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Development of multiple W/O/W emulsions as dermal carrier system for oligonucleotides: Effect of additives on emulsion stability

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ABSTRACT

Multiple water-in-oil-in-water (W/O/W) emulsions are of major interest as potential skin delivery systems for water-soluble drugs like oligonucleotides due to their distinct encapsulation properties. However, multiple emulsions are highly sensitive in terms of variations of the individual components. The presence of osmotic active ingredients in the inner water phase is crucial for the generation of stable multiple emulsions. In order to stabilize the emulsions the influence of NaCl, $MgSO_4$, glucose and glycine and two cellulose derivatives was investigated. Briefly, multiple W/O/W emulsions using Span 80 as a lipophilic emulsifier and different hydrophilic emulsifiers (PEG-40/50 stearate, steareth-20 and polysorbate 80) were prepared. Stability of the emulsions was analyzed over a period of time using rheological measurements, droplet size observations and conductivity analysis.

In this study we show that additives strongly influence the properties stability of multiple emulsions. By increasing the concentration of the osmotic active ingredients, smaller multiple droplets are formed and the viscosity is significantly increased. The thickening agents resulted in a slightly improved stability.

The most promising emulsions were chosen and further evaluated for their suitability and compatibility to incorporate a DNAzyme oligonucleotide as active pharmaceutical ingredient.

1. Introduction

Multiple water-in-oil-in-water (W/O/W) emulsions represent complex systems consisting of both, water-in-oil (W/O) as well as oil-in-water (O/W) emulsions. For the generation of such a system, a primary W/O emulsion is dispersed into a continuous water phase and stabilized using a hydrophilic emulsifier. Due to their distinct structure and properties, multiple emulsions are of special interest for several drug delivery approaches, including carriers for the dermal application of pharmaceutical drugs in pharmaceutical products (Ferreira et al., 1995; Fukushima et al., 1987; Khopade and Jain, 1999; Lindenstruth and Müller, 2004), cosmetics (Tadros, 1992; Vasudevan and Naser, 2002) and the encapsulation of flavours in food (Garti and Benichou, 2004). However, multiple emulsions are thermodynamically unstable due to the excess of free energy associated with the two present interfaces. Compared to simple emulsions, both interfaces have to be stabilized. Therefore, two different emulsifiers are required: one with a low HLB to stabilize the inner W/O interface combined with a second one exhibiting a high HLB to stabilize the outer O/W interface. Both, the concentration and the chemical structure of the emulsifier strongly influence the properties of the formulation (Geiger et al., 1998; Jager-Lezer et al., 1997; Jiao and Burgess, 2003; Schmidts et al., 2009; Tirnaksiz and Ozlem, 2005). Water migration across the oil membrane is just one among several factors contributing to changes in the emulsion properties and leading to a destabilization process over the time. It has been demonstrated that the addition of electrolytes to the water phase in W/O emulsions results in a stabilizing effect, which can be explained by a counteraction of Laplace pressure (Aronson and Petko, 1993; Koroleva and Yurtov, 2003). Other additives in the aqueous phase, such as proteins, sugars and drugs, can also exert an osmotic effect (Hino et al., 2000; Ueda and Matsumoto, 1991). Stabilization hereby depends on the chosen concentration of the osmotic active ingredient, previously added to the inner water phase. There are several factors which can influence the migration of osmotic active ingredients, e.g. partition coefficient, ionisation, charge density, molecular weight and molecular mobility of the molecule (Sela et al., 1995). Water molecules passing from the outer into the inner water phase due to osmotic disequilibrium can also lead to swelling and potential bursting of inner water droplets (Jager-Lezer et al., 1997). In order to obtain a stable formulation, the concentration of electrolytes has to be high enough to allow for the regulation of Laplace pressure but at the same time suffi-

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ciently low to avoid osmotic effects. Another alternative to improve the stability of multiple emulsions can be achieved by increasing the viscosity of the inner water phase (Omotosho, 1990). Hence, the diffusion of electrolytes and water molecules between both water phases is hampered and modifications of the emulsion occur significantly slower.

DNAzymes of the 10–23 family are single-stranded DNA antisense oligonucleotides that exhibit a direct catalytic activity following binding of a corresponding RNA molecule. By sequence-specific cleavage of mRNAs they can suppress the expression of proteins that are involved in therapeutically relevant pathways, such as inflammatory processes (Breaker and Joyce, 1994) as well as active pharmaceutical ingredients (API) for the therapy of inflammatory diseases of the skin (Sel and Renz, 2008; Wraight and White, 2001). Their potential application in the therapy of dermal diseases is challenging since these molecules are highly sensitive to DNAses which are naturally present in and on the human skin (Santoianni and Rothman, 1961).

