JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 699–707

Received October 6, 2004
Accepted February 10, 2005
DOI 10.1211/0022357056145
ISSN 0022-3573

The effect of freeze-drying with different cryoprotectants and gamma-irradiation sterilization on the characteristics of ciprofloxacin HCl-loaded poly(D,L-lactide-glycolide) nanoparticles

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Abstract

In the present study, the influence of freeze-drying with several cryoprotective agents and gamma (γ)-irradiation sterilization on the physicochemical characteristics of ciprofloxacin HCl-loaded poly(D,L-lactide-co-glycolide) (PLGA) nanoparticles was evaluated. Nanoparticles were prepared by W/O/W emulsification solvent evaporation followed by high-pressure homogenization. They were freeze-dried in the presence of 5.0% (w/v) mannitol, trehalose or glucose, with 5.0% (w/v) or 15.0% (w/v) dextran as cryoprotectants. The nanoparticles were irradiated at a dose of 25 kGy using a ^{60}Co source. The following physicochemical properties of the formulations were investigated: the ratio of particle size before (initial) and after freeze-drying, the ease of reconstitution of the nanoparticle suspensions and the drug-release profiles of irradiated and non-irradiated nanoparticles. The antibacterial activity against *Pseudomonas aeruginosa* was measured. The freeze-drying process induced a significant increase in particle size when no cryoprotectant was employed. Similar results were observed when cryoprotectants were added to the formulation. Only when mannitol was used was no significant size increase measured. Moreover, for formulations with dextran, reconstitution after freeze-drying was difficult by manual agitation and particle size could not be determined because of aggregation. After γ-sterilization no significant difference in mean particle size was observed, but reconstitution was more difficult and drug release was influenced negatively. Ciprofloxacin HCl incorporated in the nanoparticles was still effective against the micro-organism selected after freeze-drying and γ-sterilization.

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Acknowledgements: S. Bozdag is grateful to the University of Antwerp (Belgium) for the scholarship provided. K. Dillen is a Research Assistant of the Fund for Scientific Research – Flanders (Belgium) (F.W.O. Vlaanderen). The authors wish to thank Professor D. Vanden Berghe, Dr P. Cos and Mrs R. Vingerhoets, Laboratory of Microbiology, University of Antwerp, for assistance with the microbiological tests, the Laboratory of General Biochemistry and Physical Pharmacy for assistance with the intrinsic viscosity measurements and Dr Pierre Dardenne, IBA Mediris S.A., Fleurus, Belgium, for γ-sterilization of the nanoparticles.

Introduction

Topical application of ophthalmic drugs is severely limited by physiological constraints such as basal and reflex tear secretion, and reflex blinking, resulting in considerable drug loss from the precorneal area of the eye. Thus, the most frequently used dosage forms, solutions and suspensions, are compromised in their effectiveness. In most cases, only 5% or less of the instilled drug reaches the anterior tissues of the eye (Järvinen et al 1995). A large part of the drug is removed in the naso-lachrymal duct and could cause systemic side-effects (Sasaki et al 1996). Consequently, conventional dosage forms do not provide and maintain adequate drug concentrations at the site of action for a prolonged period of time. Different strategies were proposed in order to improve the precorneal residence time and/or penetration ability of the active ingredient. Among them, one approach consists of using colloidal drug delivery systems such as microparticles, liposomes or nanoparticles (Le Bourlais et al 1998). Several studies have demonstrated that nanoparticles used as ocular delivery systems can provide increased drug bioavailability and decreased systemic side-effects (Zimmer & Kreuter 1995; Barbault-Foucher et al 2002). Despite the colloidal nature of nanoparticles, surface active agents are usually added for stabilization of the suspension by direct adsorption on the particle surface. Nevertheless, some aggregation is often observed on storage (De Jaeghere et al 1999). Hence, the improvement of physical and chemical stability represents an important issue in the development of nanoparticles (Saez et al 2000). Freeze-drying appears to be one of the

most suitable methods to stabilize and facilitate the handling of colloidal systems, which otherwise stored as suspensions would suffer alteration in a brief period of time. Few studies concerning the freeze-drying of polymeric nanoparticles are reported in literature (Chacón et al 1999; Saez et al 2000; Konan et al 2002; Sameti et al 2003). Several authors have claimed that addition of cryoprotectants is necessary for the maintenance of the initial formulation characteristics (Kristl et al 1996; Roy et al 1997).

Another limitation parameter concerns sterilization of nanoparticles, which is essential considering their ocular use. Little data about the sterilization of nanoparticles have been reported (Sommerfeld et al 1998). Common techniques, such as steam or dry heat, cannot be used in the case of biodegradable aliphatic polyesters, such as poly(lactide-co-glycolide) (PLGA) and polylactide (PLA), since they alter the physical and chemical properties of the polymer (Hausberger et al 1995; Sintzel et al 1997). Consequently, gamma (γ)-irradiation seems to be an alternative for sterilization. A potential disadvantage of terminal γ-sterilization, however, is the radiolytic degradation of the incorporated drug and polymer matrix (Bittner et al 1999; Calis et al 2002). Controversial reports about the effects of γ-irradiation on microparticulate systems are found in literature (Montanari et al 1998). The drug-release rate from biodegradable drug delivery systems was demonstrated to increase (Bittner et al 1999; Calis et al 2002) or decrease (Volland et al 1994) compared to non-irradiated controls, depending on the active ingredients, matrix type and irradiation dose employed.

