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Mucoadhesive dexamethasone acetate-polymyxin B sulfate cationic ocular nanoemulsion – novel combinatorial formulation concept

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Dexamethasone acetate (DEX) and polymyxin B sulfate (polymyxin B) were formulated as a cationic nanoemulsion for the treatment of ophthalmic infections. As novel concept, the positive charge to achieve mucoadhesion was not generated by toxicologically and regulatorily problematic cationic lipids or polymers, but by using a positively charged drug in combination with positively charged preservatives. The preservative also acts as co-surfactant to stabilize the emulsion. Nanoemulsions with the lipid phase Eutanol G-Lipoid S 100 (70%:30%) containing 0.05% (w/w) DEX were produced by high pressure homogenization, followed by dissolving the hydrophilic molecules in the water phase, e.g. polymyxin B (0.1%, w/w), cetylpyridinium chloride (0.01%, w/w) and glycerol (2.6%, w/w) to yield a combination product. The particles were below 200 nm with narrow size distribution. The osmolality (374 mOsm/kg), pH (5.31) and viscosity (2.45 mPa s at 37 °C) were compatible to the ocular administration. The zeta potential of the optimized formulation was shifted from approx. +9 mV to -11 mV after mucin incubation. The *in vitro* test revealed no potential cytotoxicity. The final products were stable after 180 days of storage at 4 °C and room temperature. The developed product is a viable alternative to the commercial ophthalmic suspensions. Moreover, this concept of generating the positive charge by cationic drug and/or preservative addition can be transferred to other ophthalmic products.

1. Introduction

The therapy of eye infections requires a combined administration of anti-inflammatory steroid and antibiotic drugs to reduce related inflammation. Dexamethasone, a glucocorticoid steroid, can efficiently alleviate ocular inflammation and swelling, heat, redness, and pain which are induced by infection, chemicals, and/or severe allergies (Kim and Chauhan 2008). Dexamethasone acetate (DEX), a hydrophobic drug, was the most effective in suppressing inflammation in the cornea comparing to water soluble dexamethasone derivatives such as dexamethasone sodium phosphate (Leibowitz et al. 1978). Polymyxin B sulfate (polymyxin B), a hydrophilic drug, is a cationic polypeptide antibiotic. It has strong antibacterial capacity against Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* (Zavascki et al. 2007). The combination of DEX and polymyxin B is indicated to treat ocular inflammation such as chronic blepharitis and conjunctivitis (Shulman et al. 1996; van Endt et al. 1997). This study comprises the encapsulation of DEX and polymyxin B in a single ophthalmic product.

Conventional ophthalmic solutions, ointments and suspensions account for 90% of the commercially available dosage forms (Manish and Kulkarni 2012). However, it is well known that these conventional products can be rapidly removed from the eye surface by ocular defense mechanisms following instillation, which limit the drug residence time, thus reducing drug absorption. Besides this, the tear film and cornea compose main barriers contributing to the poor ocular bioavailability of drug as well (Achouri et al. 2013). Aiming to overcome these barriers, newer drug-delivery systems are being explored, such as controlled-release drug-delivery systems, viscosity enhancers, utilization of mucoadhesive agents, liposomes, micro- and nano- technology (Achouri et al. 2013; Bucolo et al. 2008).

Among currently available approaches, nanotechnology with mucoadhesive agents (e.g., cationic nanoemulsion) is efficient to increase the residence time on the ocular surface, by allowing electrostatic interactions between the mucosal layer with negative charges covering the cornea and cationic formulation. This strategy can be considered a promising ocular delivery system, since it has the potential to significantly enhance the bioavailability of ophthalmic drug (Hagigit et al. 2010, 2012, Rabinovich-Guilatt et al. 2004). Different strategies using cationic lipids and cationic polymers have been reported to prepare cationic lipid nanoparticles, for instance, cationic lipids including stearylamine (Klang et al. 1994), oleylamine (Tamilvanan et al. 2005), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (Fraga et al. 2011), N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium salts (DOTAP) (Hagigit et al. 2012), cetyltrimethylammonium bromide (CTAB) and dimethyldioctadecylammonium bromide (DDAB) (Fangueiro et al. 2014) as well as cationic polymers such as chitosan (Gallarate et al. 2013) and polyethyleneimine (PEI) (Wang et al. 2010a). As reported by two excellent articles (Daull et al. 2014; Lallemand et al. 2012), most of the cationic lipids and polymers hindered either by toxicity (e.g. DOPE and PEI), stability (e.g. oleylamine), regulatory issues (e.g. stearylamine and chitosan) or sometimes cost, which limit their use in pharmaceutical products.