In this study, the influence of several modifications of the inner water phase on the properties and stability of multiple emulsions was investigated. Two groups of additives were studied: osmotic active ingredients as glucose, glycine, NaCl and $MgSO_4$, as well as thickening agents derived from cellulose derivatives.

Based on published data on delivery systems for sensitive drugs (Silverman, 2005; Singh et al., 1997) in the inner water phase of multiple emulsions, promising candidates were chosen and evaluated for their potential applicability for DNAzyme oligonucleotides.

2. Materials and methods

2.1. Materials

The oil heavy paraffin was supplied by Fagron (Fagron GmbH Co. KG, Barsbüttel, Germany). The lipophilic surfactant sorbitan monooleate 80 (Span^{TM}80) was kindly provided by Croda GmbH (Kaldenkirchen, Germany). The hydrophilic surfactants polysorbate 80 (Caelo GmbH, Hilden, Germany) as well as PEG 40/50 stearate and steareth-20, both from Croda GmbH, were used. NaCl (Merck, Germany), $MgSO_4 \cdot 7H_2O$, glycine and glucose (Caelo, Germany) were applied as osmolytes in the inner water phase. The thickeners hydroxyethylcellulose and sodium carboxymethyl cellulose were purchased from Caelo GmbH (Hilden, Germany). A 10–23 DNAzyme was generated and kindly provided by sterna biologicals GmbH & Co. KG, Germany, representing the Na-salt of single-stranded DNA molecule composed of 34 deoxynucleotide bases with a molecular weight of 10.6 kDa.

2.2. Preparation of emulsions

Multiple emulsions were prepared using a two-step procedure (Ueda and Matsumoto, 1991). Briefly, the primary W/O emulsion was prepared and then gently dispersed (40 wt.%) in the external water phase containing one of the following hydrophilic emulsifiers: polysorbate 80, PEG 40/50 stearate or steareth-20.

Regular W/O emulsions were prepared by adding an aqueous phase containing different concentrations of osmotic active ingredients to the oil phase. In detail, both phases were heated to approx. 70–75 °C before the water phase was added to the oil phase. The mixture was subsequently homogenized using a rotor/stator homogenizer (Diax 600, Heidolph Germany) at 9500 rpm for 2 min. The obtained primary emulsion was cooled down to room temperature and then slowly added to the outer water phase while the system was stirred at 1200 rpm using a EUROSTAR digital stirrer (IKA® Werke GmbH 6 Co. KG, Staufen, Germany), until a homogeneous emulsion was obtained. The compositions of the multiple emulsions are shown in Table 1.

2.3. Conductometric analysis

Conductivity measurements were carried out using a WTW Microprocessor Conductivity Meter LF 96 (WTW, Germany) at room temperature. Measurements were performed directly in the undiluted emulsion (mean ± S.D., $n = 3$). The standard deviation was negligible and therefore not plotted in the graphs.

2.4. Microscopic observation

The W/O/W multiple emulsions were analyzed using an optical immersion microscope TR 300 connected to a DV 2B (VWR, Germany) camera at ×1000 magnifying power (oil immersion). This method was used to allow a standardized quality control as well as a verification of the multiple emulsions.

2.5. Droplet size measurement

Mean water droplet size (*z*-average) in the primary W/O emulsion was determined by dynamic lights scattering (High Performance Particle Sizer (HPPS), Malvern Instruments, UK). Samples were diluted 1:1000 using light paraffin oil (viscosity: 33 mPa s) prior to measurement.

Oil droplet size and distribution in multiple W/O/W emulsions were determined using a laser diffraction particle size analyser (Mastersizer S, Malvern Instruments, England). The fundamental size distribution obtained with this technique is based on a volume distribution. The particle size distribution was calculated according to the Mie theory. Measurements were performed directly after samples have been diluted in distilled water (mean ± S.D., $n = 3$). The standard deviation was negligible and therefore not plotted in the graph. Microscopic observations revealed that the bimodal particle size distribution obtained by static light scattering measurements can be attributed to the occurrence of simple oil droplets without an encapsulated water phase and oil droplets with multiplicity (containing inner water droplets). Thus, peak maximum of the larger multiple droplets were chosen for characterisation of the W/O/W multiple emulsions.

2.6. Rheological measurement

Rheological analysis was performed at 25 °C using a RheoStress 300 Rheometer (Thermo Haake, France) with cone and plate geometry, diameter of two centimetres and an angle of 2°. The apparent viscosity was measured over a shear rate of 0.1–100 s^{-1}. The results are presented as mean values (mean ± S.D., $n = 3$). The standard deviation was negligible and therefore not plotted in the graphs.

