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Evaluation of ciprofloxacin-loaded Eudragit® RS100 or RL100/PLGA nanoparticles

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Abstract

The objective of present study was to prepare positively charged ciprofloxacin-loaded nanoparticles providing a controlled release formulation. The particles were prepared by water-in-oil-in-water (w/o/w) emulsification and solvent evaporation, followed by high-pressure homogenisation. Two non-biodegradable positively charged polymers, Eudragit® RS100 and RL100, and the biodegradable polymer poly(lactic-co-glycolic acid) or PLGA were used alone or in combination, with varying ratios. The formulations were evaluated in terms of particle size and zeta potential. Differential scanning calorimetry measurements were carried out on the nanoparticles and on the pure polymers Eudragit® and PLGA. Drug loading and release properties of the nanoparticles were examined. The antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* was determined. During solvent evaporation, the size and zeta potential of the nanoparticles did not change significantly. The mean diameter was dependent on the presence of Eudragit® and on the viscosity of the organic phase. The zeta potential of all Eudragit® containing nanoparticles was positive in ultrapure water (around +21/+25 mV). No burst effect but a prolonged drug release was observed from all formulations. The particles' activity against *P. aeruginosa* and *S. aureus* was comparable with an equally concentrated ciprofloxacin solution.

Keywords: Eudragit® RS100; Eudragit® RL100; PLGA; Nanoparticles; Ciprofloxacin

1. Introduction

Most ocular infections are treated by topical application of antibiotic solutions administered as aqueous eye drops. A low bioavailability is observed due to rapid and extensive precorneal loss; consequently, instillation of highly concentrated solutions or frequent administration is required, resulting in poor patient compliance. New drug delivery systems for ophthalmic administration, such as microparticles, liposomes or nanoparticles, have been developed and studied over the last decades and were designed to combine prolonged ophthalmic action with the ease of application of liquid eye drops (Zimmer and Kreuter, 1995; Le Bourlais et al., 1998). After administration, colloidal drug carriers can remain at the application site (cul-de-sac) and the prolonged release of the active ingredient starts by particle degradation or erosion, drug diffusion, or a combination of both, depending on the biodegradable or inert nature of the polymer (Soppimath et al., 2001).

Poly(DL-lactic-co-glycolic acid) or PLGA, a copolymer of lactic and glycolic acid is one of the most biocompatible, biodegradable and non-toxic materials used for preparing nano- and microparticles (Zimmer and Kreuter, 1995; Bala et al., 2004). Blends of Eudragit® and PLGA have been described in the preparation of heparin-loaded nanoparticles as potential oral carriers (Hoffart et al., 2002; Jiao et al., 2002).

Eudragit® RS100 (RS) and RL100 (RL) polymers have been proposed as ocular delivery systems with prolonged release and improved ocular availability, but mainly for non-steroidal anti-inflammatory drugs (Pignatello et al., 2002a,b). Such drug-loaded carrier systems showed good stability properties and size distribution and a positive surface charge, which makes them potential ocular drug delivery systems. This charge can allow a prolonged residence time of the nanoparticles on the corneal surface because of interactions with anionic mucins present in the

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tear film. Microspheres made of RS or RL containing gentamicin or acetazolamide have also been described as ophthalmic delivery systems (Safwat and Al-Kassas, 2002; Haznedar and Dortunç, 2004).

Desgouilles et al. (2003) proposed two hypotheses according to the mechanism of particle formation during polymer precipitation: one nanoparticle arising from one emulsion droplet, after shrinkage, or from fusing of several emulsion droplets. The physicochemical parameters size and zeta potential were followed during the process of solvent evaporation to determine whether a reassembling of the polymers at the particles' surface occurred. Such a rearrangement could occur in Eudragit® RS or RL containing nanoparticles, due to their amphiphilic properties.

Ciprofloxacin, a powerful broad-spectrum fluoroquinolone antibiotic, useful in the treatment of infections of the outer eye such as bacterial conjunctivitis and keratitis, has been chosen as a model drug for incorporation in the formulation (Blondeau, 2004). It has been reported that fluoroquinolones possess an in vitro efficacy against gram-negative as well as gram-positive ocular pathogens, superior to other antibiotics tested (Armstrong, 2000; Egger et al., 2001). Moreover, the frequency of spontaneous resistance to ciprofloxacin is very low (Hwang, 2004). Besides providing a controlled release system, nanoparticles can be a promising alternative for eye drops, since during intensive dosing of ciprofloxacin containing eye drops or ointments, corneal precipitates and crystalline deposits at eyelashes and in dropper bottle tips may occur (Wilhelmus and Abshire, 2003).

Pseudomonas aeruginosa and *Staphylococcus aureus*, some of the most common ocular pathogens causing bacterial infection of the human cornea (keratitis and cornea ulceration), especially ulcers underneath contact lenses, were selected as test microorganisms during the determination of antimicrobial activity of the formulations prepared (Armstrong, 2000; Bourcier et al., 2003).

The aim of present study was to evaluate the particle formation process, the physicochemical properties and activity of ciprofloxacin·HCl-loaded nanoparticles prepared from mixtures of poly(lactide-co-glycolide) (PLGA) and Eudragit® in different ratios.

Moreover, the objective was to prepare positively charged nanoparticles which could interact with the anionic mucins present in the mucus layer of the tear film.

2. Materials and methods

2.1. Materials

The PLGA polymer Resomer® RG 503 was obtained from Boehringer Ingelheim (Ingelheim am Rhein, Germany) and Eudragit® RS100 and RL100 from Röhm Pharma (Darmstadt, Germany). Poly(vinylalcohol) or PVA (MW 30,000–70,000) was supplied by Sigma Chemicals Co. (St. Louis, USA), ciprofloxacin hydrochloride monohydrate by Roig Farma (Barcelona, Spain) and D-mannitol by Federa Co. (Brussels, Belgium). Dichloromethane was purchased from Sigma–Aldrich (Steinheim, Germany) and acetonitrile (HPLC grade) from Acros Organics (New Jersey, USA). Salts for the preparation of Simulated Lachrymal Fluid (SLF) were of pro analysis quality. SLF is an electrolyte solution composed of 1.7893 g/l KCl, 6.3118 g/l NaCl, 2.1842 g/l $NaHCO_3$, 0.0670 g/l $CaCl_2{\cdot}2H_2O$, 0.1572 g/l $MgCl_2{\cdot}6H_20$, adjusted with 0.1N HCl to a pH of 7.4 ± 0.1 (Van Haeringen, 1981). Filtered (Porafil 0.20 Membranfilter, Düren, Germany) purified Milli Q water (Millipore, Mollsheim, France) was used throughout all experiments.

