Influence of Preparation Conditions on Acyclovir-Loaded Poly-*d,l*-Lactic Acid Nanospheres and Effect of PEG Coating on Ocular Drug Bioavailability

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Purpose. The evaluation of nanosphere colloidal suspensions containing acyclovir as potential ophthalmic drug delivery systems was carried out. The influence of polymer molecular weight and type and concentration of various surfactants on nanosphere properties was studied. The ocular pharmacokinetics of acyclovir-loaded nanoparticles was evaluated *in vivo* and compared with an aqueous suspension of the free drug.

Methods. Nanospheres were made up of poly-*d,l*-lactic acid (PLA). The colloidal suspension was obtained by a nanoprecipitation process. The surface properties of PLA nanospheres were changed by the incorporation of pegylated 1,2-distearoyl-3-phosphatidylethanolamine. The mean size and zeta potential of the nanospheres were determined by light scattering analysis. The acyclovir loading capacity and release were also determined. *In vivo* experiments were carried out on male New Zealand rabbits. The ocular tolerability of PLA nanospheres was evaluated by a modified Draize test. The aqueous humor acyclovir levels were monitored for 6 h to determine the drug's ocular bioavailability for the various formulations.

Results. A reduction of the mean size and a decrease of the absolute zeta potential of PLA nanospheres resulted from increasing the surfactant concentration. The higher the polymer molecular weight, the smaller the nanosphere mean size. PEG-coated and uncoated PLA nanospheres showed a sustained acyclovir release and were highly tolerated by the eye. Both types of PLA nanospheres were able to increase the aqueous levels of acyclovir and to improve the pharmacokinetics profile, but the efficacy of the PEG-coated nanospheres was significantly higher than that of the simple PLA ones.

Conclusions. PEG-coated PLA nanospheres can be proposed as a potential ophthalmic delivery system for the treatment of ocular viral infections.

KEY WORDS: acyclovir; poly-*d,l*-lactic acid; nanospheres; ocular delivery system; ocular tolerability; bioavailability.

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INTRODUCTION

Acyclovir is an antiviral drug with a significant and highly specific activity against herpes viruses and is widely used in the treatment of various ocular viral diseases (1–4). In particular, herpes simplex keratitis (in the most severe cases) is characterized by the spread of the virus into the deeper corneal layers, leading to damage of the stromal cells. Therefore, treatment requires a suitable permeation of the antiviral drug through the epithelium in order to reduce the virus load. The topical application of acyclovir is limited by the low corneal penetration of the drug and by its poor water solubility (0.2 mg/ml).

Many attempts have been made to improve the ocular bioavailability and the therapeutic effectiveness of acyclovir, e.g., chemical modification of the drug (5) and its incorporation into colloidal systems such as liposomes (6–8) or nanoparticles (9). Nanoparticles have been used as ophthalmic delivery systems because they are able to penetrate into the corneal (10) or conjunctival (11) tissue by an endocytotic mechanism.

Nanoparticles, because of their polymeric nature, present some important advantages over other colloidal carriers for ophthalmic applications, that is, a high storage stability, controlled release of the encapsulated drug, and a prolonged residence time in the precorneal area, particularly in the case of ocular inflammation and/or infection (11). Furthermore, the presence of poly(ethylene glycol) (PEG) on the surface of nanospheres can modulate the interfacial properties of the carrier (12–14) and, hence, can positively influence the ocular application potentialities both in terms of mucoadhesion and improved drug permeation. For this reason, in a previous work (15), we investigated the possibility of coating the surface of acyclovir-loaded polyalkyl-2-cyanoacrylate nanospheres with PEG moieties by a simple adsorption process. Although the polyalkyl-2-cyanoacrylate colloidal carrier provided a higher ocular bioavailability of the drug, no significant difference was observed between coated and uncoated nanospheres (15).

Because those findings may have resulted from a week interaction of PEG molecules with the surface of the colloidal carrier, in this paper we investigate the preparation and characterization of a colloidal carrier in which PEG moieties are firmly anchored to the surface of nanospheres. In particular, poly-*d,l*-lactic acid (PLA) is used as the polymeric matrix for nanosphere colloidal suspensions containing acyclovir. PEG-coated PLA nanospheres were prepared by using pegylated 1,2-distearoyl-3-phosphatidylethanolamine (DSPE-PEG), which could be able to firmly anchor PEG moieties to the surface of the colloidal carrier by inserting the lipophilic phospholipid part into the hydrophobic core of PLA colloidal matrix.

