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Research paper

Development of cationic nanocrystals for ocular delivery

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ABSTRACT

A cationic nanocrystal formulation containing dexamethasone acetate nanocrystals (0.05%) and polymyxin B (0.10%) for ophthalmic application was produced using a self-developed small scale method for wet bead milling. The formulation developed offers the advantage of increased saturation solubility of the drug (due to the nano-size of the crystals) and increased residence time in the eye (due to small size and increased mucoadhesion by the cationic charge) resulting ultimately in potential increased bioavailability. Characterization of the nanosuspensions by photon correlation spectroscopy (PCS) and transmission electron microscopy showed that the production method was successful in achieving dexamethasone crystals in the range of about 200–250 nm. The physical stabilization of the nanocrystals and generation of the positive charge were realized by using cetylpyridinium chloride (CPC) and benzalkonium chloride (BAC) at the concentration of 0.01%. In contrast to other cationic excipients, they are regulatorily accepted due to their use as preservatives. The drug polymyxin B also contributed to the positive charge. Positive zeta potentials in the range +20 to +30 mV were achieved. Isotonicity was adjusted using NaCl and non-ionic excipients (glycerol, sorbitol, dextrose). Physical and chemical stabilities were monitored for a period of 6 months at room temperature, 5 °C and 40 °C. Particle size of the bulk population assessed by PCS remained practically unchanged over 6 months of storage for the various formulations without isotonicity agents, and for the CPC-containing formulations with non-ionic isotonicity excipients. The chemical content also proved stable after 6 months for all 3 temperatures evaluated. *In vitro* investigation of mucoadhesion was tested using mucin solutions at different concentrations, and the generated negative zeta potential was used as a measure of the interaction. The zeta potential reversed to about −15 mV, indicating distinct interaction. The results show the potential of increased mucoadhesion of such cationic nanocrystals compared to standard eye drop formulations. The positively charged nanocrystal formulation also showed no *in vitro* cytotoxicity as assessed on fibroblast cell culture. In summary, 3 formulation candidates were identified being a promising alternative for ocular delivery with increased performance compared to what is presently available.

1. Introduction

Drugs formulated as conventional ophthalmic preparations, such as eye drops, are quickly removed from the ocular surface as a consequence of the protective mechanisms of the eye, i.e. reflex blinking, lacrimation and lacrimal fluid turnover. Therefore, the retention time of drugs on the eye is very limited and consequently, bioavailability is very low - normally less than 5%. After instillation, the excess volume of the instilled liquid is drained by the nasolacrimal duct. Additionally, the constant turnover of the lacrimal fluid (around 1 μL/min) associated with potential systemic absorption from the conjunctival sac capillaries contributes to the low concentration of drug on the eye surface [1]. Prolonged release dosage forms may discretely increase the bioavailability, but in clinical practice, these systems have not yet been widely accepted [2]. Permeation across corneal and conjunctival epithelial barriers is very limited (even for modern prolonged delivery); therefore, when the drug target is the posterior segment of the eye (retina, vitreous choroid), an alternative is to administer high doses of the drug by intravenous or intravitreal route [3].

Nevertheless, these invasive administration routes are not practical and only effective for a limited number of diseases (and drugs). Thus, currently, there are some formulation strategies are in development to increase the duration of action of the applied drug, e.g. gels, gelifying formulations, ointments, inserts [4]. The high viscous formulations can cause blurred vision after application, and user-

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unfriendly are also inserts as the Ocusert® pilocarpine system (Ocusert Pilo) for the sustained topical ocular delivery of pilocarpine. It disappeared from the market. An optimum ocular drug delivery system should be one which can be delivered in the form of eye drops, causing no blurred vision or irritability and would need no more than one to two administrations per day [5]. This was the aim of the present study using nanocrystal suspensions.

Micro and nanoparticulate polymeric systems have been proposed as alternatives for ocular delivery [6]. However, one of the major problems related to these polymeric systems is that the drug release sometimes takes longer than the ocular residence time of the polymeric particles themselves. Liposomes have also been extensively investigated, but problems associated with their irritation potential and formulation stability are the main disadvantages [6]. Some other delivery systems currently being investigated for ocular delivery include dendrimers, cyclodextrins, nanoemulsions, niosomes and solid lipid nanoparticles (SLN) [7,8].

Problems associated with the previous delivery systems can be overcome by nanonization of the pure drug powder, resulting in drug nanocrystals (preferentially approx. 100–500 nm for maximum adhesiveness). Nanocrystals possess increased saturation solubility, dissolution velocity and additionally increased mucoadhesion [9]. After application to the eye fluid, they start immediately to dissolve fast (burst release), and the increased saturation solubility leads to an increased concentration gradient and thus increased diffusive flux into the eye surface. Not completely dissolved nanocrystals stay adhered to the eye surface for a longer time, thus acting as depot from which constantly new drug dissolves. From the regulatory point of view, in contrast to other nanoparticulate systems, nanocrystals have the distinct advantage that they are composed purely of drug; there is no matrix material such as polymer or lipid matrix. This is especially important for ocular delivery, since excipients legally authorized by regulatory agencies for ocular use are very limited [9]. In addition, because the drug loading of nanocrystals is 100% (i.e. they consist of pure drug), the instilled volume can be reduced/kept low, which contributes to a longer retention of the applied doses on the eye surface.

