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Cytotoxicity and anti-inflammatory activity of cyclosporine A loaded PLGA nanoparticles for ocular use

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Cyclosporine A loaded poly(lactide-co-glycolide) nanoparticles were prepared using the o/w emulsification solvent evaporation method and the effect of four preparation parameters on particle size and zeta potential was investigated. Release properties of the nanoparticles were examined and *in vitro* experiments were performed in order to evaluate the cytotoxicity and anti-inflammatory activity of the nanoparticles developed. Particle sizes varied between 191 and 303 nm depending on the different preparation parameters and all nanoparticle dispersions were monodisperse. The nanoparticles showed negative zeta potential values varying between -16 and -35 mV and 57 to 70 % of the amount of loaded cyclosporine A was released after 24 h. None of the nanoparticle formulations showed significant cytotoxicity compared to the negative control using human epithelial cells (HaCaT). Cyclosporine A incorporated in the various nanoparticle formulations retained its anti-inflammatory activity as significant suppression of interleukine-2 secretion in concanavalin A stimulated Jurkat T cells was measured. As the overall influence of the freeze-drying process on the characteristics of nanoparticles was limited, trehalose and carnitine should be preferred as cryoprotectants in ocular formulations for treatment of dry eye disease.

1. Introduction

The bioavailability of ophthalmic drugs is quite low due to reflex blinking and lachrymation, drainage and the barrier function of the cornea and conjunctiva (Lee and Robinson 1986; Ludwig 2005). Several strategies have been developed in order to improve the bioavailability and to prolong the residence time of topically applied drugs. Colloidal drug delivery systems such as liposomes and nanoparticles (NPs) do not cause blurred vision and retain the ease of application like eye drop solutions. However, the potential of liposomes as a topical drug delivery system is limited because of their relative low stability and short half-life on the corneal surface. Colloidal systems of biodegradable polymers hold significant promise for ophthalmic drug delivery and are suitable for poorly water soluble drugs (Nagarwal et al. 2009; Zimmer and Kreuter 1995). NPs are frequently prepared using poly(lactic acid) or its copolymer with glycolic acid (PLGA), due to the biocompatibility and biodegradability of these materials (Sintzel et al. 1996). The NPs need to be retained in the ocular cul-de-sac and release the drug at an appropriate rate in order to achieve prolonged therapeutic activity.

Cyclosporine A (CyA) was selected for incorporation in the NPs. There is a consensus that the core mechanisms of dry eye syndrome are driven by tear hyperosmolarity and tear film instability. Tear hyperosmolarity activates a cascade of inflammatory events causing damage to the surface epithelium. Apoptosis, a loss of goblet cells and disturbance of mucin expression lead to tear film instability. This tear film instability exacerbates ocular surface hyperosmolarity and completes the vicious circle (Pflugfelder 2004). CyA is effective in the treatment of dry eye disease as it will interrupt the vicious circle of inflammation and cell damage on the ocular surface and the lachrymal glands (Pflugfelder 2004). The cyclosporine-cyclophilin A complex binds to calcineurin, inhibiting its dephosphorylating activity and preventing translocation of the Nuclear Factor of Activated T-cells (NF-AT) to the nucleus and thereby preventing increased transcription of the interleukine-2 (IL-2) gene and other genes involved in T-cell activation. CyA binding to cyclophilin D is thought to be primarily responsible for its inhibition of apoptosis. This complex prevents opening of the mitochondrial permeability transition pore, hereby avoiding release of specific proteins such as nucleases and cytochrome c which are involved in the apoptotic pathway (Donnenfeld and Pflugfelder 2009; Waldmeier et al. 2003). CyA eye drops are widely used to treat ocular surface inflammation and its efficacy has been demonstrated in several controlled clinical trials. In 2003, a CyA ophthalmic emulsion (Restasis®) was approved by the FDA to treat dry eye disease (Donnenfeld and Pflugfelder 2009). As Restasis® is often associated with side effects like ocular burning (17 %) and blurred vision, there is still room for formulation improvement. L-Carnitine may play a crucial role in protecting ocular surfaces from hyperosmolarity-induced damage as it can protect against stress activation of corneal epithelial cells cultured in hyperosmolar media (Corrales et al. 2008) and it was able to increase the survival of human corneal epithelial cells when exposed to hyperosmolar solutions (Garrett et al. 2010). In addition, carnitine can be actively transported to the ocular cells (Xu et al. 2010). Moreover, Pescosolido et al. (2009) showed that tear car-