Under these circumstances, an alternative is needed to provide the positive charge. Benzalkonium chloride (BAC) or cetalkonium chloride (CAC), the typical preservatives in ophthalmic preparations, were used as cationic surfactants in the development of the technology Novasorb. Despite of these quaternary ammonium compounds toxicity, their use as surfactants is possible when used in concentrations lower or equal those when used as preservative. This technology delivered a product in the market, Cationorm, a preservative-free cationic nanoemulsion indicated for the symptomatic treatment of dry eye. The product showed higher efficacy compared to the conventional products by providing enhanced comfort and quality of life for the patients who were treated with 1-2 drops twice daily (Ousler et al. 2015). It should be noted that, the cationic

agent, i.e. BAC or CAC, is encapsulated in the oily phase of cationic nanoemulsion (Daull et al. 2014; Lallemand et al. 2012).
Unlike Novasorb technology, in the present study, the positive charge of nanodroplets is generated by preservative and polymyxin B which are dissolved in the aqueous phase of the cationic nanoemulsion. This approach could not only avoid the use of the cationic lipids or polymers, but also preserve the formulation for multiple applications. In this study, a cationic nanoemulsion containing both, hydrophobic and hydrophilic active ingredients, DEX and polymyxin B, respectively, was developed. According to our knowledge, this type of combinatorial cationic nanoemulsion without cationic lipid or polymer has not been reported before. Additionally, parameters such as particle size, distribution, zeta potential, osmolality, pH, viscosity, mucoadhesion, cytotoxicity and long-term stability were evaluated.

2. Investigations, results and discussion

2.1. Liquid lipid screening

Drug loading content and entrapment efficiency of nanoemulsions are limited by the solubility of the drug in the lipid. Thus, the screening is a critical step in the formulation development. The liquid lipid screening yielded the DEX solubility in eleven liquid lipids. By visual evaluation, it was found that the highest amount of DEX, i.e. 0.5% (w/w), could be dissolved in Eutanol G. This was supported by polarized light microscope investigations (data not shown). No DEX crystals were found in Eutanol G loaded with 0.5% (w/w) drug indicating complete dissolution of the drug in this oil. Furthermore, the presence of crystals having particle sizes of approx. 157 µm was detected in 1.0% (w/w) drug loaded oil. Eutanol G is widely used in topical pharmaceutical formulations, and it is generally regarded as nontoxic and nonirritant at the levels employed as an excipient (Rowe et al. 2009). This branched saturated fatty alcohol presents neither ocular irritation nor minimal transient irritation in the eyes of rabbits (Elder 1985).

2.2. Liquid lipid to Lipoid S 100 ratio screening

Despite the solubility in Eutanol G, DEX crystals were observed after 2 days of storage using light microscopy in formulation containing 0.05% (w/w) DEX and 20% (w/w) Eutanol G stabilized with 4% (w/w) Poloxamer 407 (data not shown). Aiming to improve the drug solubility, Lipoid S 100 was added to the lipid phase as a solubilizer. A screening study was performed to determine the optimal amount of Lipoid S 100 in the oil phase. Lecithin such as Lipoid S 100 benefits increasing the drug entrapment efficiency due to the fact that its high phase transition temperature avoids drug leaking from lipid matrix (Li et al. 2014). The blend of Eutanol G and Lipoid S 100 chosen for evaluation as well as their ability to dissolve DEX examined by visual inspection are shown in Table 1. Eutanol G:Lipoid S 100 at ratio of 70%:30% increased the DEX solubility to 1.0% (w/w) in the oil phase. The solubility was not improved with further increase of the Lipoid S 100 proportion. The results were confirmed by polarized light microscope evaluations (data not shown). No crystals in the blend (70% Eutanol G:30% Lipoid S 100) with 1.0% (w/w) DEX were found by polarized light microscopy, proving a well solubility of the drug in this oily phase. Apparent crystals having particle size of 28-72 µm were detected in 1.5% (w/w) DEX loaded sample inferring the oily phase was overloaded. Therefore, Eutanol G:Lipoid S 100 (70%:30%) was selected as lipid phase.

2.3. Selection of the surfactant and addition of the cationic agents

The appropriate selection of surfactant is a crucial part in the nanoemulsion development, specifically due to the intrinsic toxicity of this component. The order of surfactant toxicity is well known as cationic > anionic > nonionic, and nonionic surfactants are preferred for ophthalmic use. Tween® 80 and Poloxamer 407 are approved by the FDA as inactive ingredients for intravenous injections and topical preparations. Furthermore, the HLB values of Tween® 80 and Poloxamer 407 are approximately 15 and 18-23, respectively, which allows the development of o/w nanoemulsions. Hence, they were chosen to conduct the surfactant screening study. The composition and the particle size measurement (photon correlation spectroscopy (PCS) and laser diffractometry (LD) data) immediately after production of nanoemulsions, using the selected surfactants, are presented in Table 2 and Table 3. Hereby, formulations B-D stabilized with increasing concentrations of Poloxamer 407 of 2%, 3% and 4% (w/w) revealed the mean particle size (z-average) lower than 200 nm, with narrow particle size distribution (polydispersity index (PI) < 0.2). Besides this, these formulations did not contain large particles or aggregates (d(v)95% < 0.3), except for formulation A (d(v)95% = 2.495) (Table 2). Formulations E-H were stabilized using Tween® 80 (Table 3). Regarding to the formulation E, the concentration of 1%

Table 1: Overview of the blends of Eutanol G and Lipoid S 100 and their ability to dissolve DEX examined by visual inspection (+: dissolved, –: not dissolved)

Blend of Eutanol G and Lipoid S 100	DEX content (%, w/w)		
	0.5	1.0	1.5
90% Eutanol G:10% Lipoid S 100	+	-	-
80% Eutanol G:20% Lipoid S 100	+	-	-
70% Eutanol G:30% Lipoid S 100	+	+	-
60% Eutanol G:40% Lipoid S 100	+	+	-
50% Eutanol G:50% Lipoid S 100	creamy	creamy	creamy