Mucin, the mucus layer that coats the corneal surface, is negatively charged. Therefore, the ideal carrier system for the eye would be a cationic particle with high adhesion to the mucosa. This principle was exploited before in other nanoparticulate systems by [10–12]. Therefore, cationic nanocrystals promoting an increase in the saturation solubility of the drug, together with increased mucoadhesiviness have the potential to distinctly improve the drug ocular bioavailability.

A problem is the selection of the electrostatic charge provider. Many cationic lipids do not possess a regulatorily accepted status for ocular administration and are often expensive. Similar problems exist for positively charged polymers such as chitosan or polyethylenimine (PEI).

In this study, positively charged dexamethasone acetate nanocrystals combined with polymyxin B sulfate in an ophthalmic formulation were developed. The problem of positive charge generation was solved by using a positively charged drug in combination with a positively charged preservative. The chemical and physical short-term stability of the ocular nanocrystal suspension (nanosuspensions) was assessed, as well as its *in vitro* mucoadhesion potential and its tolerability was confirmed by cytotoxicity investigations.

2. Materials and methods

2.1. Materials

Dexamethasone 21-acetate was purchased from TCI (Japan), polymyxin B sulfate from Biotika A.s. (Slovak Republic), benzalkonium chloride from Merck Schuchardt (Germany) and glycerol 85% from Fragon GmbH & Co. KG (Germany). Mucin type III, sodium chloride, dextrose, sorbitol and cetylpyridinium chloride were purchased from Sigma-Aldrich Chemie GmbH (Germany). Double distilled and ultrapurified water was obtained from a Milli-Q apparatus (Millipore GmbH, Germany). All other reagents were from analytical grade.

2.2. Nanosuspension production

The nanosuspension production was performed by a self-developed miniaturized wet bead milling method [13]. The coarse suspension was composed of 5% dexamethasone acetate, 1% stabilizer and optionally 1% polymyxin B sulfate (all w/w). The stabilizers tested were cetylpyridinium chloride and benzalkonium chloride.

Briefly, the coarse suspension was processed in a 2 mL glass vial containing yttria stabilized zirconium oxide beads with diameter of 0.05 mm as the grinding media. Stirring was performed on a magnetic stirring plate RCT basic (IKA-Werke GmbH & Co. KG, Germany). Milling efficiency was increased by a special arrangement of 3 stirrers located on top of each other.

2.3. Particle characterization

2.3.1. Photon correlation spectroscopy

The hydrodynamic diameter (z-average, z-ave) of the nanocrystals was determined by photon correlation spectroscopy (PCS), using a Zetasizer Nano ZS (Malvern Instruments, UK). The results are the z-average, which is the intensity weighted mean diameter of the bulk population, and the polydispersity index (PdI), which is a measure for the width of the size distribution. Samples were diluted in water to a suitable concentration and the average values were calculated from 10 single measurements.

2.3.2. Zeta potential

The zeta potential is a measure of the electrostatic charge on the surface of the particle and is a tool to predict the physical stability of colloidal suspensions. It was measured using a Zetasizer Nano ZS (Malvern Instruments, UK) in two different media: original dispersion medium of the nanosuspension (= solution with stabilizer and preservative) and Milli-Q water (adjusted to 50 μS/cm conductivity by addition of NaCl and at pH 5.5). The electrophoretic mobility was measured and converted into zeta potential by the Helmholtz-Smoluchowski equation.

2.3.3. Light microscopy (LM) and transmission electron microscopy (TEM)

To verify the presence of large particles or agglomerates, light microscopy using a microscope Orthoplan (Leitz, Germany) was performed at 160, 600 and 1000 fold magnifications. Additional particle characterization was performed by transmission electron microscopy (TEM) using a Tecnai G^2 20 S-TWIN (FEI company, USA).

2.4. Dilution and isotonicity adjustment

Nanosuspensions containing 5% (w/w) of dexamethasone acetate obtained from the milling process were further diluted to the desired concentration of the final ocular formulation and the tonicity was adjusted, resulting in a final formulation with 0.05% of dexamethasone, 0.1% of polymyxin B, 0.01% of the stabilizer and adequate concentrations of one of the tonicity agents (0.9% NaCl; 2.6% glycerol; 5.5% sorbitol; 5.0% dextrose) (all w/w).

2.5. pH

The pH was determined 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. The pH was recorded at room temperature (RT) at steady status in triplicate.

2.6. In vitro mucoadhesion evaluation

To investigate the potential improved mucoadhesion of the nanocrystals produced, the zeta potential of these nanocrystals was measured after admixing the nanosuspension to mucin solutions with increasing mucin concentrations (10 μL dexamethasone nanosuspension + 990 μL mucin solution). First, mucin was dispersed in water at a concentration of 0.5% (w/w) and left stirring overnight under refrigeration for complete hydration. The obtained solution was subsequently diluted to obtain mucin solutions with the following concentrations: 0.025, 0.050, 0.075, 0.10, 0.15, 0.20, 0.25, 0.50 and 1.00 mg/mL. The changes in the nanocrystals surface charge, i.e. turning from positive to negative (indicative of interaction of the mucin molecules with the nanocrystals) were assessed by ZP measurements.