Table 1
Compositions of multiple emulsions containing different osmolytes in the inner water phase.

Osmolyte solution	20.0%	Inner water phase
Heavy paraffin oil	15.8%	Oil phase
Span 80	4.0%	
Lecithin	0.2%	
Distilled water	ad. 100%	Outer water phase
Hydrophilic emulsifier	1%[a]/1.2%[b]	

[a] Polysorbate 80, steareth-20.
[b] PEG-40/50 stearate.

Table 2
Physical properties of W/O emulsions containing different additives in the inner water phase.

	Additive	Concentration (M)	W/O droplet size z-average (μm)	Viscosity (Pa s)
WO1a	NaCl	0.050	0.70	1.69 ± 0.02
WO1b	NaCl	0.065	0.71	1.97 ± 0.03
WO1c	NaCl	0.100	0.78	1.98 ± 0.02
WO2a	$MgSO_4$	0.050	0.55	1.93 ± 0.05
WO2b	$MgSO_4$	0.065	0.60	1.98 ± 0.06
WO2c	$MgSO_4$	0.100	0.67	1.96 ± 0.03
WO3b	Glycine	0.080	0.75	1.49 ± 0.02
WO3c	Glycine	0.200	0.80	1.81 ± 0.05
WO4b	Glucose	0.130	0.50	1.98 ± 0.04
WO4c	Glucose	0.200	0.63	1.95 ± 0.06

3. Results and discussion

3.1. Influence of osmolytes in the inner aqueous phase on the stabilization of multiple W/O/W emulsions

3.1.1. Stabilization of the primary W/O emulsion by osmotic active ingredients

It has been reported that inverse emulsions prepared in the absence of additional electrolytes are unstable with respect to flocculation and coalescence (Kent and Saunders, 2001). It was shown that the addition of $MgSO_4$ to the water phase prior to emulsification, even at very low concentration, resulted in a substantial improvement of the stability (Kent and Saunders, 2001). Increasing the osmotic pressure by electrolytes counteracts the Laplace pressure which is associated with the curvature of the droplet surface. This results in a decreased Oswald ripening (Koroleva and Yurtov, 2003).

W/O emulsions containing the osmotic active ingredients NaCl, $MgSO_4$, glucose or glycine at different concentrations were prepared. NaCl and $MgSO_4$ are strong electrolytes and dissociate almost completely in the water solution. In contrast, glycine and glucose do not dissociate in water solution. The dissociation properties of the used additives were taken into account for the preparation of W/O emulsions. Since the osmotic pressure is independent of the properties of the molecule, but depends on the number of molecules in a given volume of solvent (colligative properties) the molality of glucose and glycine in the water phase was chosen twice as high as the one of NaCl and $MgSO_4$. The physicochemical properties of the formulations were determined subsequent to preparation.

Physicochemical properties, e.g. droplet size and viscosity of the primary W/O emulsions in relation to the used osmotic active ingredients concentrations are shown in Table 2. An increased concentration of the osmotic active ingredient resulted in a slight increase of W/O droplet sizes for all prepared formulations. This is in line with other reports from the literature working on inverse W/O emulsions (Kent and Saunders, 2001; Solans et al., 1993). A potential explanation given by Kent and Saunders (2001) proposes that a delay of the surfactant adsorption at the oil–water interface in the presence of electrolytes such as NaCl or $MgSO_4$ results in higher interfacial tensions during emulsification. Despite of differences in droplet sizes, we observed little variations in the viscosity of the formulations ranging from 1.49 to 1.98 Pa s.

For all primary W/O formulations phase separation occurred within the first 24 h. Nevertheless, these primary emulsions can be used for the preparation of stable multiple W/O/W emulsions. Previous work of our group could demonstrate that stable W/O formulations with considerably higher viscosity were not appropriate for the preparation of multiple emulsions (results not shown).

3.1.2. Preparation of the multiple W/O/W emulsion

The influence of three hydrophilic surfactants on the stability of multiple emulsions was investigated using polysorbate 80 (1 wt.%), steareth-20 (1 wt.%) and PEG-40/50 stearate (1.2 wt.%). Steareth-20 is a polyethoxylated stearyl ether whereas PEG-40/50 stearate is a polyethoxylated stearyl acid ester. Polysorbate 80 is a polyethoxylated sorbitan monooleate containing both ether and ester functional groups.