Different nonionic surfactants were used to prepare PLA nanospheres because they can both influence the colloidal physicochemical properties of the colloidal carrier and improve the carrier capacity (15,16). Because the goal of this

ABBREVIATIONS: DSPE-MPEG, methoxyethylene glycol carbamate of *sn*-1,2-distearoyl-3-phosphatidylethanolamine; HPLC, high-performance liquid chromatography; LC, loading capacity; NAC, *N*-acetylcysteine; PCS, photon correlation spectroscopy; PEG, poly(ethylene glycol); PI, polydispersity index; PLA, poly-*d,l*-lactic acid; PTFE, poly-tetrafluorine-ethylene.

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work was the development of new potential formulations for ophthalmic applications, the release of acyclovir from the proposed PLA colloidal carriers should not be excessively prolonged with respect to other commercial formulations even if a controlled release and a prolonged ocular residence time can be achieved (17). Drug release from PLA-based colloidal systems is regulated both by the polymeric matrix molecular weight and physicochemical properties of the entrapped molecule (16,18). For this reason, three PLA polymeric matrices with different polymeric weights (ranging from 16 to 200 kDa) were used to prepare PLA nanospheres.

The effect of pegylation on the ability of PLA nanospheres to increase corneal penetration of acyclovir and, thereby, to improve the ocular bioavailability of the drug was investigated *in vivo* in male New Zealand rabbits.

MATERIALS AND METHODS

Chemicals

Polyoxyethylene sorbitan monoleate (Tween 80) and octylphenoxy polyethoxyethanol (Triton X100) are Sigma Chemicals Co. products (St. Louis, MO). Polylactide acid (PLA) RES 203 (MW 16,000), RES 206 (MW 109,000), and RES 207 (MW 209,000) were purchased from Boheringer Ingheleim (Germany). Polyethylenepolypropyleneglycol (Pluronic F68) and decaethylenglycol oleyl ether (Brij 96) are Fluka products (Buchs, Switzerland). Methoxyethylene glycol carbamate of *sn*-1,2-distearoyl-3-phosphatidylethanolamine (DSPE-MPEG) is a Genzyme product (Suffolk, England). Acyclovir was provided by Sigma Chemical Co. (St. Louis, MO). Double-distilled water was used throughout. All other materials and solvents were of analytic grade (Carlo Erba, Milan, Italy).

PLA Nanosphere Preparation

PLA nanospheres were prepared following the solvent deposition method (19,20). Acyclovir (165 mg) was dissolved in a solution of H_2O-EtOH (1:1 v/v) (40 ml) containing a hydrophilic surfactant (Brij 96, Pluronic F68, Triton X100, or Tween 80) at various concentrations (ranging from 0.25 to 2.00% w/v). PLA (75 mg) was solubilized in acetone (20 ml). The organic phase was poured into the aqueous solution under stirring with a magnetic anchor, thus forming a milky colloidal suspension. The organic solvent was then evaporated off under vacuum by a rotavapor. To prepare PEG-coated PLA nanospheres, DSPE-MPEG (5 mg) was dispersed in the polymer organic solution, thus allowing the insertion of this substance into the surface of the nanospheres by means of the phospholipid moiety. Various PLA colloidal formulations were purified from untrapped acyclovir and unabsorbed nonionic surfactants by means of centrifugation and pellet washing with water. PLA nanospheres were centrifuged (~15000 × *g*) for 1 h at 5°C using a Beckman (Fullerton, CA) model J2-21 centrifuge equipped with a Beckman JA-20.01 fixed-angle rotor. After washing, PLA nanospheres were resuspended in sterile water and submitted to characterization experiments.