2.2. Preparation of ciprofloxacin-loaded Eudragit®/PLGA nanoparticles

The polymeric nanoparticles were prepared with Eudragit® RS or RL, PLGA or mixtures hereof (75/25, 50/50, 25/75% (w/w)). The preparation consisted of w/o/w emulsification solvent evaporation followed by high-pressure homogenisation (Dillen et al., 2004). Each sample was made in triplicate. Briefly, 2 ml of an aqueous ciprofloxacin·HCl solution (2.50%, w/v) was emulsified in methylene chloride (10 ml) containing the polymer(s) Eudragit® and/or PLGA (500 mg) using an ultrasound probe (Branson 450-D, 102-C with microtip, Branson, Danbury, USA) for 60 s at 20 W. The resulting w/o emulsion was poured in 25 ml of a 1% (w/v) PVA stabiliser solution, and sonicated for 60 s at 30 W to obtain a multiple w/o/w emulsion, which was homogenised employing a Microfluidizer M-110L (Microfluidics, Newton, USA) at a pressure of 50 bar during three cycles. The emulsion was then diluted in 120 ml PVA solution (0.3% (w/v) in water) in order to minimise coalescence. The cryoprotectant mannitol was added in a 5% (w/v) concentration, to obtain isotonicity after reconstitution and to ease redispersion of the solid nanoparticles by manual agitation after freeze-drying. The organic solvent was evaporated under magnetic stirring (700 rpm) for 4 h at room temperature (Variomag Electronicrührer Poly 15, H + P Labortechnik GmbH, Munich, Germany). The resulting nanosuspension was subsequently cooled down to −18 °C and freeze-dried (Leybold-Heraeus D8B, GT-2A, Germany). The freeze-dried nanoparticles were gamma-irradiated and received a dose of 25 kGy, employing Co^{60} as irradiation source (Gammir I-Sulzer irradiation unicell, IBA-Mediris, Sterigenics, Fleurus, Belgium).

2.3. Evaluation of the nanoparticles

2.3.1. Nanoparticle size and zeta potential analysis

Particle size analysis was performed by Photon Correlation Spectroscopy (PCS) with a Zetasizer 3000 (Malvern Instruments, Malvern, UK). The PCS measurements were carried out at a 90° scattering angle. Nanoparticle suspensions were diluted 100 times with filtered SLF solution. The mean particle size Z_{ave} of each sample was determined three times and the average values were calculated.

Zeta potential values were determined by electrophoretic light scattering (ELS) using the same instrument. Nanoparticles were suspended either in SLF or in filtered water and diluted with the same solutions. For each preparation, three samples were injected in the capillary cell of the Zetasizer 3000 and

each of them was determined 20 times. Afterwards, the average values of three replicates were calculated.

To evaluate the influence of evaporation time on the nanoparticles' size and zeta potential, these characteristics were measured 30 min, 1, 2 and 4 h after start of solvent evaporation. Potential differences between PLGA nanoparticles and their Eudragit® containing counterparts were investigated.

2.3.2. Thermal analysis

Differential scanning calorimetry experiments were performed on the drug, the polymers PLGA, Eudragit® RS and RL, and on the different nanoparticle formulations. In addition, films made of Eudragit® RS, RL, PLGA or mixtures hereof (75/25, 50/50, 25/75% (w/w)) were analysed. The polymers were dissolved in dichloromethane at a weight ratio of 1:20, the solution was cast on Teflon moulds and the solvent was evaporated slowly in air at room temperature for 48 h. The remaining solvent was removed in an oven at 40 °C for a week. Samples (±5 mg) were weighed into aluminium pans (TA Instruments, Brussels, Belgium) and hermetically sealed. DSC runs were performed over a temperature range 25–220 °C, at 5 °C/min. Octadecane and indium standards were used to calibrate the DSC-7 calorimeter (Perkin-Elmer, Norwalk, USA).

2.3.3. Viscosity determination of the organic phase

The apparent viscosity of 5% (w/v) solutions of Eudragit®, PLGA and mixtures hereof in methylene chloride was determined using a controlled-stress rheometer (Carri-Med CSL^2 100, TA Instruments, Brussels, Belgium) equipped with a stainless steel cone and plate geometry (6 cm diameter, cone angle 1.59°, truncation 69 μm) (TA Instruments Ltd., Surrey, UK) and a solvent trap. The temperature was set at 25 °C and the shear rate was then increased linearly from 1 to 200 s^{-1} within a period of 5 min and decreased linearly from 200 to 1 s^{-1} within the same time interval. The apparent viscosity was then calculated according to the Bingham model (regression coefficient >0.99), suitable for Newtonian liquids with a yield stress (Eq. (1))

$$\sigma = \sigma_y + \eta_p \gamma \quad (1)$$

with σ is the shear stress (Pa), σ_y the yield stress (Pa), η_p the plastic viscosity (Pa s) and γ is the shear rate (s^{-1}).

2.3.4. Analysis of ciprofloxacin·HCl

The RP-HPLC system consisted of a Gilson 321 pump (Gilson, Villiers-le-Bel, France), a UV-VIS 152 detector set at 278 nm (Gilson, Villiers-le-Bel, France), a μ-Bondapack™ C_{18} 125 Å 10 μm reversed-phase column (Waters, Milford, USA) and an HP 3395 integrator (Hewlett-Packard Company, Palo Alto, USA). The mobile phase consisted of 13% (v/v) of acetonitrile and 87% (v/v) of a 0.025 M aqueous phosphoric acid solution, adjusted to pH 3 with triethylamine. The flow rate was set at 1.5 ml/min. An external calibration curve was constructed by plotting the ciprofloxacin. HCl peak areas versus the known concentrations of the drug.

2.3.5. Drug loading determination

An amount of 150.0 mg freeze-dried preparation, accurately weighed, was dispersed in 10.0 ml of purified water and placed in a sonication bath for 10 min (Julabo USR3, Julabo, Seelbach, Germany). The samples were centrifuged at 3000 rpm for 3 h (Cetra-MP4 centrifuge, International Equipment Company, Miami, USA) and the amount of unincorporated drug was determined by HPLC analysis of the supernatant. Drug entrapment was expressed as the percentage incorporation with respect to the theoretical value (actual drug loading versus theoretical drug loading).

2.3.6. In vitro release tests

Ciprofloxacin release from nanosuspensions was evaluated using diffusion cells, whereby a dialysis membrane with a molecular weight cut-off (MWCO) of 12,000–14,000 Da (Medicell International, London, UK) separated the acceptor from the donor compartment, consisting of 300.0 mg nanoparticle preparation dispersed in 5.0 ml of water. The acceptor compartment was filled with 18.0 ml SLF and stirred magnetically at 200 rpm (Variomag Electronicrührer Poly 15, H + P Labortechnik GmbH, Munich, Germany). At regular time intervals within 24 h, samples of 1.0 ml were withdrawn from the acceptor compartment and replaced by the same volume of fresh SLF solution. The drug content of the samples was determined by the previously mentioned HPLC method. Each experiment was repeated in triplicate.