Ciprofloxacin, a frequently used antimicrobial agent in ophthalmology, was selected as the model drug for incorporation in the nanoparticles. It has proven to be a powerful topical antibiotic for use as a single agent in the treatment of bacterial conjunctivitis and keratitis caused by *Staphylococcus aureus* and numerous Gram-negative bacteria. Moreover, the frequency of spontaneous resistance to ciprofloxacin is very low (Lamholt & Kilian 2003).

The purpose of the present study was to evaluate the effects of freeze-drying using several cryoprotective agents and γ-irradiation sterilization on the reconstitution and release characteristics of ciprofloxacin HCl-loaded PLGA nanoparticles.

Materials and Methods

Materials

The PLGA polymer chosen was Resomer RG 503 (Boehringer Ingelheim, Ingelheim am Rhein, Germany) with a molecular weight (MW) of 40 000 and a D,L-lactide : glycolide molar ratio of 52:48. Poly(vinylalcohol) (PVA; MW 30 000–70 000), dextran (MW 64 000–76 000) and D-(+)-dihydrated trehalose were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Ciprofloxacin HCl was supplied by Roig Pharma (Barcelona, Spain). Dichloromethane was obtained from Sigma-Aldrich (Steinheim, Germany) and acetonitrile (HPLC grade) from Acros Organics (New Jersey, USA). D-mannitol and D-(+)-monohydrated glucose were supplied by Federa Co. (Brussels, Belgium). Filtered (Porafil 0.20 Membranefilter, Düren, Germany) purified Milli Q water (Millipore, Mollsheim, France) was used throughout the experiments. Salts for the preparation of simulated lachrymal fluid (SLF) were obtained from Merck (Leuven, Belgium) (KCl and NaCl), Merck (Darmstadt, Germany) ($NaHCO_3$) and Sigma Chemicals Co. (St Louis, MO) ($CaCl_2$ and $MgCl_2$). SLF is an electrolyte solution composed of 1.7893 g L^{-1} KCl; 6.3118 g L^{-1} NaCl; 2.1842 g L^{-1} $NaHCO_3$; 0.0670 g L^{-1} $CaCl_2.2H_2O$; 0.1572 g L^{-1} $MgCl_2.6H_2O$, adjusted with 0.1 M HCl to a pH of 7.4 ± 0.1 (Van Haeringen 1981).

Preparation of ciprofloxacin-loaded PLGA nanoparticles

Preparation of ciprofloxacin solution

A solution of ciprofloxacin HCl (2.50% w/v) was prepared by dissolving the drug in filtered purified water. The solution was sonicated for 30 s at 20 W and then for 45 s at 35 W (Branson 450-D, 102-C with microtip, Branson, Danbury, CT).

W/O/W emulsification solvent evaporation method

The nanoparticles were prepared by W/O/W emulsification solvent evaporation followed by high-pressure homogenization (Dillen et al 2004). Two millilitres of an aqueous ciprofloxacin solution were emulsified by means of sonication for 1 min at 20 W (Branson 450-D, 102-C with microtip, Branson, Danbury, USA) in an organic phase consisting of 500 mg of PLGA dissolved in 5 mL of dichloromethane. The resulting W/O emulsion was dispersed in 25 mL of the first outer water phase, a 1% w/v PVA stabilizer solution, and sonicated for 30 s at 15 W in order to obtain a multiple W/O/W emulsion, which was homogenized using a Microfluidizer M-110L (Microfluidics, Newton, USA) at a pressure of 50 bar for three cycles. The emulsion was then diluted in the second outer water phase, consisting of 120 mL of an aqueous PVA dispersion (0.3% w/v) in order to minimize emulsion coalescence and particle aggregation. The organic solvent was allowed to evaporate for 4 h at room temperature under agitation (700 rpm) using a magnetic stirrer (Variomag Electronicrührer Poly 15, H+P Labortechnik GmbH, Münich, Germany). Consequently, the polymer, insoluble in the aqueous phase, precipitated as solid particles. The resulting nanosuspension was subsequently cooled down to $-18°C$. Each type of formulation was prepared in triplicate.

Freeze-drying of nanoparticles

The frozen nanoparticle suspensions were freeze-dried (Leybold-Heraeus D8B, GT-2A, Germany). The start temperature of the samples before freeze-drying was between -5 and $-10°C$. The vacuum was created by a Trivac pump (Leybold, Germany) with a volume flow rate of 8 $m^3 h^{-1}$. The freeze-drying process ended when a vacuum of 6×10^{-2} mbar was reached at a temperature of 25°C.