Droplet sizes and viscosity were determined subjected to the hydrophilic surfactant and the concentration of osmotic active ingredients immediately to preparation (Table 3). Increasing concentrations of the osmotic active ingredients resulted in a decreased droplet size for formulations containing polysorbate 80 and steareth-20. Emulsions prepared with PEG-40/50 stearate

Table 3
Physical properties of W/O/W multiple emulsions containing different additives in the inner water phase. These data was measured directly after preparation.

	Additive	Concentration (M)	Polysorbate 80		Steareth-20		PEG-40/50 stearate	
			W/O/W droplet size (μm)	Viscosity (Pa s)	W/O/W droplet size (μm)	Viscosity (Pa s)	W/O/W droplet size (μm)	Viscosity (Pa s)
WOW1a	NaCl	0.050	–	–	35.0	0.84	184.0	0.14
WOW1b	NaCl	0.065	20.5	1.01	29.5	0.94	82.0	0.24
WOW1c	NaCl	0.100	14.5	2.04	20.0	1.20	17.5	0.77
WOW2a	$MgSO_4$	0.050	36.0	0.36	31.7	0.75	No homogenous emulsion	
WOW2b	$MgSO_4$	0.065	27.0	0.43	25.0	0.97	31.0	0.51
WOW2c	$MgSO_4$	0.100	21.0	1.06	16.0	1.37	57.0	0.37
WOW3b	Glycine	0.080	15.0	0.65	27.5	0.86	No homogenous emulsion	
WOW3c	Glycine	0.200	14.5	1.60	18.0	1.27	26.0	0.95
WOW4b	Glucose	0.130	49.0	0.64	31.5	0.77	78.0	0.29
WOW4c	Glucose	0.200	–	–	24.0	1.43	92.0	0.37

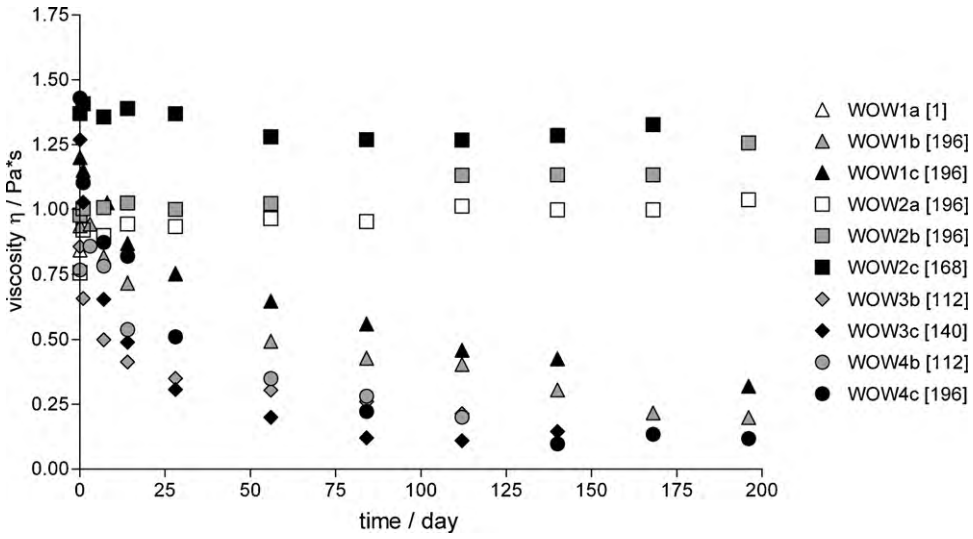

Fig. 1. Effect of different osmolytes in the inner water phase on the viscosity of multiple W/O/W emulsions. Storage at room temperature. Last measurable time point of stability presented in brackets.

showed a similar behaviour in case then NaCl was added as the osmotic active ingredient. In contrast, the other formulations prepared with PEG-40/50 showed a decreased stability which was associated with large droplet sizes and longer homogenization time (30 min) compared to the other systems (5 min) studied. The larger droplet sizes for the PEG-40/50 system can be attributed to a slower adsorption of the emulsifier to the interfacial layer. However, Hino et al. (2000) reported an inverse correlation between droplet size and the osmotic active ingredient NaCl. These contrary observations could be explained by the different ingredients that were used and an average smaller droplet size (<5 μm) that was obtained by this author (Hino et al., 2000).

An increasing osmolyte concentration led to an increased viscosity of the multiple emulsions which is in line with published data (Hino et al., 2000; Ohwaki et al., 1992). This phenomenon is most likely caused by an outflow of water from the outer into the inner water phase during the emulsification process. In accordance with the Fick's law, the flow of water is proportional to the concentration gradient between both water phases. High osmolyte concentrations therefore lead to an increased passage of water molecules between the two phases. The resulting higher viscosities with higher concentrations of osmotic active ingredients can be contributed to the change in the ratio of dispersed-to-continuous phase during preparation (Sherman, 1962).