Physicochemical Characterization of Nanospheres

PEG-coated and uncoated PLA nanosphere mean size was determined by photon correlation spectroscopy (PCS) (Zetamaster, Malvern Instruments Ltd, Worcs, England). The experiments were carried out using a 4.5-mW laser diode operating at 670 nm as light source. Size measurements were carried out at a scattering angle of 90°. To obtain the mean diameter and polydispersity index of colloidal suspensions, a third-order cumulant fitting correlation function (21,22) was performed by a Malvern PCS submicron particle analyzer. The real and imaginary refractive indexes were set at 1.59 and 0.0, respectively. The following parameters were used for experiments: medium refractive index 1.330, medium viscosity 1.0 mPa·s and a dielectric constant of 80.4. The samples were suitably diluted with filtered water (Sartorius membrane filters 0.22 μm) to avoid multiscattering phenomena and placed in a quartz cuvette. The size analysis of a sample consisted of 30 measurements, and the result is expressed as mean size ± SD. Typically, the size determination of a PLA nanosphere formulation consists in the measurement of the mean size of five different batches (30 measurements per batch), and the result is the average ±SD.

Electrophoretic mobility and zeta potential distribution were measured with the Zetamaster particle electrophoresis analyzer setup equipped with a 5-mW HeNe laser (633 nm). Also in this case, samples for zeta potential measurements were suitably diluted with filtered water. Zeta limits ranged from –120 to 120 V. Strobing parameters were set as follows: strobe delay –1.00, on time 200.00 ms, off time 1.00 ms. A Smoluchowsky constant F (Ka) of 1.5 was used to achieve zeta potential values from electrophoretic mobility.

Nanosphere Entrapment Capacity

The pellet coming from the purification process was resuspended in 10 ml water and freeze-dried. To determine the amount of entrapped acyclovir, lyophilized nanospheres (40 mg) were dissolved in CH_2Cl_2 (10 ml), and the drug was completely extracted at room temperature with pH 7.4 phosphate buffer (10 ml) for 2 h. The extraction procedure was carried out three times. The aqueous solution was filtered through 0.2-μm PTFE membrane filters and analyzed by HPLC for acyclovir content. The HPLC apparatus was a Hewlett Packard model 1100 equipped with DAD operating at 254 nm. A Hypersil ODS reversed-phase column (150 mm × 4.6 mm i.d., Alltech, Milan, Italy) thermostated at 27°C was used. The mobile phase was 0.7 M CH_3COONa (pH 6.0). The flow rate was 1.0 ml/min. Acyclovir solutions in pH 7.4 phosphate buffer ranging from 1.25 to 10 μg/ml were used for the calibration curve. The linear regression coefficient was 0.99997. The method sensitivity was 0.05 μg/ml. The entrapment of acyclovir within PLA nanospheres is expressed both as entrapment yield and loading capacity. The entrapment yield is the percentage of acyclovir added during the PLA nanosphere preparation that becomes entrapped within the colloidal carrier, whereas the loading capacity is the amount of acyclovir (mg) per 100 mg of PLA.

Drug Release from PLA Nanospheres

After the separation of the untrapped drug and washing procedure by centrifugation, the nanosphere pellet was made up to 10 ml with isotonic pH 7.4 phosphate buffer (polymer concentration 1 mg/ml). The *in vitro* release experiments were carried out at 37°C because even though the corneal temperature is around 35°C (34.3°C at the center and 35°C at

the periphery), the eye drop is instilled in the conjunctival fornix, where the temperature is 37°C. The experiments were carried out with continuous stirring by a magnetic anchor. At predetermined time intervals, 500-μl samples were withdrawn and centrifuged for 8 min at 100,000 × *g*. The supernatant was filtered (0.2-μm membrane filters) and assayed for acyclovir by HPLC. The analysis of the drug release was carried out as previously described using the following equation (23):

$$M_t/M_\infty = k\ t^n$$

where M_t/M_∞ is the drug fraction released at time *t*, and *k* and *n* are the constant and the kinetic exponent of the drug release, respectively. The value of the kinetic exponent *n* indicating the mechanism of the drug release process is dependent on the geometry of the systems (24). As concerns the nanoparticles, we considered the shape to be spherical. The fitting of the experimental release data to the model was evaluated using a nonlinear least-squares method and the χ^2 test, which shows the goodness of fit.