Blank or drug-loaded nanoparticle suspensions were freeze-dried in the absence of cryoprotectant and in the

presence of 5.0% (w/v) glucose, trehalose, dextran, mannitol or 15.0% (w/v) dextran in order to investigate the influence of presence and type of cryoprotectant on the freeze-dried nanoparticles characteristics. According to Chacón et al (1999) the presence of at least 5% cryoprotectant is essential to maintain the initial particle size. Moreover, a concentration of 5% (w/v) of the different sugars employed provides an isotonic nanoparticle dispersion after reconstitution of the freeze-dried powder with the required amount of sterile vehicle.

Gamma irradiation of nanoparticles

Drug-loaded nanoparticles and PLGA polymer were γ-irradiated using ^{60}Co as irradiation source (Gammir I-Sulzer irradiation unicell, IBA-Mediris, Fleurus, Belgium) and received a dose of 25 kGy. According to the European Pharmacopoeia this dose is adequate for the purpose of sterilizing pharmaceutical products when the bioburden is not known.

Nanoparticle size and zeta potential analysis

The mean particle size of the nanoparticles prepared was determined by photon correlation spectroscopy with a Zetasizer 3000 (Malvern Instruments, Malvern, UK). Part of the freeze-dried or γ-irradiated nanoparticles was redispersed in the SLF with mild manual shaking. The initial nanoparticle suspension or redispersed suspension was then diluted 100 times with SLF. The mean particle size of each sample was determined three times and the average value was used for calculations. Afterwards the mean particle size of three replicas was calculated and used to determine the ratio of initial particle size (S_i) and particle size after freeze drying (S_f) or γ-irradiation (S_γ).

The zeta potential of the non- or γ-sterilized nanoparticles was measured using electrophoretic light scattering. Five milligrams of each replica of the freeze-dried nanoparticles was suspended in 10 mL of SLF. This dispersion was injected in the capillary of the Zetasizer 3000 and the zeta potential value was determined 20 times. Afterwards, the average value was used for calculations.

Reconstitution/aggregation

By mild manual shaking, the ease of the reconstitution of the nanoparticle suspension was evaluated after freeze-drying and γ-sterilization. Particle aggregation was quantified by the following numerical scale: (0) absent, (1) scarce and (2) significant aggregation.

Drug loading determination

Twenty milligram portions of freeze-dried, non- or γ-irradiated nanoparticles not protected by cryoprotectant were accurately weighed. When a cryoprotectant was present, a mass equivalent to 20 mg of PLGA nanoparticles was weighed. The nanoparticles were dispersed in 10.0 mL of purified water and gently sonicated for 1 min (Julabo USR3, Julabo, Seelbach, Germany). Drug entrapment was determined indirectly by measuring the amount of ciprofloxacin HCl that was not encapsulated. The samples were centrifuged at 4400 rpm for 5 h (Cetra-MP4 centrifuge, International Equipment Company, Miami, USA) and the ciprofloxacin HCl concentration in the supernatant was determined by a validated HPLC method. The system consisted of a Gilson 321 pump (Gilson, Villiers-le-Bel, France), a UV-VIS 152 detector (Gilson, Villiers-le-Bel, France), a μ Bondapack C_{18} 125 Å 10 μm column (Waters, Milford, USA) and an HP 3395 integrator (Hewlett-Packard Company, Palo Alto, USA). The mobile phase and flow rate used corresponded to the monograph in the *European Pharmacopoeia* (1997). Ciprofloxacin HCl was detected at 278 nm and the concentration was calculated using a calibration curve.

The entrapment efficiency (EE) of the samples was calculated using the following equation: EE (%) = (actual drug loading/theoretical drug loading) $\times$ 100% (Dillen et al 2004).

In-vitro release tests

Fifty milligrams of freeze-dried non- or γ-irradiated nanoparticles without cryoprotectant were accurately weighed. The amount of non- or γ-irradiated nanoparticles with cryoprotectant was calculated as mentioned previously (see drug loading determination) and accurately weighed.

The in-vitro release experiments were carried out using vertical diffusion cells and a dialysis membrane with a cut-off of 12 000–14 000 Da (Medicell International, London, UK). In the donor compartment nanoparticles, dispersed in 5.0 mL water (for formulations with cryoprotectants) or an aqueous mannitol solution 5.07% (w/v), were introduced. The acceptor compartment was filled with 18.0 mL of SLF and stirred magnetically at 200 rpm (Variomag Electronicrührer Poly 15, H + P Labortechnik GmbH, Münich, Germany). At suitable time intervals samples of 1.0 mL were withdrawn from the acceptor compartment and replaced by the same volume of SLF solution. The drug content of the samples was determined by the previously described HPLC method.

To calculate the release rate constant of ciprofloxacin HCl, the percentage released-vs-time profile was evaluated by curve-fitting analyses.

In order to study the possible influence of the rheological properties of the media on drug release, the kinematic viscosity of the release media was determined using a capillary viscometer (Schott Geräte GmbH, Germany) at 25°C as described in the European Pharmacopoeia.