Table 2: Formulations A-D stabilized with Poloxamer 407: composition of nanoemulsion with 0.05% (w/w) DEX; PCS data (z-average (nm), PI) as well as LD diameters (d(v)50%, d(v)90%, d(v)95%) after production

Code	Lipid* (w/w)	Poloxamer 407 (w/w)	PCS data	LD diameters		
			z-average (nm) PI	d(v)50% (µm)	d(v)90% (µm)	d(v)95% (µm)
A	20.00%	1.00%	501 ± 18 0.409 ± 0.130	0.573	1.498	2.495
B	**20.00%**	**2.00%**	**166 ± 3** **0.097 ± 0.035**	**0.153**	**0.236**	**0.264**
C	20.00%	3.00%	151 ± 3 0.119 ± 0.045	0.149	0.212	0.232
D	20.00%	4.00%	122 ± 2 0.115 ± 0.019	0.136	0.195	0.213

* Lipid: Eutanol G:Lipoid S 100 (70%:30%)

Table 3: Formulations E-H stabilized with Tween® 80: composition of nanoemulsion with 0.05% (w/w) DEX; PCS data (z-average (nm), PI) as well as LD diameters (d(v)50%, d(v)90%, d(v)95%) after production

Code	Lipid* (w/w)	Tween® 80 (w/w)	PCS data	LD diameters		
			z-average (nm) PI	d(v)50% (µm)	d(v)90% (µm)	d(v)95% (µm)
E	20.00%	1.00%	1029 ± 131 0.969 ± 0.052	1.445	4.062	17.335
F	20.00%	2.00%	312 ± 10 0.349 ± 0.045	0.425	0.844	1.051
G	**20.00%**	**3.00%**	**302 ± 8** **0.168 ± 0.051**	**0.400**	**0.839**	**1.071**
H	20.00%	4.00%	282 ± 3 0.294 ± 0.033	0.395	0.684	0.795

* Lipid: Eutanol G:Lipoid S 100 (70%:30%)

(w/w) of Tween® 80 was not sufficient to obtain an emulsion in the nanosize range. Formulation F containing 2% (w/w) Tween® 80 did not continue stable after 2 days indicating ineffective stabilization. Formulations G and H containing 3% and 4% (w/w) of Tween® 80, respectively, showed similar sizes and stability. For both formulations, z-average was approximately 300 nm with PI less than 0.3 and d(v)95% ≤ 1 µm. Such findings concur with that reported by Koppolu and his collaborators (Koppolu et al. 2010) who found that the surfactant concentration is inversely proportional to the particle size.
Nevertheless, an optimized surfactant concentration in the formulation is desirable aiming to reduce the potential ocular irritation of these agents. Thus, the formulations presenting relatively small particle size (z-average ≤ 300 nm) and lower surfactant concentration, i.e. formulations B and G, were selected to conduct further development.
Cationic components, i.e. polymyxin B and the preservative, were added to the prepared nanoemulsion instead of the aqueous phase prior to high pressure homogenization. It could avoid the neutralization between the anionic and cationic components during production, thus generating precipitate. The particle size and zeta potential (ZP) results before and after the addition of cationic components are shown in Table 4. Neither PCS nor LD results were affected by the addition of cationic components. The original nanoemulsions (Table 4, formulations B and G) possessed slightly negative ZP values, i.e. approx. -5 mV, which can be elucidated by the shift of the shear plane as a result of the steric layer formed by the stabilizers. The addition of cationic ingredients did result in charge reversal to positive ZP values.

Poloxamer 407 presents higher molecular weight (9,840–14,600 Dalton) and longer chain, which can keep particles separated by steric force, but lower electrostatic repulsion. In contrast, Tween® 80 has a lower molecular weight (1,310 Dalton) and a shorter chain, resulting in higher ZP. As expected, charge reversal was more pronounced for formulations stabilized with Tween® 80 (Table 4, formulations G-1, G-2 and G-3), leading to a positive ZP of about +20 mV in original medium. After dilution, the formulation G-1 (ZP measurement in water) still presented a positive ZP of approximately +11 mV. The measurement in water resembles dilution of the nanoemulsions when administered into the eye, which means after the dilution the positive charge should still be as high as possible. As a conclusion, this formu-

Table 4: Particle size and ZP of formulations B and G before and after addition of cationic components (0.1% (w/w) polymyxin B and 0.01% (w/w) preservative)