Table 1: Effect of different independent factors on the properties of CyA loaded PLGA NPs

		Main effects			
Response	Mean value	A	B	C	D
Zave (nm)	241.2	**−23.6**	−1.2	**−10.3**	**−56.7**
PI	0.06	**0.02**	0.00	0.00	0.02
ζ potential (mV)	−25.0	1.9	−1	**2.8**	**3.9**

Statistically significant effects are printed in bold ($p < 0.05$).A, HP-β-CD; B, ratio CyA/PLGA; C, volume DCM ; D, homogenisation.

nitine concentrations in dry eye patients are lower than in healthy subjects. L-Carnitine could be a beneficial compatible solute in topically applied formulations as one of the key factors in dry eye disease is an increase in tear osmolarity.

Trehalose solution was effective and safe for the treatment of patients with moderate to severe dry eye syndrome (Matsuo et al. 2002). In experimental murine dry eye trehalose could improve the appearance of ocular surface epithelial disorders due to desiccation through suppression of apoptosis (Chen et al. 2009).

The aim of present study was to investigate the anti-inflammatory activity of the various NP formulations loaded with CyA by performing *in vitro* experiments in Jurkat T cells. A cytotoxic assay was carried out in order to determine the tolerability of the different nanoparticle formulations. Moreover, the effect of freeze-drying using several cryoprotective agents such as trehalose and carnitine on the characteristics of PLGA NPs was studied.

2. Investigations, results and discussion

2.1. NMR

Hydroxypropyl-β-cyclodextrin (HP-β-CD) was included in the formulation in order to enhance ocular bioavailability of CyA. In a CyA/HP-ß-CD inclusion complex it is expected that the lipophilic side chains of CyA will enter the hydrophobic cavity of HP-ß-CD and that shielding of methyl groups will be observed. Therefore the aliphatic region (0.7–1.7 ppm) of the NMR spectrum of the CyA/HP-ß-CD mixture was compared in detail to the spectrum of a CyA solution (Kessler et al. 1985). No substantial differences (< 0.003 ppm) could be detected hence there is no evidence for complexation. Considering the large structure of CyA containing 8 lipophilic side chains, a possible explanation may be that a dynamic equilibrium, whereby all different lipophilic side chains only enter the hydrophobic cavity for a relative short time (i.e. on the average 1/8 of the total time), leads to a proportional reduction of the shielding effect.

2.2. Characteristics of CyA loaded nanoparticles

The influence of four preparation parameters on the NPs' properties was evaluated using a 2^4 full factorial design. The effect of the different factors of the design on the properties of the NPs is summarized in Table 1. A mean diameter of 212.9 nm was obtained for homogenised NPs, which was significantly smaller ($p < 0.05$) than when no homogenisation was applied (269.6 nm). This trend of size reduction can be explained by the formation of smaller emulsion droplets during high pressure homogenisation, resulting in the formation of smaller NPs (Vandervoort et al. 2004).

The factor HP-β-CD has an effect of -23.6 nm on particle size, which means that in this case, the mean particle size of 241.2 nm was reduced with 23.6 nm in the presence of HP-β-CD. To the best of our knowledge, this effect has not been reported before but in the present study HP-β-CD seems to work at the level of the interface in an unknown way, but not related to surface tension, resulting in a lower mean particle size.

Increasing the volume of DCM led to a small, although significant shift towards lower particle sizes ($p < 0.05$). A decrease in viscosity of the inner organic phase increases the diffusion of the inner phase into the external aqueous phase leading to the formation of smaller droplets and thus smaller NPs (Li et al. 2008). Furthermore, a significant interaction between homogenisation and volume DCM was recorded ($p < 0.05$) as the effect of homogenisation changed over the levels of volume DCM and *vice versa* (nonparallel data lines). This interaction is graphically presented in Fig. 1. The trend of size reduction when homogenization was applied was more pronounced at the lower level (2.5 ml) of volume DCM. On the other hand, increasing the volume of DCM led to a shift towards lower particle sizes only when homogenisation was not applied (- level).

Polydispersity index (P.I.) of all dispersions was lower than 0.15 and they therefore could be regarded as monodisperse. Furthermore, no significant difference ($p > 0.05$) was observed between the particle size and P.I. of non-freeze-dried and freeze-dried NP's.