Ocular Bioavailability

Male New Zealand rabbits (Charles River, Calco, Italy) weighing from 1.8 to 2.2 kg, free of any signs of ocular inflammation or gross abnormality, were used. Animal procedures conformed to the ARVO (Association for Research in Vision and Ophthalmology) resolution on the use of animals in research. Animals were handled according to the Principles of Laboratory Animal Care (NIH publication # 85-23). Acyclovir levels in aqueous humor were monitored 30, 60, 120, 240, and 360 min after a single instillation (50 μl) of the various formulations into the conjunctival sac. Before paracentesis, the rabbits were anesthetized by intravenous injection of 25 mg/kg ketamine HCl (Parke-Davis, Milan, Italy). Aqueous humor (150 μl) was withdrawn through the limbus by a syringe with a 26G needle and stored at –20°C. The aqueous samples were treated with a solution of 2% (w/v) $ZnSO_4 \cdot 7H_2O$, in order to deproteinize the aqueous humor, and then vortex-mixed and centrifuged. The supernatant was filtered (0.2 μm PTFE membrane) and analyzed by HPLC. No interfering peak was observed in the blank aqueous humor chromatograms. When desired, *N*-acetylcysteine (NAC) (0.1 M) was instilled (50 μl) every 5 min for 55 min (11 instillations) in order to remove the mucus from the conjunctival and corneal surfaces. Various ocular PLA formulations were administered 5 min after the last NAC instillation (25).

The ocular bioavailability of various nanosphere formulations was compared with that of a free drug formulation prepared by dispersing acyclovir in sterile pH 7.4 isotonic phosphate buffer. The free drug dispersion was sonicated for 40 min at 10°C with a Bransonic model 2200 bath sonifier. The acyclovir-loaded PLA nanosphere formulations to be investigated for ocular drug bioavailability were prepared by suspending the purified PLA nanospheres in sterile pH 7.4 isotonic phosphate buffer. For the ophthalmic administration, both the free drug suspension and various PLA nanosphere formulations presented a final acyclovir concentration of 1% (w/v).

Ocular Tolerability

The potential ocular irritancy and/or damaging effects of the formulations were evaluated according to a modified Draize test (26). A slit lamp (mod. 4179T Sbisà, Florence, Italy) was used. The congestion, swelling, and discharge of the conjunctiva were graded on a scale from 0 to 3, 0 to 4, and 0 to 3, respectively. Iris hyperemia and corneal opacity were graded on a scale from 0 to 4. Formulations (50 μl) were topically administered in the right eye every 30 min for 6 h (12 treatments). At the end of the treatment, two observations at 10 min and 6 h were carried out to evaluate the ocular tissues. Methylene blue staining was used to evaluate the corneal integrity, which allows an accurate determination of the extent of epithelial damage because of its poor diffusion through the stroma.

Statistical Analysis

Data are expressed as mean ± SD. Statistical comparisons were made by ANOVA for repeated measures and *post-hoc* Dunnett's multiple comparison test with differences of $p < 0.05$ being considered significant (GraphPAD software, San Diego, CA).

RESULTS AND DISCUSSION

Nanosphere Preparation and Characterization

PLA nanospheres were prepared in a single step by nanoprecipitation of the polymer. The influence of some formulation parameters, i.e., the type and concentration of surfactant and the molecular weight of the polymer, on nanosphere physicochemical properties was investigated. PLA nanospheres were influenced by the surfactant concentration rather than by the type of surfactant (Fig. 1). All the surfactants allowed the formation of nanosphere colloidal suspensions with a mean size lower than 200 nm.

The presence of a nonionic surfactant is not essential for nanosphere formation, even if its presence can influence the mean size of colloidal nanospheres (Fig. 1) by reducing the dynamic interfacial tension and hence the rate of organic

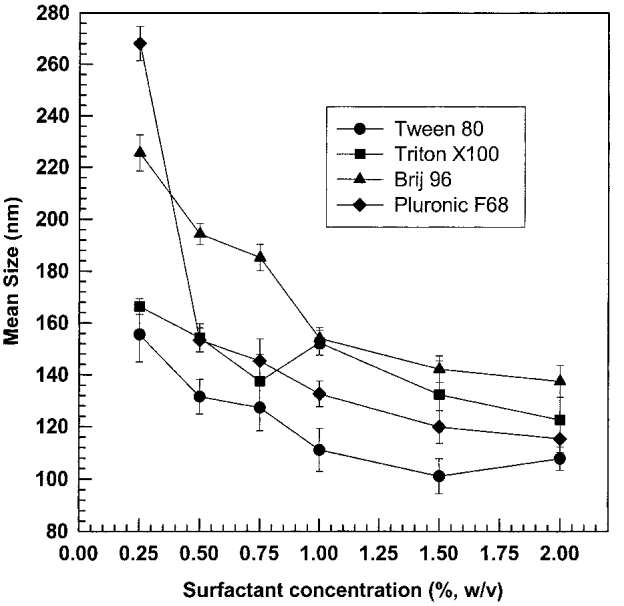

Fig. 1. Influence of the nonionic surfactant concentration on the mean size of PLA nanospheres. Data are the average of five different experiments ± standard deviation. For all preparations the polydispersity index was < 0.2.