Code	Cationic components	PCS data	LD diameters			ZP (mV)	
		z-average (nm) PI	d(v)50% (µm)	d(v)90% (µm)	d(v)95% (µm)	Original medium	Water (50 µS/cm)
B		166 ± 3 0.097 ± 0.035	0.153	0.236	0.264	-5.2 ± 0.2	-20.3 ± 0.1
B-1	Polymyxin B + CPC	167 ± 3 0.074 ± 0.056	0.155	0.230	0.253	+3.1 ± 0.2	+13.8 ± 1.5
B-2	Polymyxin B + BAC	165 ± 2 0.099 ± 0.05	0.154	0.231	0.255	+3.9 ± 0.3	+7.7 ± 0.6
B-3	Polymyxin B + CAC	165 ± 3 0.074 ± 0.04	0.154	0.231	0.255	+3.2 ± 0.3	+6.5 ± 0.6
G		302 ± 8 0.168 ± 0.051	0.400	0.839	1.071	-4.1 ± 0.4	-17.2 ± 0.5
G-1	Polymyxin B + CPC	321 ± 6 0.226 ± 0.069	0.411	0.887	1.111	+22.3 ± 1.1	+11.2 ± 0.7
G-2	Polymyxin B + BAC	296 ± 6 0.225 ± 0.063	0.403	0.851	0.978	+20.8 ± 0.5	+7.9 ± 0.4
G-3	Polymyxin B + CAC	304 ± 9 0.203 ± 0.087	0.431	0.878	1.084	+18.0 ± 0.4	+4.7 ± 0.2

*cetylpyridinium chloride (CPC); benzalkonium chloride (BAC); cetalkonium chloride (CAC)

Table 5: Formulations I and J applied in this study: composition of nanoemulsion with 0.05% (w/w) DEX, PCS data (z-average (nm), PI) as well as LD diameters (d(v)50%, d(v)90%, d(v)95%) after production

Code	Lipid * (w/w)	Tween® 80 (w/w)	PCS data	LD diameters		
			z-average (nm) PI	d(v)50 % (µm)	d(v)90% (µm)	d(v)95 % (µm)
I	**15.00%**	**2.00%**	**187 ± 5 0.103 ± 0.046**	**0.206**	**0.371**	**0.427**
J	15.00%	4.00%	139 ± 2 0.113 ± 0.043	0.142	0.208	0.229

* Lipid: Eutanol G:Lipoid S 100 (70%:30%)

Table 6: Particle size and ZP of formulation I before and after addition of cationic components (0.1% (w/w) polymyxin B and 0.01% (w/w) preservative)

Code	Cationic components	PCS data	LD diameters			ZP (mV)	
		z-average (nm) PI	d(v)50% (µm)	d(v)90% (µm)	d(v)95% (µm)	Original medium	Water (50 µS/cm)
I		187 ± 5 0.103 ± 0.046	0.206	0.371	0.427	-4.7 ± 0.2	-10.3 ± 0.2
I-1	Polymyxin B + CPC	197 ± 4 0.154 ± 0.043	0.194	0.371	0.431	+21.0 ± 0.6	+10.6 ± 0.5
I-2	Polymyxin B + BAC	191 ± 3 0.133 ± 0.037	0.193	0.375	0.437	+21.9 ± 0.7	+5.4 ± 0.0
I-3	Polymyxin B + CAC	192 ± 3 0.124 ± 0.062	0.194	0.379	0.445	+21.9 ± 0.6	+4.0 ± 1.0

*cetylpyridinium chloride (CPC); benzalkonium chloride (BAC); cetalkonium chloride (CAC)

lation has potential application as drug delivery system for the front of the eye due to its adhesive properties. Additionally, Tween® 80 showed the potential to increase the formulation's long-term stability.

2.4. Selection of total lipid content

Adhesion increases with increasing charge and decreasing size. Two approaches can be applied to obtain small droplet sizes in industrial emulsion production, i.e. increasing the pressure in the production process and reducing the lipid concentration. However, increasing the pressure (e.g. 1,000 bar) is unfavorable for industry. As an alternative, reduction of lipid concentration allows to maintain the process conditions (same pressure), thereby saving cost. In this study, reduction of particle size was achieved by decreasing the lipid concentration from 20 to 15% (w/w). The compositions and characteristics (PCS and LD data) are depicted in Table 5. The obtained results show no significance between formulation I with 2% (w/w) Tween® 80 and formulation J with 4% (w/w) Tween® 80.

Comparing formulations F (20% w/w of lipid phase) and I (15% w/w of lipid phase), which contained the same concentration of Tween® 80, it was observed that an decrease of 5% (w/w) of oil reduced the z-average from 312 to 187 nm. This result was in agreement with a previous study revealing that the mean particle size significantly increases when more oil is incorporated to the formula (Morsi et al. 2014).

For ocular delivery it is important to avoid the excessive use of surfactant, hence the cationic components were added to formulation I with lower Tween® 80 concentration. The results obtained for particle size and ZP in original medium were practically the same for all the combinations (Table 6). Moreover, polymyxin B along with CPC resulted in the highest ZP using Milli-Q water (50 µS/cm) (Table 6). Therefore, this combination (code I-1) was chosen for further investigations.

2.5. Selection of osmolality adjusting agents

Osmolality was adjusted by adding either 0.9% (w/w) NaCl or 2.6% (w/w) glycerol into the selected cationic nanoemulsion (formulation I-1), which is roughly equivalent to tear fluids osmolality. As expected, phase separation was detected for the preparation with NaCl at 40 °C after 1 week storage. This is due to the fact that NaCl as strong electrolyte decreases the stabilizing repulsive electrostatic charge of the emulsion droplets. In contrast, glycerol as nonionic agent did not lead to phase separation for the identical storage period. Hence, glycerol was chosen to adjust the osmolality.