The size of the effects of the different parameters on the zeta potential is shown in Table 1. Zeta potential values varied between -15.7 mV and -35.1 mV. In the present study, homogenisation and a larger volume of DCM resulted in smaller NPs as discussed earlier in section 2.2. These particles possessed less negative zeta potential values as a positive effect of both homogenisation and volume of DCM on the zeta potential is shown in Table 1. These results are similar to those previously reported by Vandervoort et al. (2004), who observed a clear correlation between particle size and zeta potential of pilocarpine HCl loaded PLGA NPs.

The presence of HP-β-CD and the ratio of CyA/PLGA did not significantly affect the zeta potential of the NPs prepared ($p > 0.05$). Furthermore, zeta potential values of freeze-dried NPs had a tendency to be less negative than those of non-freeze-dried NPs ($p < 0.05$).

Loading efficiencies between 44% and 95% were recorded for all CyA loaded NPs and were determined in order to calculate the cumulative release percentages. Inclusion of a homogenisation step significantly decreased drug loadings ($p < 0.05$).

2.3. In vitro drug amount released

The amount of CyA released from four different PLGA NPs in PBS pH 7.4 containing 0.01% (w/V) polysorbate 80 as solubi-

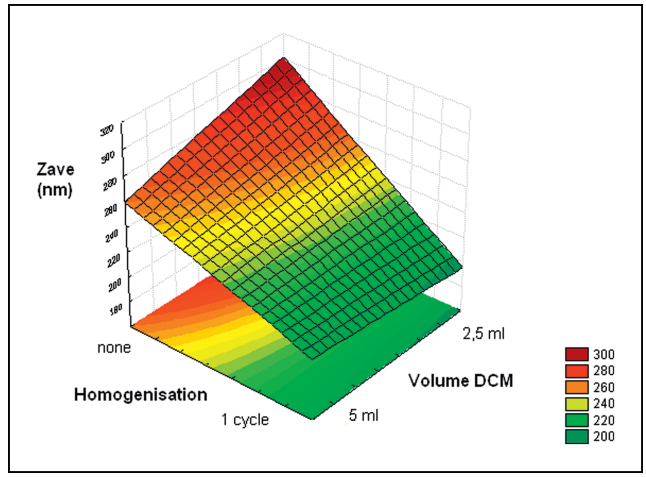

Fig. 1: Respons surface plot of Zave of CyA loaded PLGA NPs. Influence of high pressure homogenisation and volume organic solvent (DCM).

Table 2: Cumulative release of CyA from PLGA NPs in PBS pH 7.4/polysorbate 80 0.01% (w/V) after 1, 3, 6 and 24 h (n = 4)

	Amount of CyA released ± SD (%)			
Formulation	1 h	3 h	6 h	24 h
1 (+)(+)(+)(+)	51,9 ± 1,1	60,5 ± 0,9	60,4 ± 0,5	61,0 ± 0,4
2 (+)(+)(+)(–)	40,0 ± 1,5	52,9 ± 0,6	57,3 ± 1,4	57,3 ± 0,7
10 (–)(+)(+)(–)	39,7 ± 0,4	58,1 ± 4,3	60,7 ± 1,9	62,9 ± 2,6
14 (–)(–)(+)(–)	70,0 ± 1,2	72,2 ± 1,3	69,7 ± 0,7	70,1 ± 1,2

(a)(b)(c)(d) with (a) stands for HP-β-CD, (b) for ratio CyA/PLGA, (c) for volume of DCM and (d) for homogenisation.

lizer is summarized in Table 2. A cumulative release of CyA after 24 h between 57% and 70% was determined. Significantly higher release percentages ($p < 0.05$) were observed from NPs with a lower CyA/PLGA ratio (formulation 14): a burst release of about 70% of the amount of loaded CyA after 1 h was obtained without additional release. In contrast, an initial burst effect followed by a slower release rate was observed for the other nanoparticle formulations. Release of CyA from homogenised NPs (formulation 1) showed a significant larger burst effect ($p < 0.05$) as these particles are smaller and therefore possess a larger surface area, which can lead to a faster release of the drug incorporated. However, similar release percentages than those of non-homogenised NPs (formulation 2) were obtained after 6 and 24 h ($p > 0.05$). HP-β-CD seemed to have a negligible impact on CyA release when release percentages of formulation 2 were compared with those of formulation 10. The absence of an improvement of CyA release in the presence of HP-β-CD could be explained by taking the NMR results into account that showed no evidence for the formation of a CyA/HP-ß-CD inclusion complex with a higher aqueous solubility.