The in vitro release profiles were fitted to the zero-order model (Eq. (2)), first-order model (Eq. (3)), Higuchi square root model (Eq. (4)) and Hixson–Crowell cube root model (Eq. (5)), some of the most common kinetic profiles used (Hughes, 2005)

$$Q_t = Q_0 + k_0 t \quad (2)$$

$$Q_t = Q_0 \, e^{-k_1 t} \quad (3)$$

$$Q_t = k_H \sqrt{t} \quad (4)$$

$$\sqrt[3]{Q_0} - \sqrt[3]{Q_t} = k_{HC} t \quad (5)$$

with Q_t is the total amount of drug released after time t (%), Q_0 the initial amount of drug (%), k_0 the zero-order release rate constant (% h^{-1}), k_1 the first-order release rate constant (h^{-1}), k_H the rate constant obtained according to the Higuchi equation (% $h^{-1/2}$), and k_{HC} is the rate constant obtained according to the Hixson–Crowell equation (% h^{-1}).

Excel 2002 (Microsoft® Corporation, Redmond, WA, USA) was used for the calculation of the release rate constants (k_x) with the Solver tool and the determination of the correlation coefficients (R^2). As the best fitting model, the one with the highest correlation coefficients between the observed and the fitted data was selected.

2.3.7. Antimicrobial activity determination of ciprofloxacin-loaded nanoparticles

The antimicrobial activity of the loaded nanoparticles was investigated with respect to the free drug activity and compared with previously studied drug-free nanoparticles by determining

the minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) on *P. aeruginosa* (ATCC 9027) and *S. aureus* (ATCC 6538).

MIC values were determined by a microdilution test in Tryptone Soy Broth (E & O Laboratories, Bonnybridge, Scotland) in 96-well Cell Culture Clusters (Corning Incorporated, Corning, USA). For each determination, 11 wells at various ciprofloxacin·HCl concentrations were prepared (14–0.014 μg ml^{-1}). Two replicas were prepared, to the first one a microorganism suspension (2×10^5 cfu ml^{-1}; cfu = colony forming units) was added the same day, to the second replica after 24 h. Inoculated wells were incubated for 24 h at 37 °C, after which they were inspected for turbidity. MIC was defined as the lowest antibiotic concentration inhibiting visible growth after this incubation period. A positive control (growth) was formed by culture broth with microorganisms, a negative control (sterility) by broth without microorganisms. The MBC values (μg/ml) were determined by subculturing the MIC dilution and three following dilutions on Tryptic Soy Agar plates without antibiotic and inspecting for formation of colonies after 24 h of incubation at 37 °C. In this experiment, drug concentrations at which growth of organisms is inhibited but bacteria were not killed can be detected.

2.3.8. Statistical analysis

The statistical significance of the differences in particle size and zeta potential values, entrapment efficiency percentages, release rate constants and MIC and MBC values between the different nanoparticle formulations was tested by one-way analysis of variance (ANOVA) followed by multiple comparison with Newman–Keuls post hoc evaluation using the Statistica® (Statsoft, Tulsa, USA) software.

Differences were considered to be statistically significant at a level of $p \leq 0.05$.

3. Results and discussion

3.1. Characterisation of the nanoparticles

3.1.1. Physical characterisation: particle size and zeta potential measurements

All batches showed a small mean size, well suited for possible ocular application to prevent patient discomfort and provide a good drug diffusional release. Particle size for ophthalmic applications should not exceed 10 μm because with larger sizes a scratching feeling might occur (Zimmer and Kreuter, 1995). Therefore, a reduced particle size improves the patient comfort.

In Fig. 1, an overview is shown of particle sizes of nanoparticles consisting of Eudragit® and/or PLGA, before and after freeze-drying and gamma-irradiation. The size of Eudragit® RS containing nanoparticles was not significantly different from their RL counterparts, containing the same percentage of Eudragit® and PLGA ($p = 0.11$). Haznedar and Dortunç (2004) also reported that the mean particle sizes of acetazolamide-loaded Eudragit® microspheres were not affected by the polymer type. Pignatello et al. (2002a) on the other hand stated that drug-free RL nanoparticles had a slightly greater mean size than

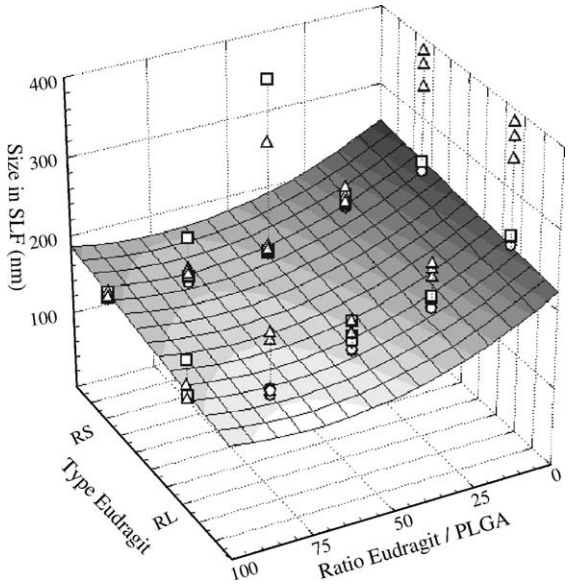

Fig. 1. Three-dimensional surface plot of particle size of all series of ciprofloxacin-loaded Eudragit®/PLGA nanoparticles. Circles: particles after solvent evaporation, squares: particles after freeze-drying, triangles: particles after gamma-irradiation.

RS nanosuspensions. A size increase from 51 to 70 nm was measured. These authors, however, used a different preparation method (quasi-emulsion solvent diffusion).