2.7. In vitro cytotoxicity evaluation

The *in vitro* cytotoxicity evaluation was performed according to the official compendium (USP 35) using culture of mammalian fibroblast cells NCTC clone L-929, general chapter ⟨87⟩, by biological reactivity of this mammalian cell culture following direct contact with the samples. The selected formulation was evaluated in triplicate. The positive and negative controls were, respectively, Latex and Whatman® filter paper (grade n° 1). The formulation was embedded in nontoxic paper disks (Whatman® filter paper, grade n° 1) of 0.5 cm diameter, and positioned over the layer of agar composed of Minimum Essential Medium, twice concentrated (MEM, Sigma) and agar (BBL, Becton Dickinson) at 1.8% (w/v), containing 0.01% (w/v) of neutral red (Merck), as vital dye, prior to its complete solidification. The diameters of the halos formed by the reactivity of the cells in contact with the samples were measured using a calibrated pachymeter (Digimatic, Mitutoyo, Japan). The cytotoxicity was presented as grades 0–4. Grade 0 shows no reactivity zone around or under the sample: 1 (slight reactivity) with a reactivity zone under the sample; 2 (mild reactivity) with a zone less than 0.5 cm beyond the sample; 3 (moderate reactivity), reactivity zone from 0.5 to 1.0 cm beyond the specimen and grade 4 (severe reactivity) with zone more than 1.0 cm beyond the sample.

2.8. Short-term physical stability

After adjustment of the tonicity with sodium chloride, glycerol, sorbitol or dextrose, samples were stored at room temperature, under refrigeration (5 °C) and 40 °C. Aliquots were drawn immediately after preparation, after 1 day, 7 days, 2 weeks, 1 month, 3 months and 6 months and particle size was assessed using PCS and light microscopy.

2.9. Short-term chemical stability

Dexamethasone chemical stability was assessed by high performance liquid chromatography (HPLC) using a KromaSystem 2000 version 1.7 (Kontron Instruments GmbH, Germany), an auto sampler model 560, a solvent delivery pump and an UV detector model 430 (Kontron Instruments SpA, Italy) at 254 nm. The analytical column was an Eurosphere-100 C18 5 μm (4.6 × 150 mm) with a flow rate of 1 mL/min at 25 °C. The samples were dissolved in methanol and the mobile phase was composed of a mixture of methanol:water at a proportion of 6:4.

3. Results and discussion

3.1. Nanosuspension production and characterization

Dexamethasone acetate is a poorly water soluble and crystalline synthetic glucocorticoid active pharmaceutical ingredient. This drug suppresses the ocular inflammation and it is indicated to treat conjunctivitis, dacryocystitis, episcleritis, keratitis and anterior uveitis. Ophthalmic preparations containing acetates are more lipophilic. Thus, these formulations penetrate through the cornea better than those formulated with phosphates, which are comparatively more hydrophilic [14]. Nanocrystals of dexamethasone acetate were obtained by a super reduced scale wet bead milling self-developed method [13]. The formulations produced had a dexamethasone acetate concentration of 5% (w/w) with different stabilization mixtures (Table 1).

Polymyxin B is an antibiotic which acts binding to the bacterial cell membrane and altering its permeability. It possesses a cyclic peptide portion, which is hydrophilic and positively charged due to the amino groups, and also a fatty acid portion, which has a hydrophobic nature. Because of the fact that it is an amphiphilic cationic molecule, it was tried to use it as the only stabilizer for the dexamethasone nanocrystals, providing not only stabilization but also positive charge to the nanocrystals. Formulation 1 (F1), which contained polymyxin B as the only stabilizer, resulted indeed in positively charged nanocrystals with zeta potential of +11 mV in original medium as shown in Table 2. When measured in water (corresponding to the dilution of the nanosuspension by the eye fluid), the charge was even higher (+17 mV).

However, the stabilization provided solely by polymyxin B was not sufficient to achieve a particle size in the nano range, which could be observed by both PCS (Fig. 1) and light microscopy. The nanocrystals formed aggregates with a PCS diameter of about 2.8 μm, nicely seen in Fig. 2A.

Addition of traditional nonionic stabilizers to F1 in a concentration of 0.1% leads to efficient de-aggregation of the aggregates and revealed nanocrystals of 491 nm (Fig. 2B) and 687 nm for poloxamer 188 and Tween® 80, respectively. However, after dilution, the zeta potential reduced to around 0 mV for both stabilizers, which is not desired. These results additionally proved that the PCS diameter measured in the micrometer range for F1 was not micro-sized crystals, but in fact aggregated nanocrystals. The aggregation was obviously due to insufficient stabilization by the polymyxin B alone.

In contrast, the formulations 2, 3, 4 and 5 were produced using a cationic stabilizer such as cetylpyridinium chloride or benzalkonium chloride alone, without polymyxin B (F3 and F5), or in combination with polymyxin B (F2 and F4). The final zeta potential had a positive value of +22 to +30 mV (in original medium) for all formulations (Table 2). In addition, the particle size achieved was in the nano range, all below 400 nm. Addition of the nanocrystals to water leads to a distinct increase in charge in the range of about +40 to +50 mV. F3 was stabilized only by cetylpyridinium chloride while F5 was stabilized only by benzalkonium chloride. Formulations 3 and 5 (F3 and F5) with combination of preservative and polymyxin B had the highest zeta potential in water, +53 mV and +46 mV, respectively, and were thus chosen to be further investigated.