2.4. In vitro assays

2.4.1. Cytotoxicity study

All particle preparations were tested for cytotoxicity towards human keratinocyte cells. After 24 h incubation, the blank formulations did not show significant differences in cytotoxicity compared to the negative control, independent of the added cryoprotectant (data not shown). Only a slight decrease in viability was measured for most CyA loaded nanoparticle preparations (Fig. 2). Some preparations had viability close to 100%, comparable to PBS treated cells (control), and others were less viable where lowest viability was noticed for homogenized NPs containing HP-β-CD and prepared with a large volume of DCM and high CyA/PLGA ratio (formulation 1). However, the differences among preparations as well as compared to control culture were not statistically significant ($p > 0.05$) (ANOVA One way; Tukey post hoc analysis). In contrast, a 0.2% (w/V) benzalkonium chloride solution significantly reduced viability of cells ($p < 0.001$). This observation is in line with previous work showing that this cationic detergent is considerably cytotoxic (Debbasch et al. 1999; Uematsu et al. 2010).
Incubation of cells with different concentrations and even undiluted NP formulation 2 showed viability comparable to PBS treated cells after 10 minutes incubation (data not shown). In contrast, Aksungur et al. (2011) reported that the cytotoxic effects of various blank NP formulations, with both a positive and negative surface charge, were time and concentration dependent. Although absence of *in vitro* cytotoxicity of pharmaceutical formulations does not fully correspond to absence of *in vivo* irritation or sensitivity, the results obtained in the present study at least indicate that the PLGA particles with negative zeta potential do not interact with cell surfaces in such a way disturbing viability.

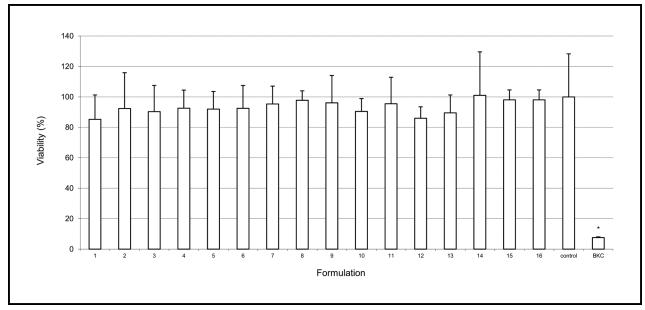

Fig. 2: Viability of keratinocyte epithelial cells (HaCaT) after incubation with CyA loaded NPs for 24 h (n = 3). BKC: 0.2% (w/V) benzalkonium chloride solution. Statisically significant lower viability of cells treated with BKC is labelled with * *(p < 0.05)*.

2.4.2. Effect of the nanoparticle formulations on IL-2 expression in activated Jurkat T cells

In order to determine the anti-inflammatory activity of the CyA loaded nanoparticle formulations, an *in vitro* screening model to identify T cell suppressive activity was developed because CyA affect T cell activation by its cyclophilin A binding activity and NF-AT inhibition (Hermans et al. 2012). Briefly, the appropriate stimulatory conditions needed to be identified. A dose-response curve with concanavalin A (ConA), a well-known aspecific T cell activator, and interleukine-2 (IL-2) as indicator for activation showed a maximum IL-2 secretion at 37.5 µg/ml ConA. This concentration was selected for all further experiments. A dose-response curve for the CyA batch used throughout this study was established by adding different concentrations CyA 45 minutes prior to ConA activation of Jurkat T cells (Baldari et al. 1991). The IC_{50} concentration calculated by regression analysis for IL-2 suppression was 11.79 ng/ml CyA.
A volume of 1 µl nanoparticle suspension was added to cell culture medium 45 minutes prior to ConA stimulation of Jurkat T cells. Next, supernatant was analyzed for the amount of IL-2 secreted by the cells. As seen in Fig. 3, inhibition of IL-2 secretion of more than 90% was observed for all nanoparticle preparations. A small but significant difference in IL-2 suppression was observed for NPs prepared with HP-β-CD (ANOVA One way; post hoc Tukey analysis). The presence of HP-β-CD resulted in an increase of IL-2 in cell culture supernatant, implicating a lower suppression capability. However, the small effect will probably not be clinically relevant. A considerable difference with stimulated cells (positive control) was noticed ($p < 0.001$). Blank NPs showed absence of suppression (data not shown), indicating that other excipients in the nanoparticle for-