phase diffusion (27,28). The presence of a nonionic surfactant is very important for the so-called "long-term" stability (29) of the nanosphere colloidal suspension, which is determined by the adsorption of hydrophilic macromolecules on the nanosphere surface, thus increasing the steric repulsion between particles. The presence of hydrophilic macromolecules on the surface of nanospheres leads to a change of the surface properties (zeta potential) of the colloidal carrier. In particular, the zeta potential of colloidal nanospheres is significantly reduced by coating with nonionic surfactants (30).

As shown in Fig. 2, the surfactant concentration also influenced the zeta potential of the PLA nanospheres; namely, the higher the surfactant concentration, the smaller was the zeta potential value. In agreement with previously reported findings (31,32), the progressive zeta potential reduction could be related to the formation of a denser surfactant film on the surface of PLA nanospheres, thus eliciting a reduced electrophoretic mobility because of an increased hydrodynamic radius. In fact, at higher surfactant concentrations than CMC, the driving force of nonionic surfactant adsorption on nanospheres is enhanced by increasing the surfactant concentration.

The adsorption of DSPE-MPEG into PLA nanospheres prepared in the presence of Tween 80 (0.5% w/v) reduced the absolute zeta potential value from –31.1 mV to –14.7 mV.

On the basis of the light-scattering results, Tween 80 was used as the nonionic surfactant for the preparation of acyclovir-loaded PLA nanospheres at the concentration of 0.5% w/v. This concentration represented a suitable compromise between the achievement of small particles and the facility to remove the excess surfactant by centrifugation and a washing procedure.

The influence of polymer molecular weight on particle size was also evaluated. In agreement with previously reported data (28,33), a significant reduction of PLA nanosphere mean size with the increase of the polymer molecular weight (Table I) was observed. This finding could be related to the increase of hydrophobicity of the polymer as a function of the molecular weight.

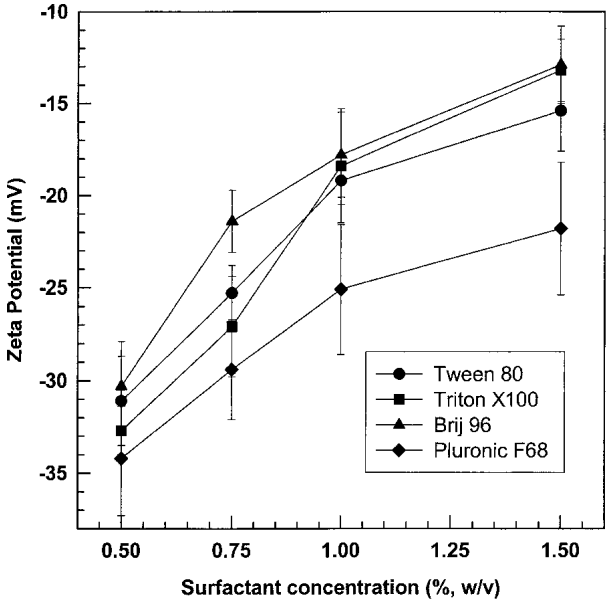

Fig. 2. Influence of the nonionic surfactant concentration on the zeta potential of PLA nanospheres. Data are the average of five different experiments ± standard deviation.

Table I. Influence of PLA Molecular Weight on Mean Size and Polydispersity Index of Nanosphere Colloidal Suspensions Prepared in the Presence of Tween 80 (0.5% w/v)

Polymer	MW[a]	Mean size (nm)[b]	PI[c]
RES 203	16,000	131.5 ± 0.3	0.112
RES 206	109,000	92.5 ± 1.7	0.177
RES 207	209,000	51.2 ± 0.1	0.291

[a] Mean molecular weight of PLA polymers.
[b] Size of PLA nanospheres is the average of five different experiments ± standard deviation.
[c] PI, polydispersity index.