3.1.3. Stability of the multiple W/O/W emulsion

In order to investigate long-time stability of multiple W/O/W emulsions, droplet size as well as viscosity and conductivity of all formulations were analyzed over a period of 6 months. The samples were stored at room temperature and 40 °C with 75% relative humidity. However, at 40 °C all preparations showed phase separation or phase inversion to W/O emulsion within the first month. Therefore, the following results correspond to room temperature only.

Instability was defined either by a loss of viscosity, a phase separation or a phase inversion. The stability of the formulations was strongly depended on the osmotic active compounds that were added, following the hierarchy: glycine < glucose < NaCl < $MgSO_4$.

Furthermore, when using polysorbate 80 as a hydrophilic emulsifier, instability occurred within 4 weeks whereas formulations prepared with PEG-40/50 stearate were stable for less than 8 weeks. Only formulations prepared with steareth-20 showed sufficient long-term stability, phase inversion and phase separation did not occur within the observation period.

Based on these observations, results obtained with Steareth-20 are further presented and discussed more in detail. With regard to the observation period, all formulations stabilized with $MgSO_4$ did not show any significant changes of droplet sizes (data not shown) compared to the starting point (see Table 3). Results from the viscosity measurement for the emulsions prepared with steareth-20 are depicted in Fig. 1. Formulations containing glucose and glycine showed a rapid decrease in viscosity within two weeks. For these formulations phase separation was observed within 16 and 20 weeks for glucose and glycine, respectively. Emulsions containing NaCl exhibited a similar behaviour; however, the decrease in viscosity was delayed. In our hands, $MgSO_4$ was the only osmotic active ingredient that formed stable formulations and resulted in a stable viscosity. Moreover, at 0.050 and 0.065 M $MgSO_4$ a weak increase of viscosity could be detected.

The observed decrease in viscosity for emulsions that were prepared in presence of glycine, glucose and NaCl can most likely be explained by the migration of water and/or additives between both water phases (Schmidts et al., 2009). Migration of water from the outer into the inner water phase or vice versa can be excluded since swelling or shrinking of droplets would result and would have been detected as changes of droplet sizes over the time. Thus, storing of the emulsions resulted in changes of viscosity caused by the migration of electrolytes from the inner to the outer water phase.

Schott and Royce (1983) have shown that electrolytes can influence the properties of emulsifiers by causing a "salting out" or "salting in" effect. Other osmolytes can account for a similar effect (Inayathullah et al., 2003). Hence, steareth-20 as a polyoxyethlyene ether might be influenced by electrolytes, such as NaCl, by disturbing the sterical configuration of emulsions for example as a result of dehydration of the polyoxyethylene chains of the surfactant. The higher the concentrations of osmolytes, the stronger the influence on the emulsifier.

As glucose and glycine do not show any electric conductivity, this hypothesis was tested by analyzing the conductivity for the emulsions containing NaCl and $MgSO_4$ (Fig. 2). Emulsion containing NaCl and $MgSO_4$ exhibited different behaviours. Whereas the conductivity of emulsions containing NaCl increased over time, the conductivity in the presence of $MgSO_4$ remained constant within the observed time period. Multiple W/O/W emulsions containing 0.100 M NaCl in the inner water phase showed a continuously

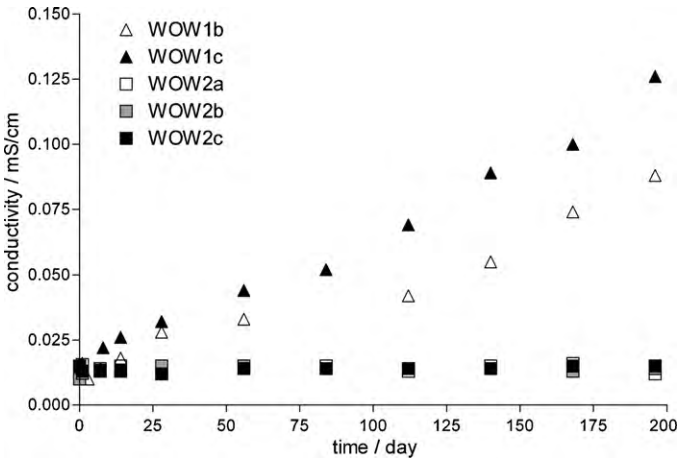

Fig. 2. Conductivity of the multiple W/O/W emulsions containing NaCl or $MgSO_4$ in the inner water phase versus time. Storage at room temperature.

higher degree of conductivity during storage than the 0.065 M which can be explained by larger amounts of ions migrating from the inner into the outer water phase. The amount of glucose and glycine in the outer water phase was not determined in this study, but Hino et al. (Hino et al., 2000) showed a considerably faster release of glucose compared to NaCl.