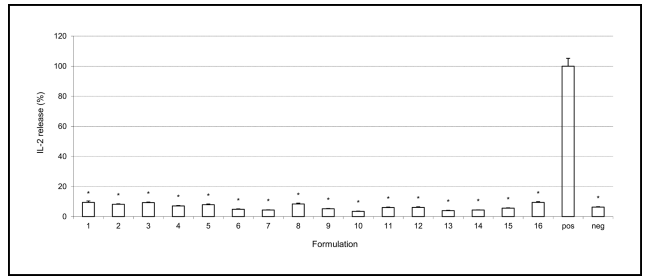

Fig. 3: Suppression of IL-2 secretion in Jurkat T cells stimulated with 37.5 µg/ml ConA by CyA loaded PLGA NPs (n = 3). pos: ConA stimulated cells; neg: unstimulated cells. IL-2 secretion significantly lower than the positive control is labelled with * (p < 0.05).

Table 3: Physicochemical properties of unloaded PLGA NPs (n = 2) before and after freeze-drying (sonication as reconstitution method)

Cryoprotectant	Sf/Si ± S.D.	Aggregation score	Zeta potential (mV)	
5% (m/V)			BF	AF
No cryoprotectant	0.953 ± 0.022	0	−11.4 ± 0.4	−20.8 ± 1.6
Mannitol	1.088 ± 0.049	0	−21.2 ± 0.8	−14.7 ± 3.8
Sorbitol	1.004 ± 0.004	0	−16.2 ± 6.3	−20.2 ± 0.6
Trehalose	0.991 ± 0.003	0	−16.4 ± 0.8	−17.8 ± 4.9
Glucose	1.029 ± 0.037	0	−18.7 ± 1.3	−23.0 ± 3.8
Carnitine	0.975 ± 0.013	0	−15.8 ± 1.1	−19.3 ± 1.8

Sf, particle size after freeze-drying; Si, particle size before freeze-drying; BF, before freeze-drying; AF, after freeze-drying. Aggregation score: (0) absent, (1) scarce, (2) significant aggregation.

mulations such as cryoprotectants had no effect, and inhibition can solely be attributed to CyA.

2.5. *Influence of cryoprotectants*

After freeze-drying, easy and rapid reconstitution and unchanged physicochemical properties are important features. Therefore, the effect of several cryoprotective agents on the characteristics of PLGA NPs was studied. In particular, trehalose and carnitine were investigated as trehalose and carnitine solutions are promising additional treatments for dry eye disease.

Table 3 summarizes the ratio of initial particle size (Si) and particle size after freeze-drying (Sf), aggregation characteristics and zeta potential values of unloaded PLGA NPs. Overall, a limited influence of the freeze-drying process on the characteristics of blank NPs was observed when sonication was used as reconstitution method. No increase in particle size was determined when no cryoprotectant was employed or sorbitol or glucose were added to the formulation. For formulations with trehalose and carnitine even a smaller particle size was determined after freeze-drying ($p < 0.05$). Here, sonication during reconstitution could have broken down agglomerates formed during preparation and freeze-drying more efficiently. On the other hand, the polydispersity index (P.I.) was not significantly altered by the reconstitution step (data not shown). Moreover, the P.I. of all dispersions was lower than 0.20 before and after freeze-drying and therefore could be regarded as monodisperse. A significant size increase was measured only when mannitol was used as cryoprotectant ($p < 0.05$). Aggregation scores were 0 for all formulations, indicating a good redispersion.

Similar particle size values and monodisperse nanoparticle suspensions were obtained when sample reconstitution was performed by adding milliQ water to the dried cake of NPs and applying a mild manual shaking instead of sonication (data not shown). Only for formulations without cryoprotective agents the reconstitution was more difficult, accompanied with the presence of a large number of agglomerates (aggregation score of 2).

Zeta potential values of freeze-dried NPs were significant more negative than those of non-freeze-dried NPs in the absence of cryoprotectant, and also when glucose, sorbitol or carnitine were used although less pronounced ($p < 0.05$). In contrast, addition of mannitol to the nanoparticle formulations resulted in less negative zeta potential values after reconstitution. Although none of the blank NPs retained their initial characteristics on reconstitution after freeze-drying, the overall influence of the freeze-drying process was limited. It can be concluded that trehalose and carnitine are preferred as cryoprotectants as no size increase was measured after reconstitution and it is known that these agents protect against ocular surface disorders such as dry eye syndrome.