Viscosimetry

The kinematic viscosity of the release media (5% w/v mannitol, trehalose, glucose or dextran and 15% w/v dextran aqueous solutions) was measured using a capillary viscosimeter (Schott Geräte, Hofheim a. Ts., Germany). The intrinsic viscosity of the PLGA polymer, dissolved in methylene chloride, was determined before and after γ-irradiation of the polymer employing an Ubbelohde suspended-level viscosimeter.

Determination of the antibacterial activity of ciprofloxacin-loaded nanoparticles

The antimicrobial effectiveness of the non- or γ-irradiated nanoparticles was assessed in comparison with an aqueous ciprofloxacin solution and blank nanoparticles by measuring the minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) against *Pseudomonas aeruginosa* (ATCC 9027). The micro-organism chosen is one of the most common ocular pathogens causing bacterial infection of the human cornea (Moreau et al 2002).

The activity of the drug-loaded nanoparticles was compared with an equivalent ciprofloxacin solution and changes in MIC and MBC related to the dosage form or sterilization process were examined. We also investigated whether or not blank nanoparticles, and thus the PLGA polymer or its acid degradation products, exhibited any antibiotic effect that could interfere with the activity determination.

The MIC values ($\mu g\,mL^{-1}$), being the lowest concentrations of antibiotic inhibiting visible growth after 24 h of incubation at 37°C, were determined in Tryptone Soy Broth (E & O Laboratories, Bonnybridge, UK) in 96-well cell culture clusters (Corning Incorporated, Corning, USA) after serial two-fold dilutions of the drug solution and nanoparticle dispersions. The dilutions were prepared on day 1, inoculation with micro-organisms occurred on day 1 or 2, and the wells were inspected for turbidity 24 h after inoculation. The MBC values ($\mu g\,mL^{-1}$) were determined by subculturing the negative dilutions on TSA plates without antibiotic and inspecting for formation of colonies after 24 h of incubation at 37°C. The MBC technique establishes the lowest level of a bactericidal agent that will kill at least 99.9% of the organisms in a standard inoculum, whereby growth of organisms which were inhibited but not killed can be detected.

A positive control for growth consisted of *P. aeruginosa* in broth and a negative control for sterility consisted of uninoculated broth.

Statistical analysis

The effect of freeze-drying and γ-irradiation on the physicochemical properties of the nanoparticles was analysed by *t*-test using Statistica (Statsoft, Tulsa, USA) software.

Results and Discussion

Physical characterization

Reconstitution and size of freeze-dried nanoparticles

The physicochemical properties of nanoparticles have an effect on their efficiency in drug delivery (Ding 1998). After freeze-drying, easy and rapid reconstitution and unchanged particle size of the product are important features. In order to choose the appropriate cryoprotectant, which effectively prevents particle aggregation, a screening of sugars was performed.

Carbohydrates are favoured as freeze-drying excipients since they are chemically innocuous and can be easily vitrified during freezing. Some carbohydrates, however, notably mannitol and lactose, can also separate from a frozen solution in the form of crystalline phases (Franks 1998). Glucose, trehalose and dextran were shown to form amorphous masses at very low temperatures (Saez et al 2000).

Table 1 summarizes the particle size, polydispersity index (PI), S_f/S_i ratio and aggregation characteristics of unloaded and ciprofloxacin HCl-loaded nanoparticles

Table 1 Physicochemical properties of unloaded and ciprofloxacin HCl-loaded PLGA nanoparticles before and after freeze-drying

Cryoprotectant (% w/v)	Unloaded NP				Loaded NP			
	Mean particle size (nm) ± s.d. (PI ± s.d.)		$S_f/S_i \pm$ s.d.	Aggregation score	Mean particle size (nm) ± s.d. (PI ± s.d.)		$S_f/S_i \pm$ s.d.	Aggregation score
	Before freeze-drying	After freeze-drying			Before freeze-drying	After freeze-drying		
No cryoprotectant	239.9 ± 2.60 (0.11 ± 0.01)	246.4 ± 2.28 (0.09 ± 0.02)	1.027 ± 0.007	0	226.1 ± 1.30 (0.08 ± 0.02)	231.6 ± 1.45 (0.05 ± 0.01)	1.024 ± 0.001	0
Mannitol (5%)	273.8 ± 4.51 (0.06 ± 0.03)	275.9 ± 4.45 (0.13 ± 0.01)	1.007 ± 0.006	0	231.5 ± 1.22 (0.08 ± 0.01)	238.5 ± 5.36 (0.11 ± 0.06)	1.030 ± 0.023	0
Trehalose (5%)	244.5 ± 0.56 (0.09 ± 0.02)	250.7 ± 0.95 (0.04 ± 0.01)	1.025 ± 0.002	0	230.0 ± 4.87 (0.09 ± 0.01)	232.9 ± 3.94 (0.05 ± 0.01)	1.012 ± 0.004	0
Dextran (15%)	253.1 ± 5.97 (0.42 ± 0.10)	290.0 ± 22.53 (0.52 ± 0.28)	1.146 ± 0.101	0	260.0 ± 6.48 (0.260 ± 0.01)	ND	ND	2
Dextran (5%)	262.4 ± 5.08 (0.25 ± 0.05)	346.2 ± 61.58 (0.60 ± 0.07)	1.321 ± 0.252	0	249.5 ± 2.35 (0.22 ± 0.02)	ND	ND	2
Glucose (5%)	259.2 ± 4.10 (0.09 ± 0.01)	264.8 ± 4.16 (0.16 ± 0.08)	1.021 ± 0.005	0	227.3 ± 3.77 (0.08 ± 0.01)	232.1 ± 3.35 (0.08 ± 0.02)	1.021 ± 0.006	0