The use of benzalkonium chloride and cetylpyridinium chloride is an elegant strategy to stabilize such ocular nanocrystals, since these two stabilizers are also preservatives. They are normally added to multi-dose topical ophthalmic preparations to prevent

Table 1
Composition of the different formulations processed by wet bead milling (all % w/w).

Formulation	Dexamethasone acetate	Polymyxin B	Cetylpyridinium chloride	Benzalkonium chloride
F1	5	1	–	–
F2	5	1	1	–
F3	5	–	1	–
F4	5	1	–	1
F5	5	–	–	1

Table 2
Zeta potential measured in water (50 μS/cm, pH 5.5) and original dispersion medium after wet bead milling for the different formulations.

Formulation	ZP (mV) in water	ZP (mV) in original medium
F1	+17	+11
F2	+37	+29
F3	+53	+31
F4	+40	+22
F5	+46	+30

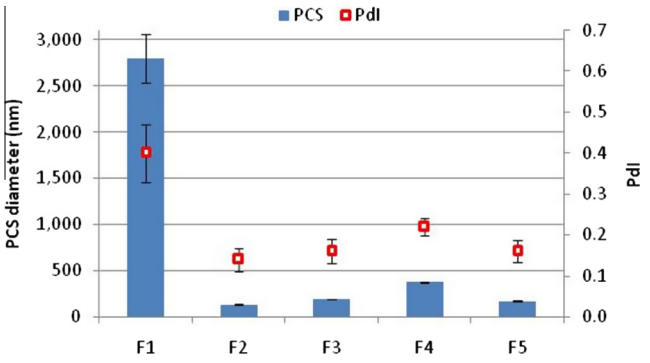

Fig. 1. PCS diameters and polydispersity indices after wet bead milling for the different formulations F1–F5 (n = 10).

the growth of, or to destroy the microorganisms introduced inadvertently during the treatment interval. In addition, they are both cationic molecules, which result in nanocrystals being positively charged. This could increase the adhesion to the ocular mucosa due to the electrostatic interaction between the positively charged nanocrystals and negatively charged mucosa. Transmission electron microscopy (TEM) of F3 confirmed the PCS results and revealed prismatic shaped nanocrystals (Fig. 3). To the best of our knowledge, no cationic ophthalmic nanosuspension combining a hydrophobic drug and hydrophilic drug has yet been reported.

Nanocrystal-based ocular drug delivery systems have the potential to improve the bioavailability (lower doses and less frequent instillation) of the ophthalmic preparation. A thermoreversible polymeric *in situ* gel-forming nanosuspension of forskolin presented significant improvement in lowering the intraocular pressure than the market product [15]. Similarly, the effect of particle size in the micron and nano-size range on the ocular bioavailability revealed that hydrocortisone nanosuspension showed AUC 0–9 h value of 30.95 ± 2.2, twofold higher than the micron range formulation (15.86 ± 2.7). The nanosuspension, with mean particle size of approximately 300 nm, containing 2% w/v of hydrocortisone, was prepared by wet milling process using 0.2% w/v of Tween®80 and 0.5% w/v of hypromellose HPMC as stabilizers [16]. Furthermore, the intraocular pressure (IOP) lowering effect of brinzolamide nanocrystals prepared by wet milling process was similar to the market product although it showed advantageous dissolution and absorption behavior. The nanosuspension containing 16% of the API was obtained using 25% w/w HPMC as stabilizer at pH 7.4 resulting in particle size of 460 ± 10 nm [17].

3.2. Dilution and isotonicity adjustment

As an ophthalmic product, these formulations have to be isotonic, or at least close to isotonicity. According to the U.S. pharmacopoeia, the lacrimal fluid is isotonic with blood, having an isotonicity corresponding to that of a 0.9% sodium chloride solution, but the eye can accommodate tonicity values as low as that of a 0.6% sodium chloride solution and as high as that of a 2.0% sodium chloride solution. Adjustment of isotonicity avoids not only the burning sensation after instillation but most important, avoids excessive lacrimation, which contributes to the drug removal from the eye surface. Therefore, the next step was to adjust the tonicity of the nanosuspensions to a physiological value and the appropriate drug concentrations of the formulations obtained.

The first choice tonicity agent would be sodium chloride, but because it can destabilize the nanosuspension, other non-ionic alternatives were also evaluated, e.g. glycerol, sorbitol and dextrose. In total, eight final formulations were prepared. The formulations F3 and F5 were first produced by wet bead milling without addition of polymyxin B. Then, polymyxin B and isotonicity agent solution were added, to achieve a final formulation with

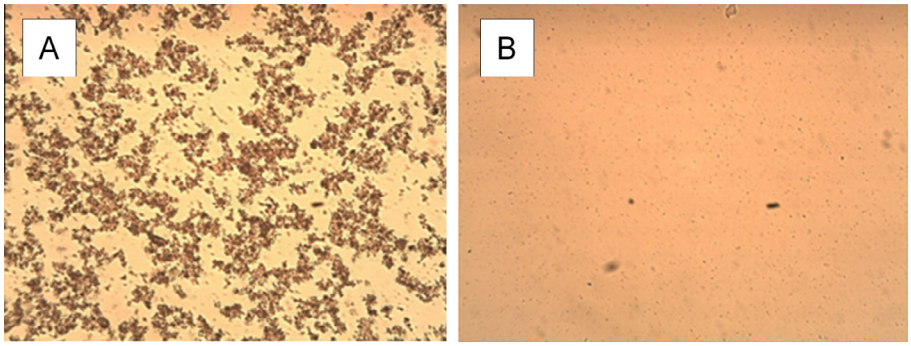

Fig. 2. Light microscopy pictures (160× magnification) of dexamethasone acetate nanosuspension stabilized with polymyxin B; A: insufficient stabilization by polymyxin B leading to agglomeration; B: dilution of the nanosuspension showed in A with 0.1% poloxamer 188 solution.