Hoffart et al. (2002) prepared RS, RL, RS/PLGA and RL/PLGA nanoparticles, unloaded or loaded with heparin. The smallest particles were obtained with Eudragit®, and especially with the highest charged Eudragit® RL, containing more quaternary ammonium groups. In agreement with their results, in present research, ciprofloxacin-loaded Eudragit® or Eudragit®/PLGA nanoparticles were significantly smaller than PLGA nanoparticles ($p = 0.0013$). Nanoparticles composed from a 25/75 mixture of Eudragit® and PLGA were significantly larger than when higher amounts of Eudragit® were employed. The viscosity of the inner phase is an important factor in the preparation of particles (Haznedar and Dortunç, 2004). The size of Eudragit® and/or PLGA nanoparticles is dependent of the inner organic phase viscosity (Hoffart et al., 2002). In Fig. 2, the

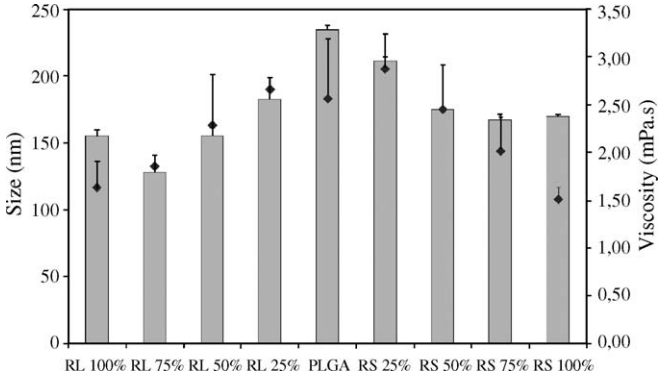

Fig. 2. Average particle size ($n = 3$) before freeze-drying of ciprofloxacin-loaded nanoparticles (grey bars) and viscosity (η_p) of the inner water phase (diamonds) for different polymer blends.

particle sizes of the nanoparticles together with the viscosity (η_p) of the organic phase containing these polymers are shown. For all polymer solutions tested, the yield stress values (σ_y) were very low (between 0 and 10 mPa). No relation between yield stress values and nanoparticle properties could be observed. During the emulsification process, the lower the viscosity of the dispersed phase, the smaller the mean diameter. A more viscous organic phase provides a higher mass transfer resistance, the diffusion of polymer–solvent phase into the external aqueous phase is reduced and larger nanoparticles are formed. A decrease in viscosity of the organic phase increases the distribution efficiency of the polymer–solvent phase into the external phase leading to formation of smaller nanoparticles (Galindo-Rodriguez et al., 2004). Increasing the polymer concentration and thus the viscosity of the inner phase led to a shift towards higher particle sizes, in the more viscous dispersion larger droplets and thus larger particles were formed.

Freeze-drying caused a significant ($p = 0.0129$) size increase in nanoparticles made of Eudragit® RS or RL or of Eudragit®/PLGA blends. This could be due to changes in the internal structure of the particles, originated during the freeze-drying process caused by the formation of ice crystals in the internal water phase or, more likely, to particle aggregation during freeze-drying resulting in more difficult redispersion. Gamma-irradiation only caused a significant size increase in PLGA nanoparticles ($p = 0.0003$) but did not influence the particle size of Eudragit® containing nanoparticles ($p = 0.44$). This can be due to particle aggregation during gamma-irradiation or to chain scission and subsequently cross-linking taking place.

The zeta potential values of the nanoparticles prepared, measured in SLF or in water, are presented in Table 1. All Eudragit® containing formulations exhibited strongly positive zeta potential values in water. Compared to previous studies with drug-free particles, the presence of the active compound did not influence the zeta potential in a significant way. The drug ciprofloxacin·HCl seemed to be uniformly dispersed in the nanoparticles.

Only PLGA nanoparticles were negatively charged, the zeta potential of these particles was significantly different from the positively charged Eudragit® containing particles ($p = 0.0001$). The zeta potential of Eudragit®/PLGA mixtures was not dependent of the percentage Eudragit® incorporated ($p = 0.63$). Hoffart et al. (2002) on the contrary reported that blends of PLGA with Eudragit® showed a strong positive surface potential but lower than that obtained with Eudragit® alone.

The zeta potential of Eudragit® RL containing particles was significantly higher than that of RS containing particles ($p = 0.0110$). The zeta potential data reflect the charges of native polymers, the polycationic Eudragit® bearing positive charges, RL being more positive than RS, and the uncharged PLGA displaying negative zeta potentials. A positive charge can facilitate an effective adhesion to the cornea surface and account for a strong interaction with the negatively charged mucosa of the conjunctiva and anionic mucins present in the tear film, prolonging the effective residence time of the formulation. Ubrich et al. (2005), however, prepared cyclosporine A loaded Eudragit® RS or RL nanoparticles and observed no significant differences in surface potential for all formulations tested.

Freeze-drying caused a significant decrease in zeta potential ($p = 0.0001$) and gamma-irradiation a significant increase ($p = 0.0012$), in a way that zeta potential after gamma-irradiation was not significantly different from before freeze-drying ($p = 0.07$). Since freeze-drying is necessary for stability reasons and irradiation for sterility, the end value of the zeta potential is comparable to the starting value.

While the zeta potential remained in a range of positive values for samples measured in water, slightly negative values were obtained in simulated lachrymal fluid. This can be explained by the presence of salts in SLF, which neutralise electrical charges present at the particle surface ("shielding"), leading to a decrease of the zeta potential value. Hoffmann et al. (1997) also noticed that positive zeta potential values were observed for methylmethacrylate copolymer nanoparticles measured in water, however negative values were obtained when NaCl solution was used. Smaller values than the original zeta potential

Table 1
Zeta potential values of ciprofloxacin-loaded nanoparticle preparations (mean ± S.D., $n = 3$)

Polymer	Zeta potential in water (mV)			Zeta potential in SLF (mV)		
	After evap.[a]	After FD[b]	After γ[c]	After evap.[a]	After FD[b]	After γ[c]
PLGA	−12.1 ± 3.1	−18.2 ± 3.6	−10.1 ± 1.8	−6.2 ± 0.3	−8.3 ± 0.8	−8.0 ± 0.2
RL:PLGA						
100:0	+21.2 ± 0.6	+23.1 ± 7.1	+28.1 ± 5.0	−5.8 ± 1.6	−5.1 ± 1.8	−4.2 ± 0.7
75:25	+26.1 ± 1.1	+24.3 ± 2.9	+29.1 ± 1.9	−4.8 ± 0.7	−4.3 ± 1.7	−3.8 ± 2.7
50:50	+27.8 ± 1.9	+19.9 ± 0.6	+24.6 ± 4.6	−3.8 ± 2.0	−4.2 ± 1.2	−5.2 ± 2.5
25:75	+28.8 ± 3.8	+22.1 ± 1.8	+21.9 ± 0.8	−4.1 ± 0.5	−4.0 ± 0.7	−4.1 ± 0.3
RS:PLGA						
100:0	+28.9 ± 1.5	+14.5 ± 2.7	+23.0 ± 0.2	−4.4 ± 0.5	−6.6 ± 1.5	−8.9 ± 2.0
75:25	+28.5 ± 0.6	+14.7 ± 2.2	+23.6 ± 1.7	−5.4 ± 1.7	−8.3 ± 2.1	−9.9 ± 3.2
50:50	+28.0 ± 1.1	+16.3 ± 1.7	+22.8 ± 2.4	−5.4 ± 2.2	−11.3 ± 4.8	−9.0 ± 0.9
25:75	+25.7 ± 3.3	+21.1 ± 0.9	+20.0 ± 0.9	−7.4 ± 0.3	−6.5 ± 0.7	−7.0 ± 0.6

[a] Solvent evaporation.
[b] Freeze-drying.
[c] Gamma-sterilisation.

of negatively charged PLGA nanoparticles were observed after adsorption of positive ions present in simulated blood fluid, such as Na^+, K^+ and Mg^{2+} ions, decreasing the electrostatic repulsive forces between particles (Kim et al., 2005).