Values are mean ± s.d., n = 3. S_f, particle size after freeze-drying; S_i, particle size before freeze-drying; PI, polydispersity index. Aggregation score: (0) absent, (1) scarce, (2) significant aggregation. ND: not determined.

before and after freeze-drying. Aggregation scores were 0 for all formulations, indicating a good redispersion. The size measurements show, however, that a small amount of aggregation occurs during freeze-drying, resulting in a size increase. Significant ($P < 0.05$) increases were measured in the absence of a cryoprotectant, and when trehalose and glucose were used, both for blank as well as for ciprofloxacin HCl-loaded nanoparticles (see Table 1). In most cases the increase, although significant, was rather small. The highest S_f/S_i ratio was obtained when dextran was employed, with size increases of 37 nm (for dextran 15% w/v) and 84 nm (for dextran 5% w/v), respectively, accompanied by a rise in PI, showing that the formulations were not monodisperse (Barbault-Foucher et al 2002). In general, higher PI values were obtained after freeze-drying compared to the values of the initial formulations.

In the case of ciprofloxacin HCl-loaded nanoparticles, manual redispersion of dextran-added formulations became impossible and particle size could not be measured. All other formulations had an aggregation score of 0. Reconstitution of nanoparticles to which glucose was added was more difficult compared to the other formulations because of the sticky nature of the freeze-dried cake, while all other formulations showed fluffy porous cakes after freeze-drying. This phenomenon was also observed by Konan et al (2002). They tested different sugars as lyoprotectants: trehalose, lactose and mannitol resulted in a fluffy, shelf-stable cake, whereas shrinkage was observed with glucose. After freeze-drying, condensed and salt-like residues were obtained for glucose while residues appeared voluminous and snow-like for trehalose and mannitol (Sameti et al 2003). Saez et al (2000), on the contrary, reported brittle cakes for mannitol, trehalose and dextran, but a porous cake when employing glucose.

None of the samples retained their initial characteristics on reconstitution, but the S_f/S_i ratios were the smallest when 5.0% (w/v) mannitol (for blank nanoparticles) and 5.0% (w/v) trehalose (for drug-loaded nanoparticles) were employed, although mannitol is known to show polymorphism and has a strong tendency to crystallize (Yu et al 1998). Our findings are partially in contrast to those of Chacón et al (1999), who reported that the best results with ciclosporin-loaded PLGA nanoparticles were obtained with 5% glucose and trehalose, maintaining the Tyndall effect, but not providing satisfactory reconstitution properties. In another study, only glucose, sucrose and trehalose containing nanoparticle formulations preserved the Tyndall effect on redispersion, suggesting that at least some of the particles retained their nanometric size, while this did not occur when dextran or mannitol were incorporated; however, only 20% concentrations of these cryoprotectants provided the required results (Saez et al 2000). Konan et al (2003) also employed trehalose as a cryoprotectant for meso-tetra(4-hydroxyl phenyl)-porphyrin-loaded PLGA and PLA nanoparticles and reported that the aggregation problem was overcome by using trehalose. Furthermore, trehalose, a non-reducing disaccharide of glucose, has previously exhibited satisfactory cryoprotective effects for pharmaceutical and biological materials (De Jaeghere et al 1999). The cryoprotective effect was attributed to the ability of sugar additive to form a glassy amorphous matrix around the particles, preventing the particles from sticking together during removal of water (Konan et al 2002). Not all solutes forming a vitreous mass, however, are able to stabilize nanoparticle suspensions, suggesting the contribution of additional physicochemical processes (Saez et al 2000).

Chacón et al (1999) reported that when a higher cryoprotectant percentage was used, no changes in the S_f/S_i ratios were observed after freeze-drying. In our study, however, small positive changes were detected when the percentage of dextran was increased.

Reconstitution, size and zeta potential of γ-irradiated nanoparticles

Numerous studies have addressed the effects of γ-sterilization on microparticulate drug delivery systems (Lalla & Sapna 1993; Volland et al 1994; Bittner et al 1999; Calis et al 2002), but little is known about nanoparticles (Alléman et al 1993; Masson et al 1997).

The particle size, PI, S_γ/S_f ratio and aggregation characteristics of freeze-dried drug-loaded nanoparticles before and after sterilization are given in Table 2. No statistically significant difference was observed between the particle size of the γ-sterilized and the non-sterilized formulations. However, the PI was higher than 0.25 for γ-sterilized nanoparticle preparations without cryoprotectants. According to Barbault-Foucher et al (2002), this indicates that formulations were not monodisperse. In contrast to non-sterilized preparations, higher PI values were observed after γ-sterilization. Particle size could not be measured in the sterilized sample with dextran because manual reconstitution was difficult and aggregation occurred (see Table 2).