3. Experimental

3.1. *Materials*

Cyclosporine A (CyA) (MW 1 203) was purchased from Brook Chemical Limited (Shanghai, China). The poly(lactic-co-glycolic acid) (PLGA) polymer employed was Resomer® RG 503 (Boehringer Ingelheim, Ingelheim am Rhein, Germany) with a molecular weight of 40 000 and a D, L-lactide/glycolide molar ratio of 52/48. Poly(vinylalcohol) (PVA) (MW 30 000-70 000), D-(+)-trehalose dihydrate, D-(+)-glucose anhydrous and D-sorbitol were purchased from Sigma Chemicals Co. (St. Louis, USA). Dichloromethane (DCM), acetonitrile (ACN) and hydroxypropyl-β-cyclodextrin (HP-ß-CD) (MW 1 380) were obtained from Sigma-Aldrich (Steinheim, Germany), D-mannitol from Sigma-Aldrich Chemicals (Buchs, Switzerland), L-carnitine from Fagron (Waregem, Belgium) and denatured ethanol 96% (v/v) from Laboratoires Lohmann & Rauscher (Liège, Belgium). Salts for the preparation of phosphate buffered saline (PBS pH 7.4) were of pro analysis quality. PBS pH 7.4 is an electrolyte solution composed of 8.2 g/l NaCl, 0.3 g/l $NaH_2PO_4.2H_2O$ and 1.54 g/l $Na_2HPO_4.2H_2O$. Throughout the experiments filtered (Sartorius 0.2 μm membrane filter, Goettingen, Germany) milliQ (Millipore, Mollsheim, France) water was used.

3.2. *Factorial design of experiments*

The influence of 4 different parameters on the NPs' properties was evaluated using a 2^4 full factorial design composed of 4 factors which were set at two levels each. The 4 factors investigated were the addition of HP-β-CD, ratio CyA/PLGA, volume organic phase (DCM) and the use of a high pressure homogenizer. The different preparations were made in duplicate in order to estimate the experimental error. The design required 2^4 experiments (Table 4) with one replica each or a total amount of 32 nanoparticle preparations. The lower and higher value can be represented by a (-) or (+) level, which is shown in Table 5. The statistical analysis of the data was performed with Statistica® software (Statsoft, Tulsa, USA).

Table 4: Factorial design of experiments: 2^4 full factorial design for the preparation of CyA loaded PLGA NPs

Formulation	HP-β-CD	Ratio CyA/PLGA	Volume DCM	Homogenisation
1	+	+	+	+
2	+	+	+	–
3	+	+	–	+
4	+	+	–	–
5	+	–	+	+
6	+	–	+	–
7	+	–	–	+
8	+	–	–	–
9	–	+	+	+
10	–	+	+	–
11	–	+	–	+
12	–	+	–	–
13	–	–	+	+
14	–	–	+	–
15	–	–	–	+
16	–	–	–	–

Table 5: The values for the lower (–) level and upper (+) level of the factors investigated in the factorial design

Factor	(–) level	(+) level
HP-β-CD:CyA (molar ratio)	0	10:1
Ratio CyA:PLGA (mass ratio)	1:10	1:5
Volume DCM	2.5 ml	5 ml
Homogenisation	none	1 cycle

3.3. Preparation of CyA loaded PLGA nanoparticles

CyA loaded NPs were prepared by the o/w solvent evaporation method. Briefly, 250 mg PLGA and CyA (25 or 50 mg) were dissolved in DCM (2.5 or 5 ml), optionally supplemented with HP-β-CD depending on the experiment of the factorial design (Table 4). In this case, NPs were prepared using an amount of CyA/HP-β-CD mixture (1/10 molar ratio) containing 25 or 50 mg CyA. The organic solution was then emulsified in 12.5 ml of a 1% (w/V) aqueous PVA solution by ultrasonication (45% amplitude, ± 27 W, 60 s; Branson digital sonifier 450-D, Danbury, USA) and optionally pushed once through a high-pressure homogenizer at an operating pressure of 500 bar (M-110L, Microfluidics, Newton, USA) in order to reduce the droplet size and to obtain a more narrow size distribution (Vandervoort et al. 2004). The resulting o/w emulsion was diluted in 60 ml of a 0.3% (w/V) aqueous PVA solution. The solvent was allowed to evaporate at room temperature for 4 h under magnetic stirring at 700 rpm (Variomag Electronicrührer Poly 15, H + P Labortechnik GmbH, Münich, Germany). To the resulting suspension, 5% (w/V) mannitol was added and samples were freeze-dried (FreeZone® 1 liter benchtop freeze dry system, Labconco, Kansas City, USA). Each type of formulation was prepared in duplicate.

