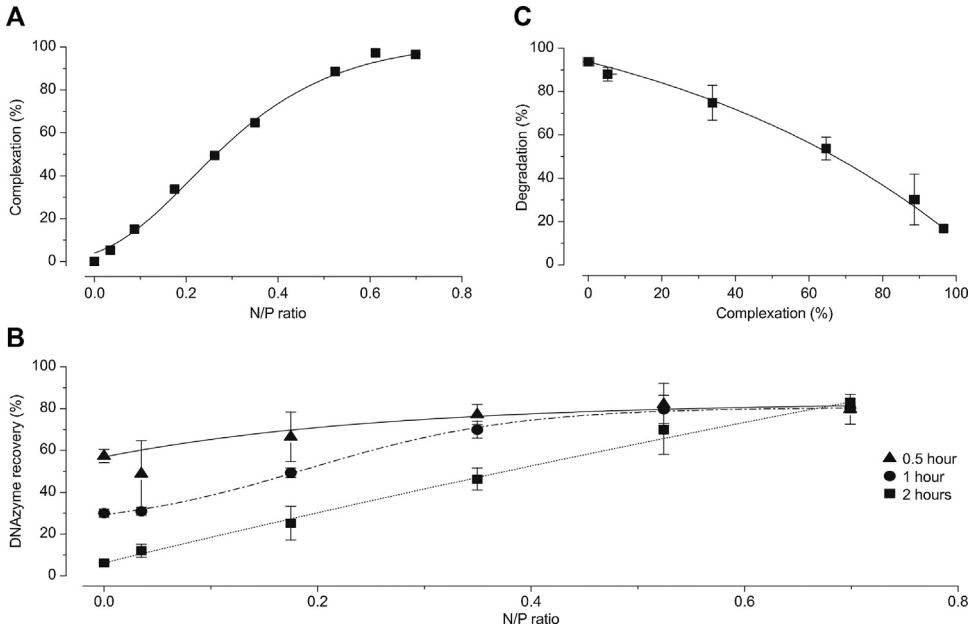

Fig. 3. Polyplex samples with a fixed Dz13 concentration in sodium acetate buffer analysed by AEX-HPLC (mean ± SD, $n = 3$). A: Complexation efficiency between Dz13 and chitosan at different N/P ratios. B: DNAzyme degradation assay with Dz13 in polyplexes at different N/P ratios. C: Correlation between DNAzyme degradation after 2 h and degree of complexation of Dz13 and chitosan.

Table 5
DNAzyme degradation assay of different samples with Dz13 (mean ± SD, $n = 3$). Protective DDS consisted of SME with propylene glycol and polyplexes (N/P: 0.7).

	DNAzyme recovery (%)		
Samples	0.5 h	1.0 h	2.0 h
Aqueous solut.	44 ± 1	22 ± 2	7 ± 0
Acetate buffer	57 ± 3	30 ± 2	6 ± 0
SME	25 ± 1	10 ± 1	2 ± 0
Protective DDS	101 ± 5	84 ± 6	70 ± 3

a time-dependent degradation of Dz13 was observed. The ingredients of the SMEs might led to temporary destabilisation and dissociations of the polyplex compounds. Bordi et al. (2013) observed a similar destabilisation of the electrostatic interaction between chitosan and DNA in the presence of a high ionic strength medium. Furthermore, the efficiency of enzymatic reactions is generally sensitive towards the reaction medium. In the present study, matrix effects of the protective DDS influenced DNase I activity. A decreased pH value by the sodium acetate buffer of polyplex samples (Mori et al., 2001) had an inhibitory effect on the DNAzyme degradation. In contrast, a high magnesium concentration (Pan et al., 1998) of the SMEs increased the enzymatic activity. Nevertheless, the data clearly demonstrated that incorporating the polyplexes into the SME compensated for the SME's lack of a protective characteristic, resulting in a protective DDS. Additionally, the approach using polyplexes could be adaptable for other DDSs where the DNAzyme is unprotected (Schmidts et al., 2011).