For all other samples, no aggregation was observed, but reconstitution took a certain time before obtaining homogeneous dispersions.

The zeta potential of the nanoparticles was examined in SLF, which possesses a similar electrolyte composition as tear fluid. Results are given in Table 2. All zeta potential values measured were slightly negative, between −7.6 and −12.3 mV. The sterilization process did not have a significant influence on the zeta potential values of the nanoparticles.

Drug loading and ciprofloxacin HCl release

The entrapment efficiency of the non- and γ-sterilized nanoparticles prepared was between 44 and 54%. Freeze-drying in the absence or presence of cryoprotectants and sterilization did not influence drug loading. These results are in agreement with the work of Bittner et al (1999), who observed no effect of γ-sterilization on the drug loading of PLGA nanoparticles with tetracycline HCl.

Drug release before γ-sterilization

Release profiles of ciprofloxacin HCl from the nanoparticle preparations with or without cryoprotectant are shown in Figure 1. No burst release was observed for any of the freeze-dried formulations. Kim & Park (2004), however, reported that freeze-drying of microspheres might cause

Table 2 Physicochemical properties of freeze-dried ciprofloxacin HCl-loaded PLGA nanoparticles before and after γ-sterilization

Cryoprotectant (% w/v)	Mean particle size (nm) ± s.d. (PI ± s.d.)		$S_\gamma/S_f \pm$ s.d.	Aggregation score	Zeta potential (mV) ± s.d.	
	Before γ-irradiation	After γ-irradiation			Before γ-irradiation	After γ-irradiation
No cryoprotectant	231.6 ± 1.45 (0.05 ± 0.01)	211.7 ± 20.26 (0.25 ± 0.23)	0.914 ± 0.091	0	−8.0 ± 0.40	−8.9 ± 0.60
Mannitol (5%)	238.5 ± 5.36 (0.11 ± 0.06)	238.1 ± 12.75 (0.18 ± 0.04)	0.999 ± 0.075	0	−12.3 ± 0.94	−12.2 ± 2.36
Trehalose (5%)	232.9 ± 3.94 (0.05 ± 0.01)	217.9 ± 8.15 (0.21 ± 0.17)	0.936 ± 0.050	0	−9.6 ± 0.40	−8.3 ± 0.50
Dextran (5%)	ND	ND	–	2	−9.2 ± 0.61	−7.6 ± 0.88
Dextran (15%)	ND	ND	–	2	−8.4 ± 0.32	ND
Glucose (5%)	232.1 ± 3.35 (0.08 ± 0.02)	230.5 ± 6.73 (0.10 ± 0.07)	0.992 ± 0.015	0	−10.4 ± 0.75	−8.1 ± 0.80

Values are mean ± s.d., n = 3. S_γ, particle size after γ-sterilization; S_f, particle size before γ-sterilization; PI, polydispersity index. Aggregation score: (0) absent, (1) scarce, (2) significant aggregation. ND: not determined.

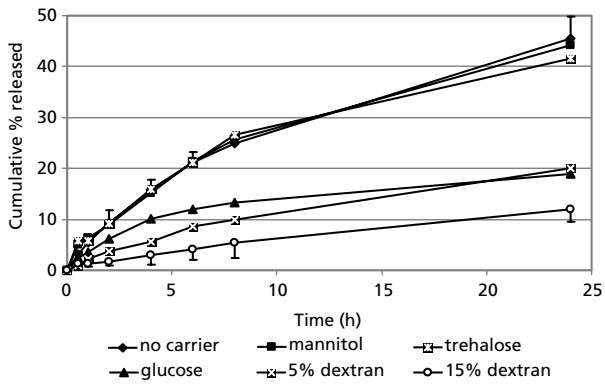

Figure 1 In-vitro release profiles of ciprofloxacin HCl-loaded, non-sterilized nanoparticle formulations with or without cryoprotectants (mean ± s.d., n = 3).

burst release because of the changes in pore size, geometry and pore interconnection formation during the freezing and subsequent drying steps. In our study the absence of burst release could be due to the size of the particles and/or the low concentration of drug present at the surface. Further studies will be performed to elucidate this release behaviour.

Table 3 summarizes the release-rate constants, intercept and correlation coefficient values of curves fitted by the Higuchi model. Fitting of the release profiles (percentage release-vs-time) proved the adequacy of Higuchi's square-root equation compared to zero-order, first-order and cube-root equations, as determined by the F test for all formulations.