When compared to previously studied drug-free nanoparticles, the incorporation of ciprofloxacin·HCl did not influence the zeta potential of the polymer particles significantly. The zeta potential of Eudragit®/PLGA nanoparticles was again independent of the percentage of Eudragit® used ($p = 0.60$). Only the zeta potential of PLGA ciprofloxacin-loaded particles was significantly lower than that of all other preparations ($p = 0.0082$). This leads to the hypothesis that the more surface active and hydrophilic polymer Eudragit® is more present at the outer surface of the particles. Like already observed in water, particles composed with Eudragit® RL give rise to higher (less negative) zeta potential values than with Eudragit RS®, when measured in SLF. The difference in content of ammonium groups between both polymers gives rise to an alteration in zeta potential, even with the masking effect of the ions present in the medium on the charge. This effect of the number of quaternary ammonium groups on the zeta potential is more pronounced when measured in water.

Freeze-drying and gamma-irradiation had no significant effect on the zeta potential of the particles measured in SLF ($p = 0.14$).

The size and surface charge were followed during evaporation of the organic solvent, to determine whether a rearrangement of the polymers occurred during evaporation, since Eudragit® has amphiphilic properties. The size ($p = 0.89$), zeta potential in water ($p = 0.91$) or in SLF ($p = 0.62$) of all samples prepared did not change significantly as a function of time of evaporation (data not shown). This is in agreement with the PLA-model of Desgouilles et al. (2003) with no droplet or particle aggregation, but only slight size shrinkage until a minimum value. The zeta potential of their PLA emulsion/nanoparticle suspension was constant over the 240 min period of the solvent evaporation process. Rosca et al. (2004) also reported that microdroplets did not coalesce or break up after the emulsification step, thus particle size distribution was determined only by the emulsification step and solvent transport determined particle morphology, encapsulation and release behaviour.

3.1.2. Differential scanning calorimetry experiments

DSC experiments were carried out to determine the miscibility of Eudragit and PLGA in the different ratios employed in the nanoparticles. The thermograms of PLGA, Eudragit® RL and RS, the cryoprotectant mannitol, and of two nanoparticle formulations are presented in Fig. 3. The T_g of the PLGA polymer was 50.5 °C (see Fig. 3a), while Eudragit® RS and RL had T_g values of 58 and 72 °C, respectively (see Fig. 3b). The T_g values of the different nanoparticles prepared were situated between the T_g's of the individual polymers. The nanoparticles with different ratios of both polymers all possessed T_g values of around 60 °C (see Fig. 3d and e), pointing at the presence of one phase. PLGA and Eudragit® RL or RS were thus miscible in all concentrations used. The results obtained suggest that the nanoparticles consisted of a homogeneous amorphous polymer matrix. The endothermic melting peak in Fig. 3d and e is due to the presence of the cryoprotectant mannitol in the freeze-dried nanoparticle formulations. As can be deducted from Fig. 3c, the melting temperature of mannitol is 166 °C.

Films made of PLGA, Eudragit® and mixtures hereof were also analysed to study the miscibility of both polymers. Films provide denser material than freeze-dried nanoparticles, giving a better thermal contact during performance of the analysis. The T_g values of the PLGA/Eudragit® films were also situated between the T_g's of the individual polymers, this leads to the conclusion that both polymers were miscible in all concentrations tested.

3.1.3. Nanoparticle drug loading and release characteristics

The entrapment efficiency percentages, shown in Fig. 4, varied between 60 and 70% for all formulations prepared. The entrapment efficiency was affected by the nature of polymers used (PLGA versus Eudragit® or blends hereof) ($p = 0.0087$) but not between different polymer ratios ($p = 0.42$). It also did not vary between the two types of Eudragit® used ($p = 0.39$), although one could have expected this since it is known that acidic compounds – like ciprofloxacin·HCl – interact with RS and RL polymers by means of electrostatic bindings between the carboxyl moiety of the drug and the quaternary ammonium groups of the polymer (Pignatello et al., 2002b). The amount of ammonium groups is higher in Eudragit® RL than in Eudragit RS®; they are absent in PLGA. Jiao et al. (2002) and Hoffart et al. (2002) reported drug loadings inside nanoparticles formulated with blends of RS or RL with PLGA up to twofold higher with regard to PLGA alone. In present research however, the differences, although significant ($p = 0.0087$) were not this pronounced. Since the pH of the 2.5% (w/v) aqueous ciprofloxacin solution amounted 6, and the pK_a of the carboxylic acid group in ciprofloxacin is 6.16, ionisation of the carboxylic group will not be very pronounced and interaction with Eudragit® rather limited (Bermejo et al., 2004). This may also explain the fact that the differences between Eudragit® RL and RS were not significant, despite of the difference in number of quaternary ammonium functions between these two polymers.

The ciprofloxacin release curves from all nanoparticle formulations are illustrated in Fig. 5. The release rate constants (k_x) calculated by the above-mentioned mathematical models and the correlation coefficients (R^2) between the observed release data and the fitted profiles are summarized in Table 2. Higuchi's square root equation showed a better fit than zero order, first order and cube root equations.

For Eudragit®, only modelling of drug release from microspheres has been described (Hombreiro-Pérez et al., 2003; Gibaud et al., 2004). Haznedar and Dortunç (2004) used different mathematical models (zero order, first order, Higuchi and Hixson–Crowell) to characterise release from Eudragit® microspheres containing acetazolamide. Release rate data were found to fit first-order kinetics. Kristmundsdóttir et al. (1996), on the other hand, prepared diltiazem-loaded Eudragit® RS and RL microparticles. They reported that drug release obeyed the Higuchi diffusion controlled model.