In this work, polymyxin B and CPC, active pharmaceutical ingredient and preservative, respectively, provided cationic property to the formulation instead of cationic lipids or polymer. In addition, unlike results reported earlier (Wang et al. 2010b; Xiang et al. 2007; Zhao et al. 2012), not any organic solvent was required to dissolve DEX and the lipid, which avoids solvent evaporation and residual solvents. The particle size, PI, ZP and content of DEX of final formulation after production were described in section 2.7 (data measured on "day 0").

2.6. Characterization of DEX and Polymyxin B loaded cationic nanoemulsion

2.6.1. Osmolality, pH value and viscosity

Osmotic pressure is a colligative property and depends upon the number of molecules in solution. The eye can usually tolerate preparations equivalent to 0.5-1.8% (w/w) of NaCl (equal to 154-554 mOsm/kg) (International Pharmacopoeia 2014). In practice, the tonicity limits may range from 185-616 mOsm/kg, without causing any discomfort to the eye. The cationic nanoemulsion showed the osmolality of 374±1 mOsm/kg (n=3), which was proved to be in the tolerance range.

Normal tears have a pH of about 7.4 and buffer capacity. The pH of ophthalmic polymyxin B and dexamethasone acetate suspension may vary from 3.5 to 6.0, according the official compendium (United States Pharmacopoeia 2012). The pH value of the formulation should be the one where the drug product is the most stable. The value of cationic nanoemulsion was 5.31±0.5 (n=3). This non-buffered formulation would be quickly diluted by the tears after instillation, and tears have the ability to rapidly restore the physiological pH of the tear film.

Viscosity is also an important quality attribute that should be evaluated aiming the increase of the product retention time in the eye. The viscosity values of cationic nanoemulsions were 3.06±0.01 mPa s at 25.0±0.5 °C and 2.45±0.01 mPa s at 37.0± 0.5 °C (n=3). High viscosity is beneficial to retard the droplets settling and decrease the elimination rate. As a consequence, mucoadhesiveness is increased, thus increasing the bioavailability of active pharmaceutical ingredients.

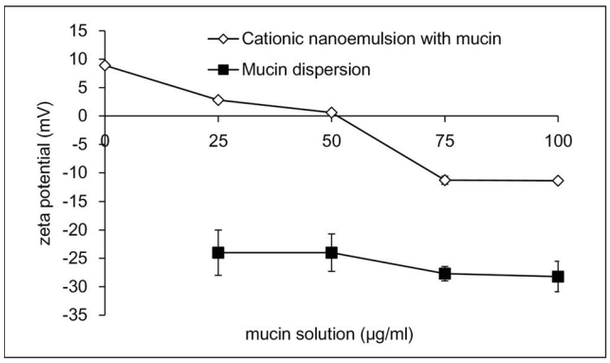

Fig. 1: Zeta potential of the cationic nanoemulsion when mixed with a series of concentrations of mucin dispersions (0, 25, 50, 75 and 100 µg/ml) (measured in Milli-Q water (50 µS/cm)). The charge of mucin dispersions were used as blank control (n=3).

2.6.2. Mucoadhesion

The ionic interaction between the negatively charged mucin and cationic formula is the most expectable mucoadhesive mechanism. The electrostatic interaction could be monitored and indicated by the alteration of ZP, as reported by Bhatta et al. (2012) and Shen et al. (2009). As shown in Fig. 1, along with the increased concentration of mucin preparation, the ZP of cationic nanoemulsion sharply declined from positive charge (approx. +9 mV) to zero, afterwards to negative charge (approx. -11 mV). This result shows the electrostatic interaction between cationic nanoemulsion and mucin dispersions. Furthermore, it shows the adhesion ability of cationic nanoemulsion onto the mucin, which is a flexible, protective layer coating the front of the eye, produced by the conjunctival goblet cells (Shen et al. 2009).

Table 7: Reactivity grade for the *in vitro* cytotoxicity test (n=3)

Sample	Reactivity grade		
	1	2	3
Formulation	0	0	0
Negative control	0	0	0
Positive control	4	4	4

2.6.3. Cytotoxicity test

After a 48-hour observation period, the cell cultures exposed to the discs containing the cationic nanoemulsion showed no signs of reactivity (grade 0) (Table 7). Thus, the formulation revealed no cytotoxicity. In a similar way, the agar diffusion test was suitable to evaluate the cytotoxicity of nanoparticles deposited on latex (Lee et al. 2014). Among the advantages, this method allows to mimic physiological conditions. The zone of diffusion represents a concentration gradient of toxic components and it requires no sample preparation (Baek et al. 2005).