3.4. Preparation and freeze-drying of blank PLGA nanoparticles

Blank nanoparticles were prepared similar as described in section 3.3. PLGA was dissolved in 5 ml DCM, supplemented with HP-β-CD and the o/w emulsion was pushed once through a high-pressure homogenizer at an operating pressure of 500 bar (M-110L, Microfluidics, Newton, USA). Blank nanoparticle suspensions were freeze-dried in the absence of cryoprotectant or in the presence of 5% (w/V) mannitol, sorbitol, trehalose, glucose and carnitine in order to investigate the influence of presence and type of cryoprotectant on the characteristics of freeze-dried NPs. Each type of formulation was prepared in duplicate.

3.5. NMR study

NMR proton spectroscopy has been used to study possible complexation of CyA by HP-β-CD. NMR spectra of a solution of the CyA/HP-β-CD mixture in CD_3OD (99.8% D) were recorded at 30 °C on a Bruker DRX 400 NMR spectrometer operating at 400 MHz and 64 scans were accumulated.

3.6. Physical characterization of nanoparticles

3.6.1. Particle size analysis

The mean particle size (Z_{ave}) was determined by Photon Correlation Spectroscopy using a Zetasizer 3000 (Malvern Instruments, Malvern, UK) after reconstitution (30 s vortex, 5 min sonication (Julabo USR3, Julabo Labortechnik GmbH, Seelbach, Germany)) of the lyophilized NPs in milliQ water. The initial nanoparticle suspension was diluted 10 times with milliQ water before measuring. The Z_{ave} of each sample was determined three times and the average values were used for further calculations. The ratio of initial particle size (Si) and particle size after freeze-drying (Sf) of blank PLGA NPs, supplemented with different cryoprotectants was determined. The ease of reconstitution was evaluated for both mild manual shaking and 5 min sonication of the nanoparticle suspensions. Particle aggregation was quantified by the following numerical scale: (0) absent, (1) scarce and (2) significant aggregation.

3.6.2. Zeta potential analysis

The zeta potential of the initial nanoparticle suspension or reconstituted NPs was determined by Electrophoretic Light Scattering (ELS) using a Zetasizer 3000 (Malvern Instruments, Malvern, UK). Measurements were carried out in milliQ water. The samples were injected in the capillary cell of the Zetasizer 3000 and the zeta potential was determined 12 times. The average values were used for further calculations.

3.7. HPLC assay

Quantitative determinations of CyA were performed by a validated HPLC assay. The HPLC system consisted of a pump (Shimadzu LC-20AT; isocratic), UV-VIS detector (Shimadzu SPD-M20A), auto sampler SIL-20A and column (LiChroCART 125–4 HPLC-Cartridge: LiChrosper 100 RP-18 (5 μm)). The column temperature was set at 61 °C, the mobile phase consisted of ACN:water (80:20) at a flow rate of 1.0 ml/min and detection was performed at 210 nm. The injection volume was 50 μl and the retention time in the set condition was 3.25 min. The HPLC assay showed good linearity ($R^2 = 0{,}999$) and the range that was validated for this method was between 1 μg/ml and 40 μg/ml. Limit of quantification and limit of detection were 0.1 μg/ml and 0.05 μg/ml respectively.

3.8. Determination of drug loading

In order to determine the drug loading or drug concentration present in the nanoparticle suspensions, an amount of 50 mg of each freeze-dried sample was accurately weighed and reconstituted in 10 ml ACN:PBS pH 7.4 (1:1 V/V) and sonicated (Julabo USR3, Julabo Labortechnik GmbH, Seelbach, Germany) for 1 h. Next, samples were centrifuged for 2 h at 4500 rpm and 4 °C (Sigma 3–16PK, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The amount of CyA in the supernatant was determined by HPLC analysis as described above. Each experiment was repeated in triplicate. The total drug loading in the nanoparticle formulations (DL) was determined employing the following equation:

$$DL(\%) = (\text{measured amount of CyA/theoretical amount of CyA in the sample}) \times 100.$$

3.9. In vitro drug release

PBS pH 7.4 containing 0.01% polysorbate 80 was selected as release medium. In the absence of a surfactant the drug release is limited by the very low aqueous solubility of CyA (± 25 μg/ml). When a high concentration of polysorbate 80 is present (well above the CMC) a burst release of 60–90% of the amount CyA loaded in NPs was measured after 1h (Hermans et al. 2012). The very hydrophobic CyA is solubilised inside micelles. This situation will not occur *in vivo*. Consequently the medium PBS pH 7.4 with 0.01% polysorbate 80 was used in the present study.