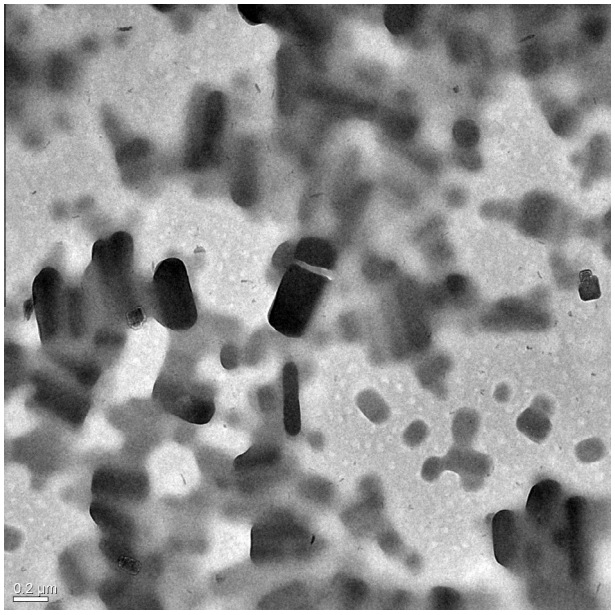

Fig. 3. TEM picture of dexamethasone acetate nanocrystals produced by wet bead milling and stabilized with cetylpyridinium chloride, formulation F3 (scale bar 0.2 µm).

0.05% dexamethasone acetate, 0.1% of polymyxin B, 0.01% of the stabilizer and adequate concentrations of one of the tonicity agents (0.9% NaCl; 2.6% glycerol; 5.5% sorbitol; 5.0% dextrose) (all w/w). Particle size and zeta potential of these formulations were assessed before and immediately after dilution with polymyxin B and the tonicity agent. The final formulations all possessed mean particle size in the range of 230–260 nm (Table 3). It has been previously reported that for three corticoid (dexamethasone, hydrocortisone and prednisolone) suspensions, the smaller the particle size, the higher the intensity of drug action (expressed as intraocular pressure evaluated in rabbits) [18]. For instance, dexamethasone suspensions with mean particle sizes of approximately 1, 2.5 and 5 µm showed increase in bioactivity as a function of decreasing particle sizes, and all of them were superior compared to the solution of the same drug. The dexamethasone suspension with mean particle size of 1 µm increased the maximum intraocular pressure by 80% compared to the same drug solution. This supports the principle that particle size reduction can increase the ocular bioavailability. In addition to that, a high positive charge on the nanocrystals surface could further increase retention time (=increased bioavailability).

Table 3 shows – as expected – a strong increase in measured size after addition of NaCl, due to the known zeta potential reduction after addition of electrolytes, leading to aggregation. All nonionic isotonicity agents did not or little affected the size.

3.3. In vitro mucoadhesion evaluation

Recently, better understanding of the properties of the tear film has been leading the formulators to develop products with the potential to shape the future of the ophthalmic drug delivery [19]. The conjunctiva and the cornea are protected by this film, a multi-layered structure, comprised of a buffered solution (pH 7.4) containing electrolytes, 1543 proteins reported so far (lysoenzyme, albumin and glycoproteins like mucin) [20] and more than 600 lipid species from 17 major lipid classes [21]. The third layer of this film, closest to the cornea, is an aqueous-mucin gel layer containing water, salts, proteins and carbohydrates besides mucins, which are high molecular weight glycoproteins heavily

Table 3
PCS diameters and zeta potential (measured in original medium) before and after dilution to the final drug concentration and addition of the different tonicity agents.

Formulation	PCS (nm) before	PCS (nm) after	ZP (mV) before	ZP (mV) after
F3-NaCl	233	240	+31	+39
F3-Glycerol	233	236	+31	+31
F3-Sorbitol	233	249	+31	+28
F3-Dextrose	233	262	+31	+29
F5-NaCl	210	1357	+30	+33
F5-Glycerol	210	228	+30	+30
F5-Sorbitol	210	259	+30	+27
F5-Dextrose	210	242	+30	+25

glycosylated (50–80%) [22]. The globlet cells of the conjunctiva mainly secrete this component. Mucin is a negatively charged molecule due to its associated sialic acid and sulfate residues [23]. Additionally, it modifies the hydrophobic corneal surface to a hydrophilic surface by adhering to the glycocalyx on the corneal microvilli allowing the hydration of the tissues. Mucin can play an important role in ocular bioavailability depending on the extent of its behavior as barrier or retention site [24].