2.7. Stability study

The stability of the DEX and polymyxin B loaded cationic nanoemulsion was investigated. As depicted in Fig. 2, particle size and PI remained stable after 180 days when stored at both 4 °C and room temperature (RT). The ZP in Milli-Q water (50 µS/cm) increased to around +15 mV at 4 °C, while the value decreased to approximately +5 mV at RT (Fig. 3). Regarding to chemical stability (Fig. 4), the amount of DEX after production was 94.1% (w/w), and the values remained unchanged, i.e. 93.9% (w/w) in the samples stored at 4 °C, while they decreased to 85.4% (w/w) in the samples stored at RT after half a year. According to the United States Pharmacopoeia (2012), the product should contain the equivalent of not less than 90.0% (w/w) and not more than 110.0% (w/w) of the labeled amount of DEX. Setting the 94.1% (w/w) of the nominal concentration after production equal to 100% (w/w), then a reduction during storage at RT to 90.8% (w/w) took place. It should be noted, that no protective measures were taken to reduce degradation. Based on this, a shelf life of more than 1 year even at RT seems feasible when taking such measures (e.g. oxygen-free water, nitrogen flushing etc.). Storage at RT is more consumer-friendly. At cooling, a shelf life of 2 years can be predicted. Furthermore, the investigation at 40 °C was terminated after 180 days of storage due to the fact that demulsification became obvious (data not shown). The ZP and amount of DEX dramatically declined over a period of 90 days at the stress temperature of 40 °C.

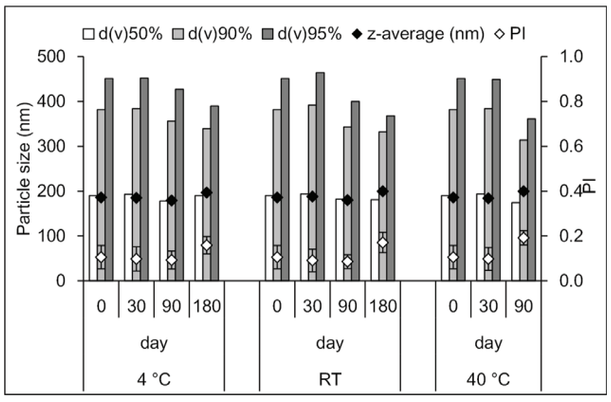

Fig. 2: Z-averages and PIs, LD diameters 50% to 95% of DEX and polymyxin B loaded cationic nanoemulsion over a period of 180 days at different temperatures (note: formulation at 40 °C showed phase separation at 180 days).

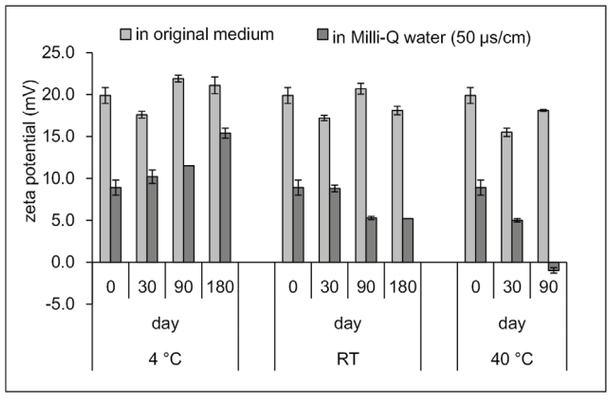

Fig. 3: Zeta potential of DEX and polymyxin B loaded cationic nanoemulsion measured in original medium and in Milli-Q water (50 µS/cm) over a period of 180 days at different temperatures (note: formulation at 40 °C showed phase separation at 180 days).

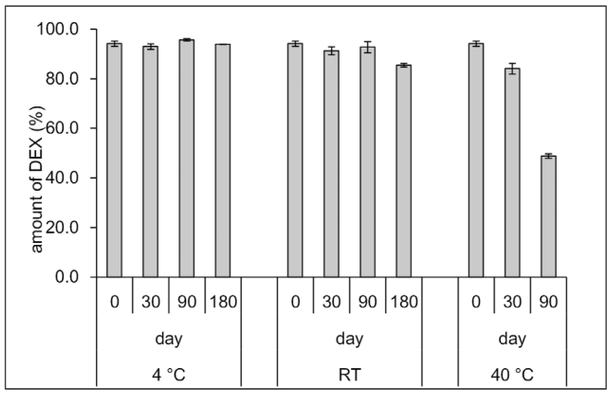

Fig. 4: Amount of DEX in DEX and polymyxin B loaded cationic nanoemulsion over a period of 180 days at different temperatures (note: formulation at 40 °C showed phase separation at 180 days).).

2.8. Conclusions

In the present study, a DEX loaded nanoemulsion has been produced by homogenization technology without the aid of organic solvents, followed by dissolving hydrophilic molecules, i.e. polymyxin B, preservative and neutral osmotic agent to yield a combination product. In this delivery system, the cationic agents are polymyxin B as well as the preservative instead of cationic lipids or cationic polymers. The particle sizes were below 200 nm with narrow size distribution (PI < 0.2) and all the evaluated parameters, i.e. osmolality, pH, viscosity, mucoadhesion and cytotoxicity test, practically allow ophthalmic administration. In addition, the physicochemical characteristics were acceptable at fridge temperature over a period of 180 days storage, with a prediction of a 2 years long-term stability. The study shows the successful development of a cationic nanoemulsion containing both hydrophilic and lipophilic active pharmaceutical ingredients to improve their ocular bioavailability for the treatment of ophthalmic infections. It can be a potentially superior alternative to commercial ophthalmic preparations containing these two drugs. Additionally, this innovative "amphiphilic cationic concept" can be applied to many other ophthalmic products.