Differences between emulsions containing NaCl and $MgSO_4$ are due to different interactions of Na^+ and Mg^{2+} ions with the emulsifiers. Bivalent cations, such as Mg^{2+}, salt-in polyoxyethylated non-ionic surfactants by complexation of the cations and the oxygen atoms of the ether. On the other hand, monovalent cations (e.g. Na^+) salt-out the surfactants by enhancing the structure of water or by dehydration (Schott, 1973). In the case of the presented formulation with steareth-20 as emulsifier, the "salting in" properties of magnesium ions had a positive effect on the stability of the emulsion in comparison to other additives. Glucose (Miyajima et al., 1983) as well as glycine (Ali et al., 2008) are both structure makers and, like Na^+ ions, therefore destabilize the surfactant.

The high decrease in viscosity for the samples containing glucose and glycine can be explained by either a faster migration into the outer water phase or stronger interactions with emulsifiers in comparison to the electrolytes. However, further investigations are needed to confirm these results.

The results presented in this study could clearly demonstrate that multiple W/O/W emulsions are very sensible systems in terms of stability and composition. Choice of appropriate additives for the inner water phase has among other factors a major impact on the stability of multiple W/O/W emulsions. When comparing the stability of the tested formulations only emulsions containing steareth-20 as emulsifier and $MgSO_4$ in the inner water phase were stable over a longer period of time (6 months).

However, irrespective of the hydrophilic emulsifiers used, similar emulsion behaviour for individual osmotic active ingredients was observed. Therefore it can be assumed, that the reported correlations could be probably applied to a majority of non-ionic surfactants.

3.2. Influence of thickeners in the inner aqueous phase on the stabilization of multiple W/O/W emulsions

In order to improve the stability of the systems using NaCl as a stabilizing electrolyte (these formulations tended to phase separation or phase inversion over the course of time) a potential stabilizing effect of thickening additives was tested.

It is known from literature, that incorporation of macromolecules into the inner water phase can improve the long-time stability of multiple W/O/W emulsions. This might be attributed to the formation of a rigid film at the interface protecting the droplets from coalescence (Law et al., 1986; Omotosho, 1990). In the present work, two different cellulose derivatives, hydroxyethylcellulose and sodium carboxymethyl cellulose, were incorporated into the inner water phase and their impact on the multiple W/O/W emulsions was tested. Most cellulose derivatives dissolve in cold water and are mainly used for controlling viscosity by gelling. The composition of the emulsions is presented in Table 4.

Table 4
Compositions of multiple emulsions containing thickeners in the inner water phase.

Water	19.80%	Inner water phase
NaCl	0.12%	
Thickener[a]	0.08%	Oil phase
Heavy paraffin oil	15.80%	
Span 80	4.00%	
Lecithin	0.20%	
Cetyl palmitate	0.80%	
Distilled water	ad. 100%	Outer water phase
Hydrophilic emulsifier	1%[b]/1.2%[c]	

[a] Hydroxyethyl cellulose or sodium carboxymethyl cellulose.
[b] Polysorbate 80, steareth-20.
[c] PEG-40/50 stearate.

As a result, the addition of the thickener into the inner water phase did not affect the physical properties of the primary W/O emulsion subsequent to production. The viscosity of formulations prepared with hydroxyethyl cellulose and sodium carboxymethyl cellulose (approximately 2 Pa s and water droplet sizes of approximately 0.6 μm), was comparable to emulsions prepared without thickeners.

However, these additives affected the formation of the multiple emulsions. Using sodium carboxymethyl cellulose as a thickener and polysorbate 80 or PEG-40/50 stearate as emulsifiers, multiple W/O/W emulsions were formed, but the using steareth-20 a simple W/O emulsion was obtained (phase inversion). In contrast, using hydroxyethyl cellulose, multiple W/O/W emulsions could be prepared exclusively with steareth-20 and simple W/O emulsions were obtained using polysorbate 80 or PEG-40/50 stearate as hydrophilic emulsifiers. The differences are probably caused by interactions between the cellulose derivatives and the chemical emulsifier groups, like ester groups (polysorbate 80 or PEG-40/50 stearate) and ether groups (steareth-20). However, in cases where multiple emulsions were obtained (hydroxyethyl cellulose with steareth-20 and sodium carboxymethyl cellulose with polysorbate 80 or PEG-40/50 stearate), no measurable differences in emulsion properties were observed in comparison to formulations prepared without a thickener subsequent to preparation. Surh et al. (2007) reported a similar observation in respect to multiple emulsions containing a whey protein isolate as a thickening additive in the internal water phase.