Loading Capacity and Drug Release

The loading capacity ranged from ~2% to ~8% (Table II). The drug entrapment within the PLA nanospheres was negatively influenced by the polymeric matrix molecular weight; in fact, the higher the molecular weight the lower the acyclovir loading capacity. This behavior could be caused by the increased hydrophobicity of the PLA nanosphere polymeric bulk as a function of the molecular weight (30,33), thus reducing the affinity for hydrophilic drugs such as acyclovir. No significant variations in the encapsulation efficiency of acyclovir were observed by changing the polymer concentration in the medium (data not shown). The insertion of DSPE-MPEG into PLA nanospheres increased acyclovir entrapment, probably through a drug–PEG moiety interaction, which retains part of the drug at the level of the nanosphere surface (Table II).

As concerns acyclovir release from PLA nanospheres (Fig. 3), the drug leakage was monitored for 8 h; a longer observation would have been useless for an ophthalmic application of these carriers because of the clearance of nanospheres by lachrymal fluid. Total drug release was not obtained during the experiment.

The acyclovir release profile from PLA nanospheres (Fig. 3) is characterized by an initial phase of rapid drug release followed by a more gradual release. The initial burst effect can be attributed to the release of the drug encapsulated near the nanosphere surface and is clearly related to the drug loading in the nanospheres. The gradual release shown in the second part of the process is the consequence of the

Table II. Acyclovir Encapsulation Efficiency of PLA Nanospheres at Different Polymeric Matrix Molecular Weights Prepared in the Presence of Tween 80 (0.5% w/v)[a]

Nanosphere	MW[b]	Entrapment yield	LC
$PLA_{RES\ 203}$	16,000	2.7 ± 0.4	5.9 ± 0.8
$PLA_{RES\ 206}$	109,000	1.9 ± 0.2	4.2 ± 0.5
$PLA_{RES\ 207}$	209,000	1.0 ± 0.3	2.2 ± 0.6
PEG-$PLA_{RES\ 203}$[c]	16,000	3.5 ± 0.1	7.7 ± 0.3

[a] Each value is the average of five different experiments ± standard deviation.
[b] Mean molecular weight of PLA polymers used for PLA nanosphere preparation.
[c] PEG-coated nanospheres prepared in the presence of DSPE-MPEG.

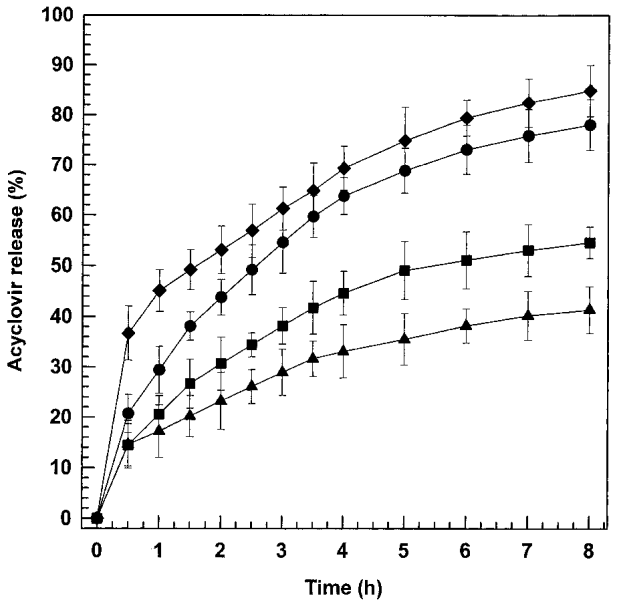

Fig. 3. Acyclovir release from various nanosphere colloidal systems made up of PLA at different molecular weights and prepared in the presence of Tween 80 (0.5% w/v) as nonionic surfactant. PLA nanospheres were suspended in isotonic phosphate buffer (pH 7.4). Release experiments were carried out at 37 ± 0.2°C, immediately after sample preparation. Each point represents the mean value of five different experiments ± standard deviation. Keys: ●, RES 203 (MW 16,000); ■, RES 206 (MW 109,000); ▲, RES 207 (MW 209,000); ♦, PEG-coated PLA nanospheres made up of RES 203.

release of the drug fraction encapsulated in the core of the nanospheres. After the burst effect, the release profiles of both the uncoated and coated nanospheres prepared with RES 203 is practically the same. The higher amount of drug released during the burst effect from the coated nanospheres is evidence of a different drug distribution during the nanoprecipitation. Furthermore, the initial higher burst effect may probably be caused by the hydrophilic surface of the carrier. Increasing the molecular weight of the polymer decreases the drug release rate from the nanospheres.