As presented in Figure 1, similar amounts of drug were released from formulations without cryoprotectant, and with mannitol or trehalose. The release-rate constants were 10.281, 9.734 and 9.089 $h^{-1/2}$ for preparations without cryoprotectant, mannitol and trehalose, respectively. In the case of 5.0% glucose, 5.0% dextran and 15.0% dextran the release rate was lower (4.036, 2.994 and 3.473 $h^{-1/2}$ respectively). The viscosity of the release medium and properties of reconstituted freeze-dried nanoparticles could possibly explain the decrease in release rate. Similar characteristics were observed for the nanoparticles in the absence of cryoprotectant and in the presence of mannitol and trehalose (see aggregation scores in Table 2). The kinematic viscosity values of SLF, mannitol and trehalose solutions (5.0% w/v) equal 1.10, 1.21 and 1.22 mPas, respectively. Although the kinematic viscosity value of the release medium was 1.21 mPas for the formulations with glucose, the release rate was slower compared to preparations without cryoprotectant, mannitol and trehalose. The sticky structure and difficult reconstitution of nanoparticles protected by glucose could be the reason. Also, nanoparticles in the presence of dextran have a lower release rate constant than the other formulations because of aggregation after reconstitution and the viscosity of the release medium (3.05 and 15.04 mPas for 5.0% and 15.0% w/v dextran, respectively). A higher dextran concentration led to a viscosity increase of the release medium and this caused a decrease in the release rate and diffusion of the drug.

Release after γ-sterilization

Figure 2 presents the release profiles of ciprofloxacin HCl from the γ-sterilized nanoparticles in the absence and presence of a cryoprotectant. Gamma-irradiation has been shown not to affect the stability of the model drug, ciprofloxacin (Weyenberg et al 2004). No burst effect was observed, neither for γ-irradiated formulations nor for non-sterilized preparations. Higuchi's square-root equation showed a significantly better fit than zero-order, first-order and cube-root equations, as determined by the F test (see Table 3).

As illustrated in Figure 2, γ-sterilized preparations without cryoprotectant and with mannitol and trehalose

Table 3 Slope, intercept and correlation coefficient values of fitted curves (Higuchi model)

Cryoprotectant (w/v, %)	Before γ-sterilization			After γ-sterilization		
	K ± s.d. (%$h^{-1/2}$)	Int ± s.d. (%)	Cor. coeff ± s.d.	K ± s.d. (%$h^{-1/2}$)	Int ± s.d. (%)	Cor. coeff. ± s.d.
No cryoprotectant	10.281 ± 0.335	−4.607 ± 1.033	0.991 ± 0.003	6.806 ± 1.460	−2.818 ± 0.414	0.991 ± 0.011
Mannitol (5%)	9.734 ± 1.684	−3.196 ± 2.544	0.988 ± 0.008	3.020 ± 1.193	−0.549 ± 1.692	0.935 ± 0.104
Trehalose (5%)	9.089 ± 0.375	−1.871 ± 1.643	0.979 ± 0.009	6.519 ± 0.660	−4.410 ± 0.862	0.988 ± 0.009
Dextran (5%)	2.994 ± 0.705	−1.288 ± 0.644	0.994 ± 0.004	3.772 ± 1.102	−1.956 ± 0.945	0.996 ± 0.002
Dextran (15%)	3.473 ± 0.322	−1.778 ± 0.718	0.983 ± 0.010	3.045 ± 0.604	−1.687 ± 0.125	0.977 ± 0.018
Glucose (5%)	4.036 ± 1.740	0.683 ± 2.520	0.891 ± 0.095	4.014 ± 0.612	−1.596 ± 0.378	0.997 ± 0.001

Values are mean ± s.d., n = 3. K = release-rate constants, Int = intercept, Cor. coeff. = correlation coefficient.

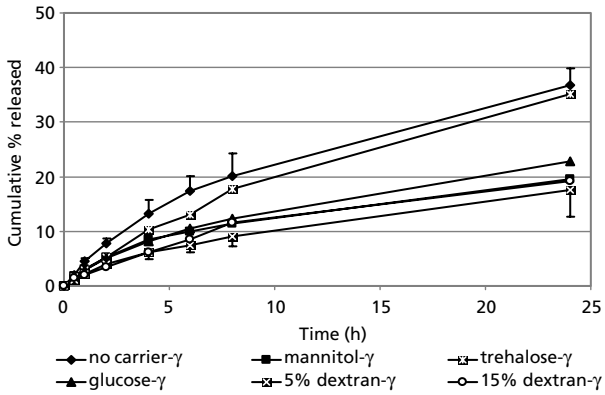

Figure 2 In-vitro release profiles of ciprofloxacin HCl-loaded, γ-sterilized nanoparticle formulations with or without cryoprotectants (mean ± s.d., n = 3).

had slower release profiles compared to non-sterilized formulations, resulting in significant ($P < 0.05$) release-rate decreases (see Table 3). On the other hand, no significant changes in the release rate after sterilization were observed for glucose and dextran (5.0% and 15.0%, w/v) protected nanoparticles (Table 3).