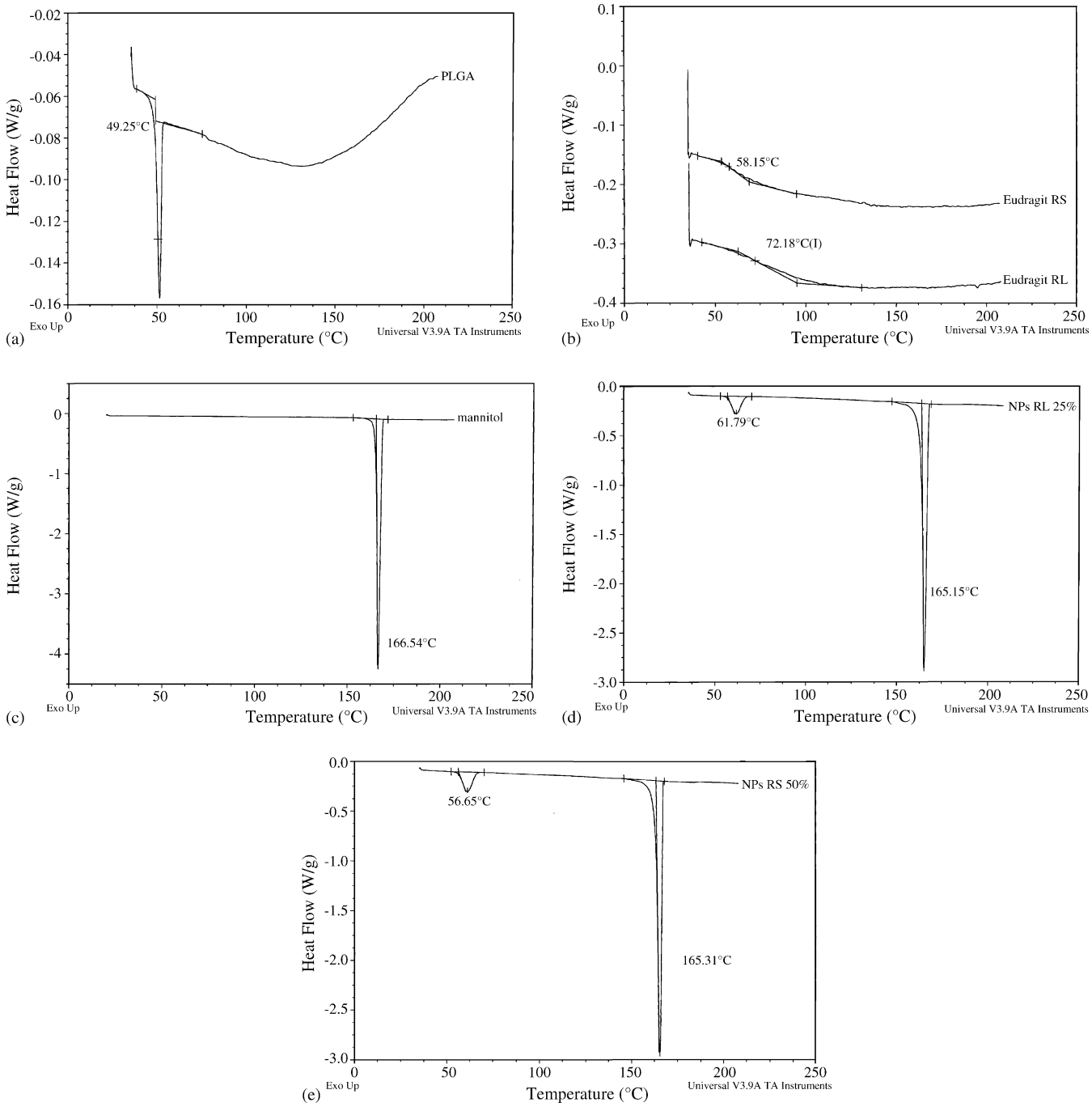

Fig. 3. DSC thermograms of PLGA (a), Eudragit® RS and RL (b), mannitol (c) and ciprofloxacin-loaded Eudragit®/PLGA nanoparticles ((d) 25% Eudragit® RL and (e) 50% Eudragit® RS).

In the case of PLGA, most drug release modelling has been performed for microspheres (Faisant et al., 2003; Siepmann et al., 2004; Zhang et al., 2003). Few studies about release modelling of PLGA nanoparticles were published. Release data of 5-fluorouracil-loaded PLGA nanoparticles were investigated by using zero order, first order, Higuchi and Hopfenberg release kinetics (Bozkir and Saka, 2005). According to the correlation coefficients, release data fitted best to the Higuchi's diffusion kinetics. The in vitro release was suggested to be controlled by a combination of diffusion with slow and gradual erosion of the particles (Bozkir and Saka, 2005). The Higuchi model describes, however, purely diffusion-controlled drug release. In addition, polymer degradation can play a crucial role in drug release. According to Panyam et al. (2003), the polymer degradation rate is faster for nanoparticles compared to microparticles. Dunne et al. (2000), on the other hand, found a linear relationship between the degradation rate and particle size of PLGA spheres. Larger particles degraded fastest due to autocatalytic effects and length of diffusion path. At an incubation temperature of 25 °C, PLGA particles showed an induction period of about 20 days

Table 2
Release rate constants k_x (mean ± S.D., $n = 3$) and correlation coefficients (R^2) calculated after fitting the release profiles obtained using different mathematical models

Polymer	Zero order		First order		Higuchi		Hixson–Crowell	
	k_0	R^2	k_1	R^2	k_H	R^2	k_{HS}	R^2
PLGA	−1.1193 ± 0.2686	0.9488	0.0131 ± 0.0036	0.9653	4.45 ± 1.01	0.9813	0.0192 ± 0.0051	0.9600
RL:PLGA								
100:0	−1.3008 ± 0.5319	0.9442	0.0158 ± 0.0071	0.9623	5.17 ± 1.90	0.9743	0.0229 ± 0.0100	0.9566
75:25	−1.2089 ± 0.2766	0.9182	0.0144 ± 0.0037	0.9410	4.90 ± 0.93	0.9897	0.0210 ± 0.0052	0.9336
50:50	−0.8879 ± 0.2058	0.9236	0.0100 ± 0.0026	0.9405	3.56 ± 0.75	0.9766	0.0149 ± 0.0037	0.9349
25:75	−1.0640 ± 0.1522	0.9300	0.0123 ± 0.0020	0.9484	4.28 ± 0.56	0.9865	0.0182 ± 0.0028	0.9424
RS:PLGA								
100:0	−1.1265 ± 0.0653	0.8684	0.0132 ± 0.0008	0.8944	4.60 ± 0.18	0.9580	0.0194 ± 0.0012	0.8858
75:25	−1.2324 ± 0.0456	0.9108	0.0147 ± 0.0007	0.9346	5.02 ± 0.23	0.9890	0.0214 ± 0.0009	0.9268
50:50	−1.1287 ± 0.0497	0.9298	0.0131 ± 0.0006	0.9499	4.48 ± 0.10	0.9844	0.0193 ± 0.0008	0.9434
25:75	−1.2156 ± 0.0424	0.9180	0.0144 ± 0.0006	0.9415	4.90 ± 0.20	0.9833	0.0211 ± 0.0009	0.9338