Therefore, a cationic particulate system has a potential increased retention time in mucosal surfaces due to the electrostatic interactions between particles and mucosa. A study by He et al. [25], using positively charged chitosan microspheres proposed the correlation of the amount of mucin adsorbed with the positive zeta potential of the microspheres. The extent of adsorption was proportional to the absolute values of positive zeta potential of chitosan microspheres and negative zeta potential of mucin. These results were confirmed by testing the adsorption of the chitosan microspheres in rat small intestine. Another study by Shen et al. [26], also correlated the interaction of mucin and cationic nanostructured lipid carriers (NLC) with mucoadhesion. The presented correlation was supported by animal studies in rabbits. Distinct increased ocular residence time of the drug was found for the positively charged NLC.

A simple method to evaluate the potential interaction between mucin and the cationic nanocrystals produced was to admix the nanosuspension with mucin solutions with increasing mucin concentrations and measure its zeta potential (ZP). The results obtained showed that as the mucin concentration was increased, the ZP reduced, and then became close to zero, and further increase in mucin concentration eventually caused charge reversal of the nanocrystals (Fig. 4). This means that the positively charged nanocrystals became negatively charged because of the adsorption of the negatively charged mucin molecules onto their surface. Similarly, chitosan-coated nanoparticle incubation in mucin dispersion for 6 h, resulted in insignificant decrease in the zeta potential. The result indicated their electrostatic interaction [27].

It can therefore be assumed that when these cationic nanocrystals get in contact with the eye mucosa, they have a higher interaction potential (= attachment strength) with the mucosal surface compared to standard formulations. This could lead to increased retention time, overcoming one of the biggest issues for ophthalmic preparations, the low retention time in the eye.

It was found that the zeta potential for blank muncin solutions at different concentrations all had similar values of around −30 mV. Therefore, it can be assumed that the measured zeta potential for the nanocrystals was influenced by mucin molecules adsorbed onto their surface. It should be noted, however, that this is just a characterization tool for mucoadhesive formulation screening. Given the complex composition of the tear film and the unique peculiarities of the eye anatomy, a definite in vivo correlation is difficult to be established. Further in vivo studies are necessary to confirm the real mucoadhesion properties.

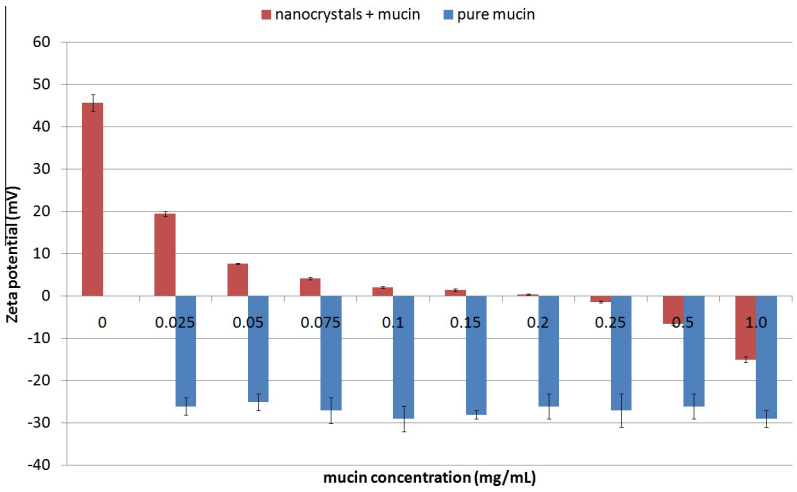

Fig. 4. Zeta potential of dexamethasone nanocrystals (F3) after incubation with mucin solutions containing increasing mucin concentrations (0.025–1 mg/mL) and pure mucin at the same concentrations as a comparison (n = 3).

3.4. In vitro cytotoxicity evaluation

A comparative study between ocular irritation test, using rabbits and *in vitro* test through agar diffusion using NCTC clone 929, FPC-IAL and SIRC cell lines revealed that the agar diffusion can be used as screening test for the detection of ocular irritation contributing for a decrease in the use of animals in tests [28]. In similar way, the agar diffusion test was suitable to evaluate the cytotoxicity of silver nanoparticles coated using hydrogen-bonded multilayer film. The test indicated that the zone inhibition of the nanoparticles increased with increasing film thickness [29].

Therefore the cationic nanosuspension containing cetylpiridinium chloride and glycerol was evaluated using this method and revealed no cytotoxicity. It presented no sign of reactivity (grade 0) after the incubation period (Table 4).

There was no inhibition halo for the investigated formulation, as opposed to the positive control where the inhibition halo is clearly evident (Fig. 5, upper). Additionally, the neutral red uptake assay confirmed the non-toxicity of the cationic nanosuspension (Fig. 5, lower). The uptake of neutral red, a weak cationic dye, by viable cells is due to its ability to incorporate the dye within lysosomes, which present lower pH compared to the cytoplasm. Damaged lysosomal membrane results in decreased uptake of neutral red. This mechanism enables it as a very sensitive indicator of cell viability. Thus it is possible to differentiate the non-viable cells from viable cells [30].

3.5. Short-term physical stability

After 6 months storage, the samples containing cetylpyridinium chloride were the ones which showed the best stability results for all 3 temperatures investigated (Fig. 6). They could be easily redispersed by manual shaking whereas the samples containing benzalkonium chloride presented irreversible caking (except for the samples containing NaCl – they could easily be re-dispersed but showed the largest particle sizes).