3.5. In vitro studies

3.5.1. Skin penetration study

DNAzyme applied with the protective DDS was subjected to a skin penetration study. In this study, aspects of degradation, penetration and distribution were examined using different analytic methods (Fig. 4). AEX-HPLC analysis of the remaining formulation revealed that polyplexes could maintain the integrity of Dz13 on the skin surface. By contrast, the unprotected Dz13 in aqueous solution were nearly fully degraded after 24 h (Fig. 4A). To calculate the distinct degradation of each sample, the ratio between the peak area of the intact Dz13 to the whole peak area of the chromatogram was compared relative to the ratio of HPLC calibration standards. The aqueous solution had a relative recovery of 6 ± 7%, the polyplexes in aqua of 97 ± 1% and the polyplexes in SME of 99 ± 2%. Similar results were observed with the FAM-DNAzyme (data not shown). The reduction of intact DNAzymes in aqueous solution is triggered by commensal bacteria and the skin. The penetration of intact Dz13 into the skin was quantified using hybridization-ELISA. The polyplexes themselves did not positively affect the penetration efficiency compared with the aqueous solution. However, the combination of polyplexes and SME as protective DDS increased the skin uptake from 0.3 ± 0.3 μg/cm² to a value of 1.0 ± 0.5 μg/cm² (Fig. 4B). The CLSM visualised the distribution of FAM-DNAzyme in the skin. The pictures of vertically cross-sectioned skin specimens revealed that the identified fluorescence signal was located within the outermost skin layers (Fig. 4C). However, the CLSM was unable to verify the integrity of the FAM-DNAzyme. Therefore, the quantified and summarized CLSM data indicated a similarly enhanced penetration efficiency of the FAM-DNAzyme from aqueous solution and the protective DDS (Fig. 4D). Degraded FAM-DNAzyme can penetrate with less difficulty into the skin due to a smaller MW, resulting in a false-positive signal. Therefore, the fluorescence analysis can only be utilised if a potential degradation of the fluorescence-labelled molecule is monitored. In conclusion, the protective DDS can protect DNAzyme from degradation on the skin surface and can enhance the penetration of intact DNAzyme into the layers of the skin.

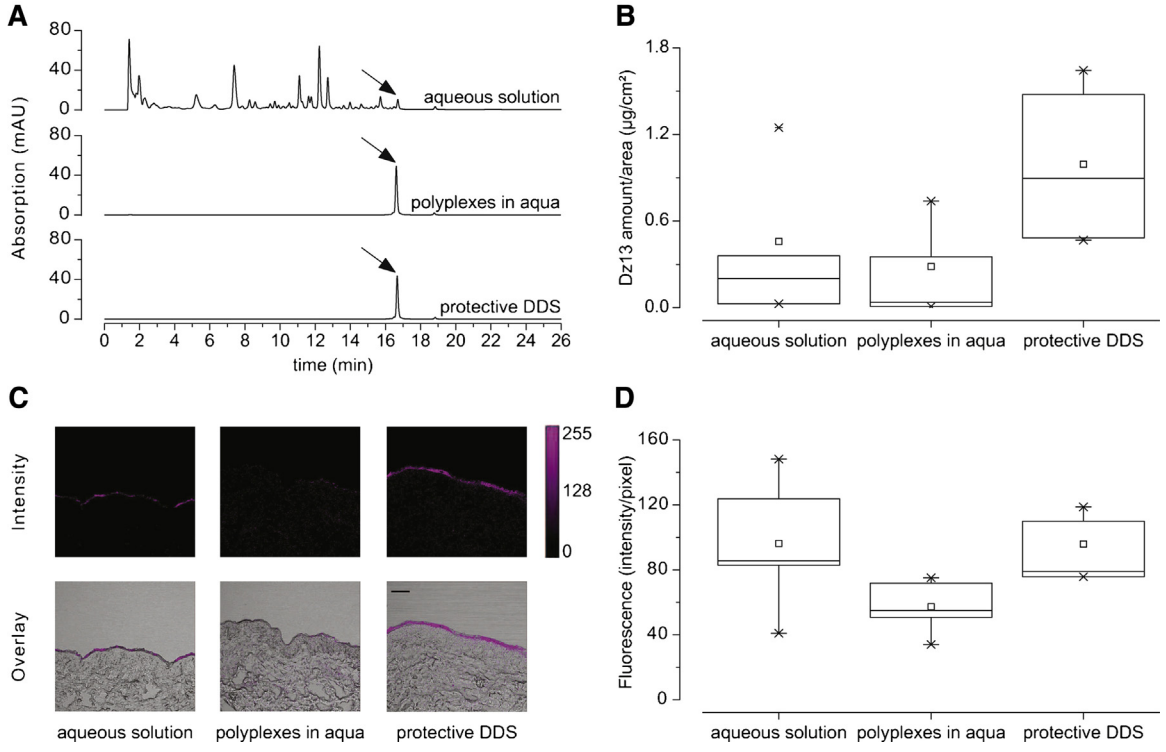

Fig. 4. Penetration study with Dz13 in aqueous solution, polyplexes in aqueous solution or polyplexes in SME. A: AEX-HPLC chromatograms of Dz13 in the applied formulations after the penetration study. Arrows indicate the intact DNAzyme. B: Intact Dz13 recovery in skin samples measured by hybridization-ELISA ($n = 5$). C: CLSM pictures of skin specimens. Spectral analysis (upper picture) and overlay with transmitted light picture (lower picture). The scale bar represents 100 μm. D: Summed data of all spectral analysis CLSM pictures ($n = 5$).