3. Experimental

3.1. Materials

Dexamethasone acetate (DEX) (TCI, Tokyo, Japan); polymyxin B sulfate (polymyxin B), potency 8106 IU/mg (Biotika A.s, Slovenska Lupca, Slovak Republic); Cetiol V and Myritol® 312 (Cognis Deutschland GmbH, Düsseldorf, Germany); cottonseed oil, soybean oil, sunflower seed oil, cetylpyridinium chloride (CPC), NaCl and mucin from porcine stomach (Sigma-Aldrich Chemie GmbH, Steinheim, Germany); acidum oleinicum (oleic acid), Eutanol G (octyldodecanol) and Miglyol® 812 (medium chain triglycerides) (Caesar & Loretz GmbH, Hilden, Germany); isopropyl myristate (Merck Schuchardt OHG, Hohenbrunn, Germany); olive oil and sesame oil, refined (Henry Lamotte Oils GmbH, Bremen, Germany); Lipoid S 100 (Lipoid GmbH, Ludwigshafen, Germany); Tween® 80 (polysorbate 80, Uniqema, Everberg, Belgium); Lutrol® F127 (Poloxamer 407, BASF SE, Ludwigshafen, Germany); benzalkonium chloride (BAC) (Merck Schuchardt, Munich, Germany); cetalkonium chloride (CAC) (Alfa Aesar GmbH & Co KG, Karlsruhe, Germany); glycerol (VWR International S. A. S, France); 0.9% (w/w) NaCl (B. Braun Melsungen AG, Melsungen, Germany); freshly obtained double distilled ultra-purified water (Milli-Q, Millipore GmbH, Germany); the other reagents were used of analytical grade.

3.2. Methods

3.2.1. Liquid lipid screening

The solubility of DEX was evaluated through a screening of eleven liquid lipids selected among those suitable for ophthalmic preparations: oleic acid, Cetiol V, cottonseed oil, Eutanol G, isopropyl myristate, Myritol® 312, Miglyol® 812, olive oil, sesame oil, soybean oil and sunflower seed oil. Briefly, increasing amounts of DEX were dissolved in 1 g of the oil at room temperature (RT) with continuously stirring for 1 h. After this period, a visual inspection and polarized light microscopy were conducted to determine the absence of any residual DEX (= complete dissolution). In the case remaining undissolved drug (= solubility exceeded at RT), the dispersion was heated up to 70 °C and maintained at this temperature for 1 h, and subsequently cooled

down to RT. Solubility of DEX after cooling was determined by visual inspection and polarized light microscopy (presence or absence of drug crystals). The selected oil was the one that showed highest solubility after the cooling phase.

3.2.2. Liquid lipid to Lipoid S 100 ratio screening

Lipoid S 100 was added to the liquid lipid to potentially increase the solubility of DEX. To determine the best ratio of selected liquid lipid to Lipoid S 100 which dissolves the highest concentration of DEX, increasing amounts of DEX were added to the melt blend and agitated for 1 h at 70 °C with 200 rpm stirring. Then after cooling down to RT, solubility of DEX in the mixture of liquid lipid and Lipoid S 100 was evaluated by visual inspection and polarized light microscopy (presence or absence of drug crystals). The blend showing the highest drug solubility was referred to as proper lipid matrix.

3.2.3. Preparation of cationic nanoemulsion

On the base of a comprehensive screening, a blend of Eutanol G and Lipoid S 100 (70%:30%, w/w) was selected as lipid phase. Tween® 80 or Poloxamer 407 was employed as emulsifying agent. DEX loaded nanoemulsions were obtained by hot high pressure homogenization. Briefly, the lipid phase (15% or 20%, w/w) was heated to around 70 °C, followed by addition and dissolution of DEX (0.05%, w/w). The heated surfactant solution (70 °C) was added into the lipid phase under high speed stirring at 8000 rpm/min for 1 min using an Ultra-Turrax T25 (Janhke & Kunkel GmbH and Co KG, Staufen, Germany). The pre-emulsion was homogenized by a Micron LAB 40 (APV Deutschland GmbH, Germany) at 600 bar using three cycles and 70 °C. Polymyxin B (0.1%, w/w), one of various preservatives (0.01%, w/w), i.e. CPC, BAC and CAC, and osmolality adjusting agent (0.9% (w/w) NaCl or 2.6% (w/w) glycerol) were admixed to the final nanoemulsion under continuous stirring at 200 rpm at RT. The final formulation was composed of 0.05% (w/w) DEX, 15% (w/w) lipid phase (Eutanol G:Lipoid S 100 = 70%:30%), 2% (w/w) Tween® 80, 0.1% (w/w) polymyxin B, 0.01% (w/w) CPC and 2.6% (w/w) glycerol.