Furthermore, no noticeable changes in the viscosity, droplet size as well as the release of electrolytes were observed until phase separation or phase inversion occurred and therefore, at least in this setting, the emulsions are not affected by the presence of these thickeners in the inner water phase as shown exemplarily using sodium carboxymethyl cellulose and polysorbate 80 (Fig. 3). However, phase separation occurred in the emulsions containing cellulose derivatives several weeks later compared to formulations without a thickener. In case of polysorbate 80 and PEG-40/50 stearate combined with sodium carboxymethyl cellulose a delay in phase separation was observed of 8 and 4 weeks, respectively. Steareth-20 stabilized with hydroxyethyl cellulose showed a delay in phase separation of 4 weeks. This is probably caused by an inhibition of coalescence or Ostwald ripening of the internal water droplets.

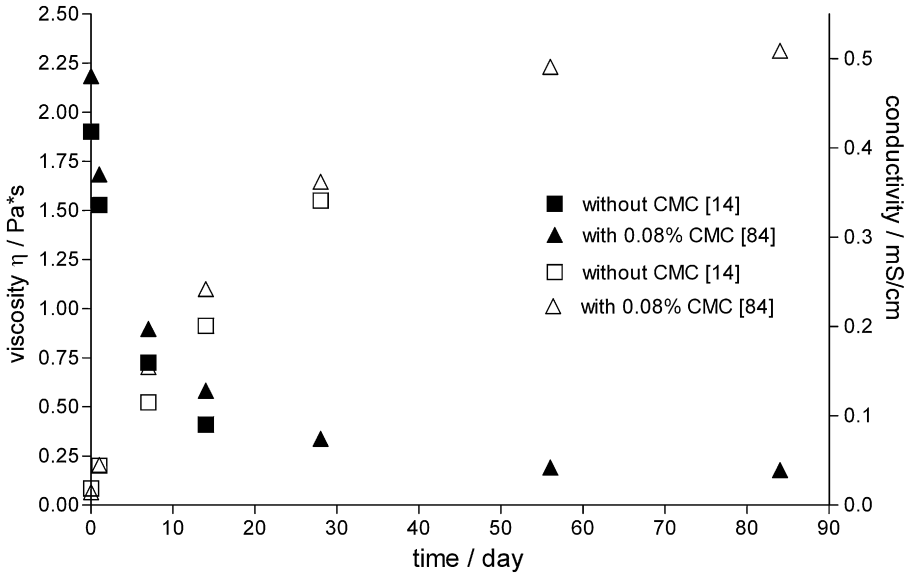

Fig. 3. Viscosity and conductivity of multiple W/O/W emulsions containing 0.08% sodium carboxymethyl cellulose, incorporated in the inner water phase and polysorbate 80 as hydrophilic emulsifier. Last measurable time point of stability presented in brackets.

Subsequently it can be concluded, that even if the addition of cellulose derivates into inner water phase of the tested formulations resulted in a delayed phase separation of the formulations, it neither essentially affected the physicochemical properties of the W/O/W emulsion nor the release of encapsulated electrolyte.

3.3. Multiple emulsions containing DNAzyme as drug in the inner water phase

DNAzymes represent a novel class of antisense molecules, which have not been yet established in the treatment of human disease. In the present study, the multiple W/O/W emulsions were developed to serve as dermal delivery systems for DNAzymes. Multiple emulsions as drug delivery systems have several advantages compared to simple W/O emulsions, e.g. better protection of encapsulated substances accompanied by an optimized permeation behaviour (Youenang Piemi et al., 1998). This is particularly important in the case of DNA-based drugs where protection against DNAses, that are located both on the skin and in somatic cells, is ensured (Santoianni and Rothman, 1961).

Based on the results discussed in chapter 3.1, two formulations containing DNAzyme in the inner water phase were prepared. Since steareth-20 resulted in the most stable formulations, it was used as a hydrophilic emulsifier. The inner water phase consisted of 0.065 M $MgSO_4$ or 0.065 M NaCl solution. Pure DNAzyme might not markedly alter the osmolarity of the solution because of its high molecular weight. However, since the active substance composition is the Na–salt of DNAzyme, the composition can potentially increase the osmotic pressure of the inner water phase. Therefore, according to previously presented results the viscosity and conductivity of emulsions containing DNAzyme is higher compared to formulations without DNAzyme. Over the time, a decrease in viscosity is observed for both formulations (Fig. 4). In general, the observed trend is similar to the one observed for emulsions prepared without DNAzyme, i.e. the viscosity decreases over the time in the presence of NaCl, whereas in the presence of $MgSO_4$ no significant changes were detected after an initial decrease. In contrast, the electrolyte release is clearly influenced by the DNAzyme due to its salt properties. Whereas in absence of DNAzyme no increase of conductivity of formulations containing $MgSO_4$ during periods of storage is observed, the presence of DNAzyme resulted in a slight