For the samples containing cetylpyridinium chloride, even at the critical storage condition of 40 °C, the integrity of the particles was still preserved for all tonicity agents, except for NaCl. Although NaCl is normally the first option tonicity agent for standard formulations, because of the peculiarities of nanoparticulated systems, in this case, it is the worst option. As expected, NaCl was the tonicity agent which produced the worst results for both cetylpyridinium chloride and benzalkonium chloride containing formulations. This is due to the destabilizing potential of ions, which adsorb on the surface of the particles, leading to zeta potential reduction and consequently physical instability.

Cetylpyridinium chloride and glycerol proved to be the most successful combination, with the smallest PCS diameters, which were 260, 255 and 269 nm for the storage temperatures of RT, 5 and 40 °C, respectively, after 6 months. In fact, glycerol was previously reported to have no influence in the stability or rutin nanosuspensions, where it was used up to a concentration of 20% and still no destabilization was found after 6 months [31]. Another example is the intravenous parenteral nanoemulsions, e.g. Lipofundin® marketed by the company B. Braun Melsungen AG, which also use glycerol as tonicity agent. Besides being a tonicity agent, it also acts as viscosity enhancer. Slightly increased viscosity is desired for ophthalmic preparations since it contributes to mucoadhesion and also acts on the stability of the nano formulation itself. Cetylpyridinium chloride, besides acting as stabilizer and conferring the cationic nature of the produced nanocrystals, also acted as preservative. The concentration used in this formulation is in the range normally used for quaternary ammonium compounds as preservative in eye drops. This is an elegant strategy to preserve such formulations, since excipients that are legally approved for ophthalmic use are restricted.

3.6. Short-term chemical stability

The chemical stability of dexamethasone in the formulation containing the combination of cetylpyridinium chloride and glycerol was investigated during 6 months at room temperature (RT), 5 °C and 40 °C. After production, 94.1% of nominal content was recovered, which was set as 100% value for the chemical stability study. The % recovery of the initial concentration is shown in Fig. 7.

After 6 months, the concentration of dexamethasone was remained practically unchanged for the three temperatures inves-

Table 4
Cationic nanosuspension 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

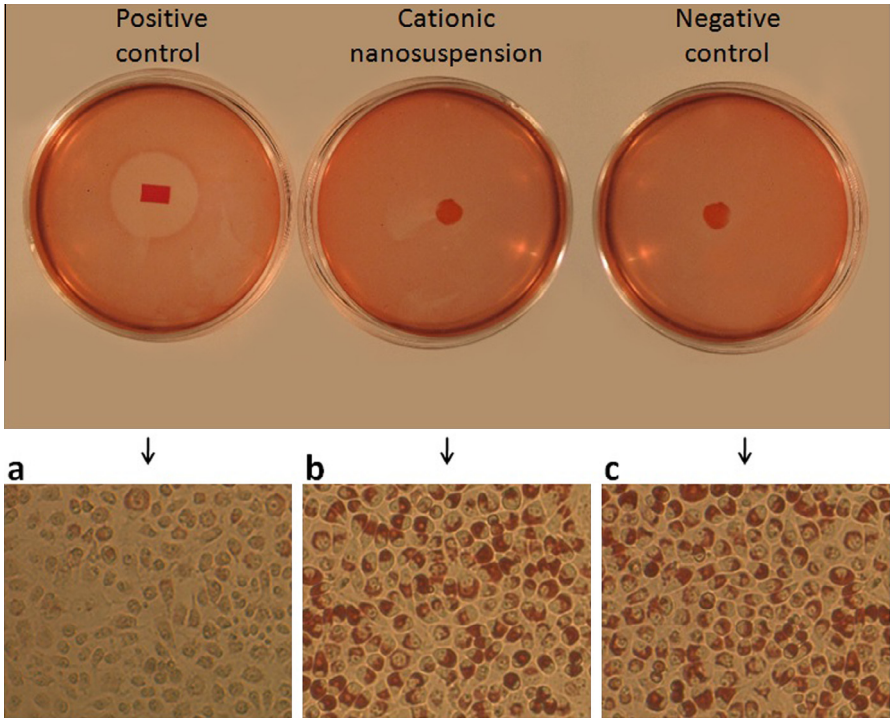

Fig. 5. Cytotoxicity evaluation of the cationic nanosuspension using culture of mammalian fibroblast cells NCTC clone L-929; Upper - left dish: positive control with clear inhibition halo, indicating reactivity grade 4 (severe reactivity); central dish: no inhibition zone for the cationic nanosuspension, indicating reactivity grade 0 (no reactivity); right dish: negative control with no inhibition halo (no reactivity). Lower - (a) non-viable cells (cytostatic effect or cell death); (b) and (c) uptake of neutral red by viable cells, light microscopy (100×).