3.2.4. Particle size and ZP analysis

The mean particle size (z-average) and polydispersity index (PI) were determined by photon correlation spectroscopy (PCS), using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). To detect potential large particles, laser diffractometry (LD) was used as additional characterization method using a Malvern Mastersizer 2000 (Malvern Instruments, UK). The obtained data is displayed by a volume percentage of particles having a given size. For instance, diameter d(v)50% represents 50% particles equal to or lower than the given size value, the same for diameters d(v)90% and d(v)95%. The calculation is done based on Mie theory with the optical parameter 1.456 as the real refractive index and 0.01 as the imaginary refractive index.
Light microscopy/polarized light microscopy (Ortophlan, Leitz, Germany) equipped with a digital camera (Moticam 3.0 MP, both from Motic Deutschland GmbH, Germany) was applied to detect the presence of potential large particles and aggregations. Photographic records were performed using the computer program Motic® Image PLUS.
Zeta potential (ZP) is applied to quantify the charge of nanoparticles and forecast the stability in dispersions. The ZP is calculated with the Helmholtz-Smoluchowski equation determined by the measurement of the electrophoretic mobility. Laser Doppler anemometry (Zetasizer Nano ZS, Malvern Instruments, UK) was used for ZP analysis. The measurements were performed in original dispersion medium (aqueous phase of the formulation) and in conductivity adjusted Milli-Q water (50 µS/cm) using 0.9% (w/w) NaCl solution. Measurements were carried out as triplicate.

3.2.5. Osmolality

The osmolality of the cationic nanoemulsion was determined by freezing point depression using a semi-micro osmometer K-7400 (Markus Meske, Berlin, Germany). The sample (100 µl) was directly introduced into the measurement vial, and then placed in the cooling cavity. Measurements were performed in triplicate.

3.2.6. pH

The determination of pH was carried out using a pH 1000 L, pHenomenal® (VWR, Germany). The pH meter was calibrated with standard pH 4.00, 7.00 and 10.00 buffer solutions prior to each measurement. A certain amount of sample was placed in a beaker and the pH was recorded at RT at steady status. Measurements were carried out as triplicate.

3.2.7. Viscosity

The viscosity of cationic nanoemulsions was analyzed with an Ubbelohde viscometer AVS 350 (SCHOTT-GERÄTE GmbH, Germany). The samples were set to equilibrium prior to each measurement and measurements were conducted at 25±0.5 °C and 37±0.5 °C in triplicate, respectively.

3.2.8. Mucoadhesion

In this study, mucoadhesive propensity of cationic nanoemulsion was carried out using mucin as the mucosal component. The commercially available mucin was hydrated in Milli-Q water (5 mg/ml) at 4 °C overnight, and then diluted to the different concentrations for further use. The solution was dispersed using a bath sonicator (BANDELIN electronic GmbH & Co. KG, Germany) for 30 min. 10 µl of sample were dispersed in 1 ml of different concentrations of mucin dispersions (0, 25, 50, 75 and 100 µg/ml) and vortex-mixed for 1 min followed by 10-fold dilution using Milli-Q water (50 µS/cm). This process of adhesion was evaluated by ZP determination. 1 ml of different concentrations of mucin dispersions (25, 50, 75 and 100 µg/ml) was diluted and measured in the same manner as blank control.

3.2.9. Cytotoxicity test

Culture of mammalian fibroblast cells (NCTC clone L-929) were maintained in Minimal Essential Medium (MEM, Sigma, USA) containing Earle's salts and supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10% bovine serum without antibiotics. The culture was incubated at 37±2.5 °C in a humidified incubator with a 5±1% CO_2 atmosphere for a minimum of 48 h.
The optimized formulation was evaluated in triplicate. Latex and Whatman® filter paper (grade n° 1) were used as positive and negative controls, respectively. The formulation was embedded in nontoxic paper disks (Whatman® filter paper, grade nº 1) of 0.5 cm diameter, and positioned over the layer of agar prior to its complete solidification. The Petri dishes were again incubated in the greenhouse with 5% CO_2 at 37±2.5 °C for additional 24 h (USP 35). After this period, the samples were analyzed macroscopically observing the presence or absence of a clear halo in or around the test sample. The diameters of these halos (n=4), when present, were accurately measured, using a calibrated pachymeter (Digimatic, Mitutoyo, Japan). The cytotoxicity was classified as grades 0 to 4. Grade 0 represents no reactivity zone around or under the sample; 1 (slight reactivity) with a reactivity zone limited to an area under the sample; 2 (mild reactivity) with a zone extending less than 0.5 cm beyond the sample; 3 (moderate reactivity), reactivity zone extending 0.5 to 1.0 cm beyond the specimen and grade 4 (severe reactivity) with zone extending more than 1.0 cm beyond the sample.

3.2.10. Stability study

The investigation of the stability was performed storing the cationic nanoemulsions at 4 °C, RT and 40 °C over a period of 180 days. The samples were analyzed at determined time intervals (day 1, 30, 90 and 180).
Physical stability was characterized by several aspects. Particle size and distribution were evaluated by PCS and LD. The phase separation was visually inspected. The drug recrystallization as well as the presence of aggregates during the storage was monitored by light microscopy. The ZP of cationic nanoemulsion was measured in original dispersion medium and in Milli-Q water (50 µS/cm).
Chemical stability of DEX in nanoemulsion was assessed by high performance liquid chromatography (HPLC). The HPLC system was composed of a KromaSystem 2000 version 1.83 (Kontron Instruments GmbH, Germany), an auto sampler model 560, a solvent delivery pump and an UV detector model 430 (Kontron Instruments SpA, Italy). The chromatograph was equipped with a Eurospher 100-5 C18 column 4.6 × 150 mm with the flow rate of 1 ml/min. The observation wavelength was set at 254 nm. The mobile phase consisted of methanol and water (60:40, v/v). Methanol was the diluent for the assay preparation.

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