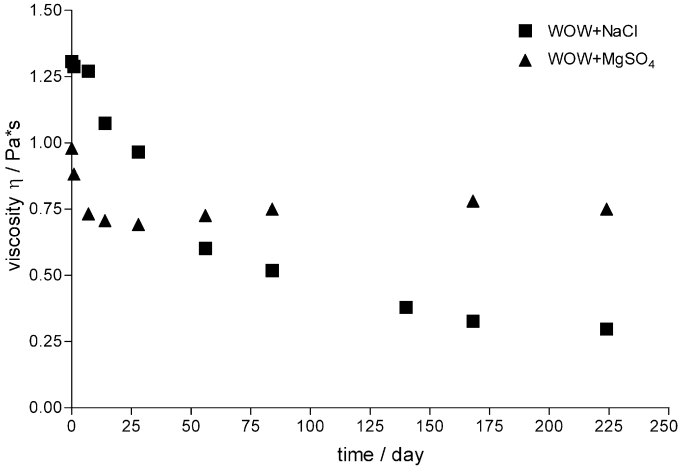

Fig. 4. Viscosity of multiple W/O/W emulsions containing DNAzyme in the inner water phase. Storage at room temperature.

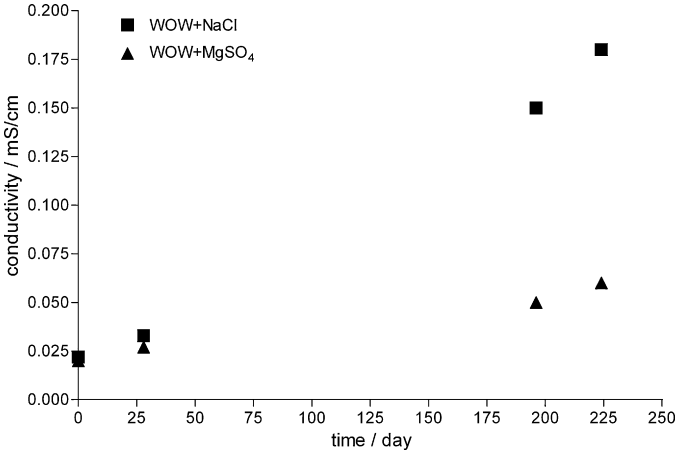

Fig. 5. Conductivity of multiple W/O/W emulsions containing DNAzyme in the inner water phase. Storage at room temperature.

increase of conductivity (Fig. 5). Formulations containing NaCl in the inner water phase showed stronger increase of conductivity in comparison to placebo formulations.

These experiments demonstrated that the multiple emulsions represent feasible drug delivery systems for the encapsulation of macromolecular active substances, such as DNAzyme oligonucleotides. The long-time stability of the formulations is not strongly influenced by addition of DNAzymes. However, further investigations regarding the encapsulation efficiency and the protection of the DNAzyme oligonucleotides against degradation in the formulations are necessary.

4. Conclusion

The aim of this work was to investigate the influence of additives in the inner water phase on the properties and stability of multiple emulsions. It has been established that the nature and amount of osmotic active ingredients significantly affects the physical properties of the emulsion directly after preparation as well as its behaviour during storage. For the individual osmotic active ingredients, irrespective of the hydrophilic emulsifiers used, similar emulsion properties were observed. It can therefore be assumed, that the presented results can be probably applied to most nonionic surfactants. The behaviour of emulsions containing $MgSO_4$ as an additive differed from the other formulations tested in this study. This is probably due to a "salting in" effect of magnesium ions on polyethoxylated emulsifiers. Glucose and glycine stabilized the formulation the least probably due to rapid migration into the outer water phase. The most stable formulations were achieved using $MgSO_4$ as an additive and steareth-20 as hydrophilic emulsifier.

The addition of cellulose derivatives did not influence the physical properties of multiple emulsions. However, a slight increase in long-time stability was observed. This is probably caused by an inhibition of coalescence and Ostwald ripening of the internal water droplets.

In this study we could show that multiple emulsions represent suitable systems for the encapsulation of macromolecular drugs. Active substances such as DNAzyme oligonucleotides can be easily incorporated into the inner water phase and did not influence the general behaviour of multiple W/O/W emulsions.

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