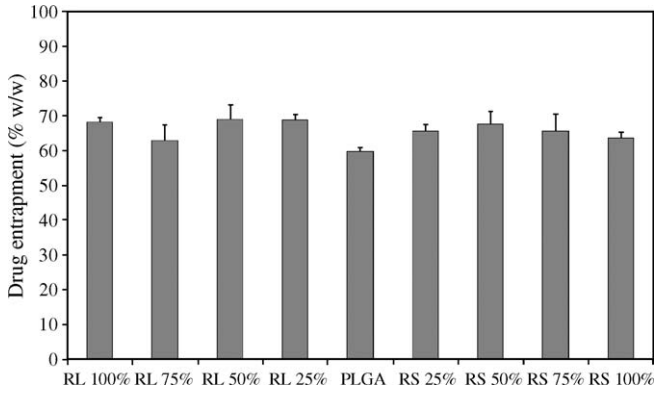

Fig. 4. Drug loading (mean, $n = 3$) of Eudragit®, Eudragit®/PLGA and PLGA nanoparticles.

after which polymer degradation proceeded. Changes in polymer molecular weight and polymer mass loss were negligible during the induction period consistent with minimal polymer degradation over this time.

Release kinetics of drugs from poly(lactide-co-glycolide) micro- and nanoparticles are controlled by diffusion and/or erosion mechanisms. From the data obtained in present study, it can be concluded that during the in vitro release study over 24 h, which corresponds to the induction period, release of the drug from PLGA nanoparticles or Eudragit®/PLGA mixtures is controlled by diffusion, as described by the Higuchi model.

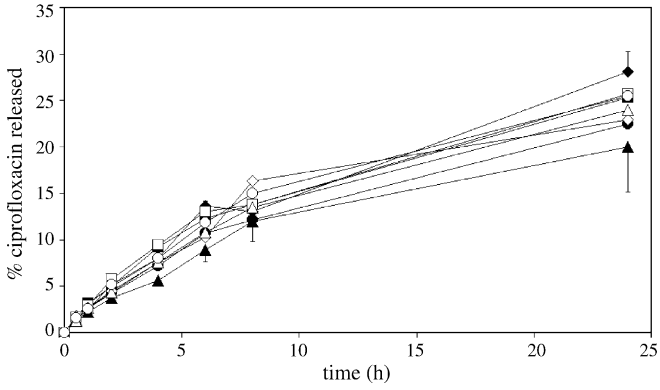

Fig. 5. Release of ciprofloxacin·HCl from Eudragit® or Eudragit®/PLGA nanoparticles (mean, $n = 3$). Open symbols: Eudragit® RS, closed symbols: RL, diamonds: 100%, squares: 75%, triangles: 50%, circles: 25% Eudragit®.

In general, all nanoparticle preparations showed a prolonged release, no burst effect could be observed (see Fig. 5). A slow release pattern was observed for all nanoparticle preparations, like in the work of Jiao et al. (2002) and Hoffart et al. (2002), however, the release in present study was not biphasic over the studied period (24 h). These authors observed a higher release from PLGA than from Eudragit® nanoparticles; a 1/1 combination Eudragit®/PLGA did not differ in drug release from pure Eudragit® particles. The explanation for the low release from Eudragit®-containing particles was a strong ionic interaction between the polycationic Eudragit® and the polyanionic drug heparin. When comparing the release rate constants (k_H), no significant difference in drug release between PLGA, Eudragit® RS and RL nanoparticles and nanoparticles made of blends thereof could be observed ($p = 0.22$). The presence of a carboxyl group in ciprofloxacin allows chemical and/or physical interactions like ion-pairs to occur with the ammonium group of RS and RL polymers (Pignatello et al., 2001). Contrary to heparin however, ciprofloxacin only possesses one carboxyl group per molecule, so the interaction is probably not that pronounced.

Different drug release profiles were observed by Haznedar and Dortunç (2004) when Eudragit® RL was used instead of Eudragit® RS. Drug release rates from Eudragit® RS microspheres were very slow and incomplete, from the same formulations prepared using Eudragit® RL as polymer different drug release profiles were observed. Release was less retarded because of the greater water permeability of the latter, due to the higher quaternary ammonium group content. The contemporary presence of two polymers created a more compact matrix, hindering drug desorption and the following diffusion into the dissolution medium. In present study, no significant difference between drug release rate constants of Eudragit® RS or RL containing nanoparticles could be observed ($p = 0.44$).

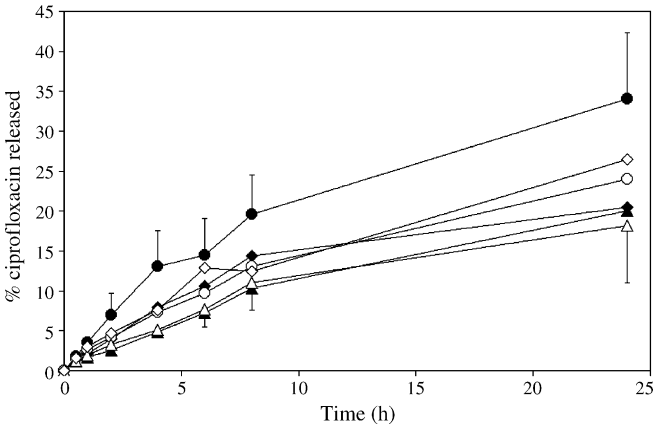

Fig. 6. Release of ciprofloxacin·HCl from PLGA, Eudragit® RL and Eudragit® RL/PLGA nanoparticles, before and after gamma-irradiation (mean, $n = 3$). Diamonds: PLGA nanoparticles, triangles: 1/1 blend, circles: Eudragit® RL nanoparticles, closed symbols: particles before sterilisation, open symbols: particles after sterilisation.

Gamma-irradiation did not cause a significant change in drug release nor from Eudragit®-containing nanoparticles, nor from pure PLGA formulations ($p = 0.95$) (see Fig. 6).

Most reports about effects of gamma-irradiation on drug release from PLGA particulate systems concern microparticles. A dose related influence of γ-irradiation on the in vitro release properties was observed, with a significant acceleration of drug release from these systems after irradiation (Mohr et al., 1999). Faisant et al. (2003) also reported an increase in release rate with increasing γ-irradiation dose. This was attributed to the irradiation induced ester bond cleavage and the resulting decrease in polymer molecular weight.

The results of present study are in agreement with the studies reported by Martínez-Sancho et al. (2004) and Song et al. (1997). Gamma-sterilisation at a dose of 25 kGy did not affect the release rate of aciclovir from PLGA microspheres (Martínez-Sancho et al., 2004). In a study about PLGA nanoparticles, no change in release of various drugs before and after irradiation could be detected (Song et al., 1997).