After instillation in the cul-de-sac, nanoparticles will interact with the mucus layer at the cornea and conjunctiva and a sustained drug release could be expected from NPs entrapped. Considering the possible behaviour of NPs *in vivo*, four time points were chosen to measure the amount of CyA released: 1, 3, 6 and 24 h. In this way a rapid, moderate and slow remove of NPs from the eye was simulated.

In order to measure the amount CyA released from PLGA NPs, 50 mg of freeze-dried sample was accurately weighed, transferred to a glass vial and dispersed into 10 ml PBS pH 7.4 with 0.01% (w/V) polysorbate 80 as solubilizer (5 min sonication (Julabo USR3, Julabo Labortechnik GmbH, Seelbach, Germany)). The vials were placed in an oscillating water bath (30 oscillations/min) at 32 °C and at specified time intervals (1, 3, 6 and 24 h) 4 ml sample was withdrawn and replaced by an equal volume of fresh release medium. The sample was centrifuged at 15300 rpm for 20 min (Sigma 3–16PK, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) and the CyA concentration in the supernatant was determined using the HPLC method as described above. Each experiment was repeated in triplicate.

3.10. Cytotoxicity assessment of nanoparticle preparations

Preparations used for ophthalmic purpose need to show minimal cytotoxicity. In order to meet this requirement, preparations were tested using keratinocyte epithelial cells (HaCaT) analogous to Dillen et al. (2007). HaCaT cells are a valuable alternative to primary epithelial cell lines in order to assess whether the NPs would affect viability of cells in general.

The evaluation of the *in vitro* cytotoxicity of the NPs was based on cell viability, determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells were cultured in RPMI 1640 GlutaMax (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 100 μg/ml Penicillin/100 U Streptomycin (Invitrogen) (Dillen et al. 2007). Nanoparticle preparations (1 μl) were diluted in growth medium and cells were further cultured for 24 h incubation at 37 °C/5 % CO_2. A 0.2% (w/V) benzalkonium chloride solution (BKC) was used as positive control for cytotoxicity. The amount of surviving cells was quantified by the MTT analysis as described by Dillen et al. (2007). Additional experiments with undiluted formulations were performed in order to mimic the *in vivo* situation on the corneal surface. Cells were incubated for 10 min with a dilution series of formulation 2 and cytotoxicity was evaluated as discussed above.

3.11. Cell culture and cytokine expression studies

In order to evaluate the influence of the preparation conditions of the NPs on the biological activity of CyA, *in vitro* experiments using Jurkat T cells were performed as T cells are involved in dry eye pathology. An established cell line was used in order to reduce the biological variation compared to primary cell culture. The biological read-out was IL-2 secretion of concanavalin A (Con A) activated cells.

Jurkat T cells were grown in RPMI 1640 GlutaMax (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 100 µg/ml Penicillin/100 U Streptomycin (Invitrogen) and incubated at 37 °C/5 % CO_2. Cells were passed every 2–3 days. For cytokine expression studies, cells were seeded at a density of $2x10^4$ cells/well in 96 well plates. After 24 h, cells were treated as described in the results section.

IL-2 in cell supernatant was measured using the DuoSet human IL-2 ELISA kit (R&D Systems, UK) following manufacturer's instructions. Quantification was performed at 450 nm on an Infinite M200 device (Tecan, Mechelen, Belgium) using the Magellan^{TM} software analysis package. In parallel, cytotoxicity on cells was determined as described for the HaCaT cells above to exclude suppression of IL-2 expression solitary due to cell death.

4. Conclusion

In conclusion, none of the blank NPs retained their initial characteristics on reconstitution after freeze-drying, although the overall influence of the freeze-drying process was limited. It can be concluded that trehalose and carnitine are preferred as cryoprotectants as no size increase was measured after reconstitution and it is known that these agents protect against ocular surface disorders such as dry eye syndrome. *In vitro* tests revealed the absence of cytotoxic effects for all NPs prepared and significant suppression of IL-2 secretion by all CyA loaded NPs in ConA stimulated Jurkat T cells was observed.

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