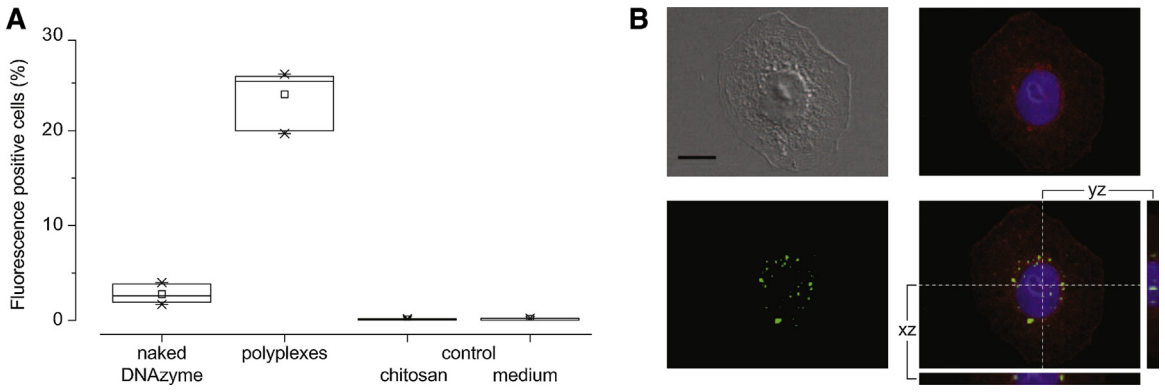

Fig. 5. Cellular uptake of the FAM-DNAzyme with polyplexes into HaCaT keratinocytes after 24 h of incubation. A: Percentage of fluorescence positive cell measured by flow cytometry (controls $n = 3$, samples $n = 9$). B: Pictures of HaCaT keratinocyte incubated with polyplexes. FAM-DNAzyme are represented in green. Nucleic DNA and F-actin filament of the cytoskeletons were stained with DAPI (blue) and Alexa Fluor 555 Phalloidin (red), respectively. The scale bar represents 10 μm. Orthogonal section yz and xz are corresponding to the cross. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5.2. Cell uptake study

On the cellular level, the penetration and distribution of DNAzyme with the polyplexes (N/P: 0.7) were compared to naked DNAzymes with HaCaT keratinocytes. To simulate the cell uptake after penetration into the skin, the concentration of FAM-DNAzyme in the cell medium was adjusted to 1.0 μg/cm^2 (according to Section 3.5.1). The results of the flow cytometry demonstrated an enhanced uptake of FAM-DNAzyme by polyplexes into the keratinocytes (Fig. 5A). The data are in accordance to the summed data of CLSM analytic (data not shown). The results indicated that polyplexes were able to cover anionic properties of DNAzymes (see Section 3.3.2), which might have enhanced the internalization progress. The concentration of the intact FAM-DNAzyme in cells was determined to be 0.12 ± 0.00 μg/cm^2 by hybridization-ELISA. The internalized FAM-DNAzyme was distributed into the cytoplasm at the side of action (Fig. 5B).

4. Conclusion

In the present study, a physicochemical stable SME with sufficient preservation was developed as an appropriate DDS for DNAzyme Dz13. To protect Dz13 against enzymatic degradation, a liposomal formulation and chitosan-DNAzyme polyplexes were developed. In a validation process, the integrity of Dz13 was monitored in different samples in the presence of degrading DNase I. The assay revealed that the protective properties of the liposomes were mainly driven by the lipid ingredients rather than by encapsulating the Dz13. The superior polyplexes indicated that the stability and charge of the complex and the protective characteristic depend on the N/P ratio. The combination of developed SME and the polyplexes was able to compensate for the SME's lack of protection. In general, using the polyplexes may be possible for other DDS with insufficient protection of DNAzymes. The developed protective DDS was examined in in vitro model systems at the skin and cellular level. The protective DDS was able to protect Dz13 and FAM-DNAzyme against degradation on the skin surface. The penetration of the intact Dz13 and FAM-DNAzyme into the skin and subsequently into keratinocytes was enhanced compared with the control samples. Topically applied FAM-DNAzyme was distributed into the outermost layers of skin, and in the case of the cell culture, FAM-DNAzyme was mainly located in the cytoplasm at the site of action. The presented results confirm that the developed protective DDS is an appropriate system for the dermally applied DNAzyme towards a therapy against actinic keratosis. Finally, the protective DDS could be adaptable for other nucleic acid-based APIs.

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