Further in vivo studies should be carried out to determine whether the release rate obtained with the nanoparticles is appropriate for therapeutic use in eye infections.

3.1.4. Antimicrobial activity

The antibacterial effectiveness of γ-sterilised ciprofloxacin-loaded nanoparticle systems against *P. aeruginosa* and *S. aureus* was assessed in comparison with an aqueous drug solution and compared to that of previously studied drug-free nanoparticles using a microbiological method. Table 3 shows the ranges (minimum–maximum value) of MIC and MBC values of the nanoparticles, determined after 1 (24 h) and 2 days (48 h) and compared to free drug (ciprofloxacin·HCl in aqueous solution). Drug-free particles showed no antimicrobial activity at all, even though acid degradation products that were formed and cationic surface-active polymers could have had an effect.

The MIC values after direct inoculation (MIC 24 h) and after inoculation after 1 day (MIC 48 h) were not significantly different ($p = 0.12$). MBC values were significantly higher than their

Table 3
Range of MIC and MBC values (μg/ml) of ciprofloxacin-loaded nanoparticle preparations ($n = 3$)

Polymer	MO[a]	MIC 24 h	MBC 24 h	MIC 48 h	MBC 48 h
cipro sol.[b]	P.a.[c]	0.11–0.22	0.22–0.44	0.11–0.22	0.22–0.44
PLGA	P.a.	0.26–0.28	0.26–0.28	0.27–0.53	0.53–0.56
RL:PLGA					
100:0	P.a.	0.31–0.62	0.62–1.21	0.31–1.20	1.20–1.25
75:25	P.a.	0.53–0.61	0.53–1.11	0.27–0.31	0.28–0.61
50:50	P.a.	0.26–0.34	0.26–0.65	0.26–0.34	0.53–0.69
25:75	P.a.	0.12–0.23	0.23–0.24	0.12–0.23	0.23–0.28
RS:PLGA					
100:0	P.a.	0.28–0.59	1.12–2.36	0.29–0.59	1.12–1.18
75:25	P.a.	0.56–0.64	0.57–1.12	0.28–0.63	0.63–1.15
50:50	P.a.	0.29–0.32	0.29–1.22	0.29–0.32	0.31–0.64
25:75	P.a.	0.22–0.46	0.22–0.91	0.22–0.46	0.22–0.46
cipro sol.[b]	S.a.[d]	0.18	0.70	0.35	0.70
PLGA	S.a.	0.40–0.41	0.79–0.83	0.40–0.41	0.79–0.83
RL:PLGA					
100:0	S.a.	0.23–0.47	0.45–0.94	0.23–0.47	0.45–0.93
75:25	S.a.	0.20–0.42	0.40–0.84	0.20–0.84	0.40–1.67
50:50	S.a.	0.40–0.52	0.52–1.59	0.20–1.03	0.80–2.07
25:75	S.a.	0.23–0.24	0.24–0.47	0.12–0.24	0.47–0.91
RS:PLGA					
100:0	S.a.	0.22–0.43	0.44–0.86	0.42–0.86	0.84–1.73
75:25	S.a.	0.42–0.48	0.84–0.96	0.42–0.96	0.84–1.93
50:50	S.a.	0.23–0.48	0.46–0.96	0.23–0.87	0.92–1.74
25:75	S.a.	0.22–0.46	0.44–0.92	0.22–0.46	0.44–0.92

[a] Microorganisms.
[b] Aqueous solution of ciprofloxacin·HCl.
[c] *Pseudomonas aeruginosa*.
[d] *Staphylococcus aureus*.

corresponding MIC values ($p = 0.000022$) and MBC 48 h was significantly higher than MBC 24 h ($p = 0.0040$). Therefore, the inhibiting effect on the microorganisms studied here remained unaltered and the concentration needed to kill the bacteria was higher than that needed to inhibit their growth. Since the MIC values against *P. aeruginosa* and *S. aureus* were comparable ($p = 0.22$ for MIC 24 h and 0.18 for MIC 48 h), the drug inhibited the growth of both bacteria to the same extent. The MBC values against *S. aureus* however were significantly higher than those against *P. aeruginosa* were ($p = 0.0138$ and 0.000017 for MBC 24 h and MBC 48 h, respectively), so the drug was less active in the killing of *Staphylococcus*.

The type of polymer used had no influence on the MIC and MBC values of the nanoparticle preparations, at least for the *P. aeruginosa* and *S. aureus* examined here, except in the case of MBC 24 h where Eudragit® RL containing particles were more active ($p = 0.0267$).

The ratio Eudragit®/PLGA influenced the activity of the nanoparticles prepared significantly ($p < 0.00003$). The activity of PLGA nanoparticles and of particles composed from 25/75 blends of Eudragit® and PLGA was comparable to that of an aqueous ciprofloxacin·HCl solution. The activity of Eudragit® nanoparticles and of particles composed from 50/50 and 75/25 blends was significantly lower than that of an aqueous

ciprofloxacin·HCl solution. The results obtained show that the antibiotic activity of drug-loaded nanoparticles was equal or slightly lower than that of free ciprofloxacin·HCl so the incorporation of the drug in the nanoparticles does not cause a loss of the drug antibiotic activity. Some of the samples prepared (25/75 Eudragit®/PLGA) show a slightly strengthened activity with respect to the free drug. This is in accordance with the results of Fontana et al. (1998) who developed polyethylcyanoacrylate nanoparticles, loaded with ampicillin.

Thus, the microbiological assay demonstrated that the incorporation of ciprofloxacin·HCl into Eudragit®/PLGA nanoparticles retained their activity against the two microorganisms studied here.

4. Conclusions

Although PLGA nanoparticles are biodegradable, they possess a negative zeta potential and probably low interactions with anionic mucus. Therefore, the possibility of producing positively charged nanoparticles by adding Eudragit to these formulations was investigated. Since the zeta potential of Eudragit®/PLGA mixtures was independent of the percentage of Eudragit® incorporated, the lowest concentration Eudragit (25%) could be selected for further research, since it contains the lowest amount of non-biodegradable polymer while retaining desired qualities such as a positive zeta potential, satisfying entrapment efficiency and a small mean size, well suited for ocular application. Eudragit® RL containing particles were more positively charged than when RS was employed. A prolonged release was obtained in all preparations and drug release did not significantly differ between the formulations. After microbiological characterisation, one can conclude that the nanoparticles are active against *P. aeruginosa* as well as against *S. aureus*, however to a lesser extent. Eudragit® RL containing particles were more active against the microorganisms selected than RS containing particles. Particles prepared with the lowest percentage (25%) Eudragit® were as active as an aqueous solution of the drug, when higher percentages were used, the activity decreased slightly but significantly.

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