Controversial reports on the effects of γ-irradiation on the release properties of biodegradable microparticulate drug delivery systems are found in the literature; increased (Lalla & Sapna 1993; Volland et al 1994; Bittner et al 1999; Calis et al 2002) or decreased (Lalla & Sapna 1993; Volland et al 1994) release rates were measured after γ-irradiation. Little information is, however, available on the influence of γ-sterilization on the release characteristics of nanoparticles (Alléman et al 1993). In our study, slower or similar release profiles were observed, possibly because of the hydrophilic nature of ciprofloxacin HCl and increased aggregation behaviour of γ-sterilized nanoparticles reducing the contact surface area with the medium. Volland et al (1994) also found a decrease of the release kinetics with increasing irradiation doses for captopril-loaded microspheres using the same polymer. They reported that this could be attributed to the hydrophilic nature of the captopril molecule that is released predominantly by pore diffusion. The decreased mechanical strength of polymers under irradiation due to polymer chain breakdown leads to a disturbed pore network within the microspheres, resulting in a decrease of free pore diffusion.

The intrinsic viscosity of PLGA decreased from 0.424 to 0.364 after irradiation of PLGA. This leads to the assumption that, although release was slightly retarded after irradiation, the chain length of the PLGA polymer decreased after γ-irradiation.

Antimicrobial activity of ciprofloxacin HCl-loaded nanoparticles

The antibacterial effectiveness of ciprofloxacin HCl-loaded nanoparticles was assessed in comparison with an aqueous drug solution and blank nanoparticles using a microbiological method. An overview of the results is presented in Table 4. The MIC and MBC values were determined over 2 days.

MIC values of non- or γ-sterilized nanoparticles were comparable with an equivalent ciprofloxacin HCl solution; these values lay between 0.110–0.437 and 0.104–0.479 $\mu g\,mL^{-1}$ for ciprofloxacin solution and for all non- or γ-sterilized nanoparticle formulations, respectively. Furthermore, there is no time influence on the MIC values determined. In general, MIC values were comparable with MBC values. The MBC value of ciprofloxacin HCl solution was 0.437 $\mu g\,mL^{-1}$ and the MBC values were situated between 0.209 and 0.959 $\mu g\,mL^{-1}$ for all non- or γ-sterilized nanoparticle formulations.

The blank nanoparticles showed no antimicrobial activity, although the acid environment after degradation and release of lactic and glycolic acid could have inhibited bacterial growth. Thus, the microbiological assay demonstrated that the incorporation of ciprofloxacin HCl into PLGA nanoparticles did not change the MIC values. The MBC values increased when nanoparticles were applied instead of the aqueous solution. The presence of cryoprotectant and γ-irradiation had no influence on the MIC and MBC values of the nanoparticle preparations, at least for the micro-organism examined here. One can conclude that although the drug has not been released by 100% after 24 hours (see Figure 1), a sufficient amount of drug was released to kill micro-organisms to the same extent as the aqueous drug solution.

Table 4 MIC and MBC values of the antimicrobial agent against *Pseudomonas aeruginosa**

Formulation	MIC ($\mu g\,mL^{-1}$)	MBC ($\mu g\,mL^{-1}$)	MIC ($\mu g\,mL^{-1}$) (24 h)	MBC ($\mu g\,mL^{-1}$) (24 h)
Ciprofloxacin HCl solution	0.110–0.437	0.437	–	–
NP in absence of cryoprotectant	0.221 (0.443)	0.221 (0.443)	0.443 (0.221)	0.887 (0.443)
NP in presence of 5% (w/v) mannitol	0.104 (0.418)	0.209 (ND)	0.209 (0.418)	0.418 (0.418)
NP in presence of 5% (w/v) trehalose	0.421 (0.210)	0.843 (0.843)	0.210 (0.421)	0.843 (0.843)
NP in presence of 5% (w/v) dextran	0.440 (0.440)	0.881 (0.881)	0.440 (0.440)	0.440 (ND)
NP in presence of 15% (w/v) dextran	0.447 (0.447)	0.447 (0.895)	ND (0.447)	1.790 (0.895)
NP in presence of 5% (w/v) glucose	0.479 (ND)	0.959 (ND)	0.239 (0.239)	0.959 (0.959)

*Results from γ-irradiated nanoparticles are shown in parentheses. NP, nanoparticles; ND, not determined.

Conclusion

In the present study the effects of freeze-drying with different cryoprotectants and γ-sterilization on the properties of ciprofloxacin-loaded PLGA nanoparticles were investigated. From the ratio of particle size before and after freeze-drying and γ-irradiation, it can be concluded that none of the samples retained their initial characteristics on reconstitution after freeze-drying. No significant difference in the particle size and the zeta potential value of the γ-sterilized formulations compared to non-sterilized samples was observed. Reconstitution after γ-sterilization appeared to be more difficult than before sterilization for all formulations.

It can be concluded that considering easy reconstitution of nanoparticle suspensions, dextran is not the most appropriate cryoprotectant for present formulation.

After γ-sterilization a slower or similar drug release was measured. Although γ-sterilization appears to be a potential sterilization procedure for drug delivery systems, it should be carefully investigated and used with caution since it might cause changes in the properties of the drug delivery formulations.

A microbiological activity test proved that although not 100% of the drug incorporated was released from the nanoparticles after 24 h, the activity against *P. aeruginosa* of the non- or γ-sterilized nanoparticles and aqueous drug solution was comparable.

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