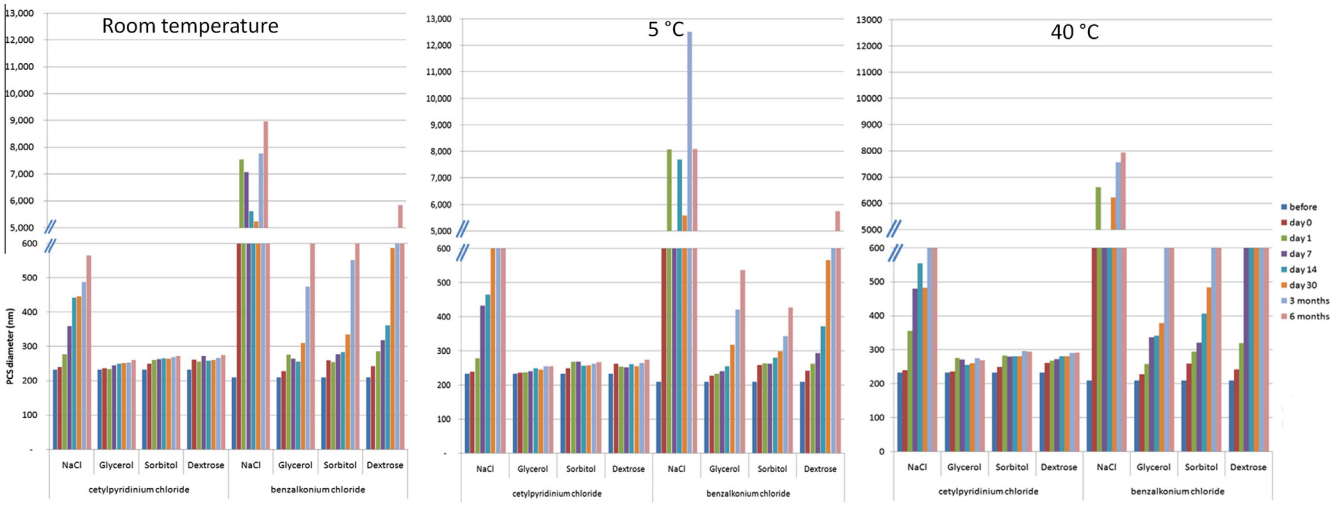

Fig. 6. PCS diameters of the isotonic formulations stabilized, additionally to polymyxin B, either by cetylpyridinium chloride or by benzalkonium chloride, stored at three different temperatures for 6 months.

tigated. It is known that for nanocrystals, the chemical stability is much less of an issue compared to physical stability [32]. While in solutions the drug is dissolved in the medium (and therefore much more exposed to chemical degradation, e.g. oxidation), in a nanosuspension the amount of drug exposed to the medium accounts for only the molecules on the surface of the nanocrystals. After an initial (possible) degradation of a drug monolayer on the nanocrystal surface, the remaining drug (nearly the totality of the drug content) is protected inside the insoluble crystals. For the present formulation, immediately after production, the amount of drug recovered was 94.1% of the nominal content. After six months, the amount recovered from the initial content was 102.9%, 98.4% and 105.1% for the samples stored at RT, 5 °C and 40 °C, respectively. This indicates an excellent chemical stability and therefore a long-term stability of at least 2 years can be predicted, which is fundamental for a product to go into the market.

Moreover, the official compendium (USP 2012) establishes for the commercial ophthalmic suspension containing polymyxin B, neomycin and dexamethasone acetate a pH range from 3.5 to 6.0. This range is supported by electrochemical studies conducted in alkaline solution, for identifying the dexamethasone oxidation mechanism, which revealed its decomposition into three ill-

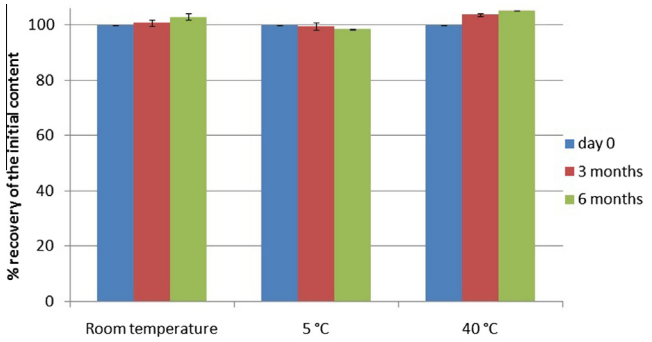

Fig. 7. Recovery (%) of the initial content of dexamethasone in the formulation containing CPC and glycerol stored during 6 months at three different temperatures (n = 3).

defined oxidation peaks [33]. The cationic nanosuspension pH value was 4.9 ± 0.1 (n = 3). The vision organ can rapidly recover the physiological pH of the lacrimal film.

4. Conclusions

The nanocrystals described in this study produced by wet bead milling were significantly smaller than what had been previously achieved by using another production method – approximately 250 nm versus approx. 1 µm using high pressure homogenization [18]. Based on this small size, from the underlying physical laws positive effects for ocular application can be predicted: adhesion to surface increases with decreasing size (= prolonged retention time), and saturation solubility increases exponentially with decreasing size (and thus concentration gradient to the eye). The positive charge is likely to create additionally charge-mediated mucoadhesion, as reported for other cationic systems [25,26]. From the stability results after 6 months for all 3 temperatures, a satisfactory long-term physical and chemical stability required for a commercial product can be predicted, and thus a formulation for patients seems feasible.

Apart from the expected increased therapeutic efficiency, patient comfort and compliance during the treatment will increase (less frequent instillation with the practicality of an eye drop). From the regulatory point, a smart approach is using preservatives as charge provider instead of cationic excipients with lack of regulatory acceptance for ocular application. This principle can be transferred to formulate other cationic formulations. Important is, that the concentration used stays in the concentration range accepted for preservation.

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