According to the kinetic analysis (Table III), the drug release from the nanospheres appears mainly controlled by the diffusion of the drug through the polymeric matrix. Hence, the different release profiles as a function of the polymer molecular weight (a slower release for higher molecular weights) can be explained with the driving force for the diffusion of drug molecules throughout the polymer generated from the different drug loading in the nanoparticles. On the other hand, the drug release does not appear affected by the mean sizes of the nanoparticles. A higher drug release can be justified by the smaller mean size of the nanoparticles, but in this study the nanoparticle dimensions significantly decrease, and thus the polymer molecular weight increases.

In light of the slower release of acyclovir from polymeric matrixes with high molecular weights, PLA with a molecular weight of 16,000 was used for the preparation of the ophthalmic colloidal carrier.

Table III. Values of the Kinetic Exponent of Drug Release (n), of the Correlation Coefficient (r), and Chi-Square (χ^2) for the PLA Nanospheres

Nanosphere	MW[a]	n	Statistical parameters
$PLA_{RES\ 203}$	16,000	0.49 ± 0.01	r = 0.9939 χ^2 = 0.07
$PLA_{RES\ 206}$	109,000	0.49 ± 0.02	r = 0.9938 χ^2 = 0.03
$PLA_{RES\ 207}$	209,000	0.41 ± 0.02	r = 0.9936 χ^2 = 0.03
PEG-$PLA_{RES\ 203}$[b]	16,000	0.30 ± 0.01	r = 0.9944 χ^2 = 0.006

[a] Mean molecular weight of PLA polymers used for PLA nanosphere preparation.

[b] PEG-coated nanospheres prepared in the presence of DSPE-MPEG.

Ocular Tolerability

To evaluate the applicability of PLA nanospheres as an ophthalmic drug delivery system, the ocular tolerability was evaluated following a modified Draize test protocol (26). PLA nanospheres caused no sign of ocular inflammation or tissue alteration in the rabbit eye (data not shown). The scores of conjunctival congestion, swelling, and discharge were zero for all the experiments. Iris hyperemia and corneal opacity scores were zero at all observations. The absence of *in vivo* irritant activity can promote the ophthalmic use of PLA nanosphere colloidal carriers.

Ocular Bioavailability

The ocular bioavailability of acyclovir-loaded PLA nanospheres was evaluated and compared with the bioavailability of both a free drug aqueous suspension and a formulation of empty nanospheres physically blended with acyclovir. The concentrations of acyclovir in the aqueous humor of rabbit eyes after a single instillation of the various formulations are shown in Fig. 4. Key bioavailability parameters describing the time profile of the acyclovir levels in aqueous humor are reported in Table IV.

PLA nanospheres showed significantly higher ($p < 0.001$) levels of acyclovir compared to the free drug formulation. PLA nanosphere colloidal suspensions containing acyclovir provided a significant sustained drug release in the aqueous humor with respect to the free drug by ensuring effective acyclovir levels for up to 6 h. The aqueous $AUC_{0\rightarrow6}$ values were significantly ($p < 0.001$) greater for the PEG-coated PLA nanospheres than for uncoated nanospheres and the free drug suspension with a 1.8-fold and 12.6-fold increase, respectively. Furthermore, we found that, when the mucus was removed from conjunctival/corneal surfaces, the acyclovir-loaded PEG-coated nanosphere formulation led to a 6.5-fold increase ($p < 0.001$) of the drug in the aqueous humor compared to the free drug suspension (Table IV, Fig. 4), whereas no significant difference was observed between PEG-coated and uncoated nanospheres. The $AUC_{0\rightarrow6}$ values for the acyclovir-loaded PEG-coated nanospheres in normal rabbit eyes and in *N*-acetylcysteine-pretreated rabbit eyes were 424 ± 24 and 221 ± 40 μg/ml/min, respectively (Table IV). The aqueous $AUC_{0\rightarrow6}$ values were significantly ($p < 0.001$) greater for the acyclovir-loaded PLA nanospheres than for the free drug suspension, with a 6.99-fold increase.

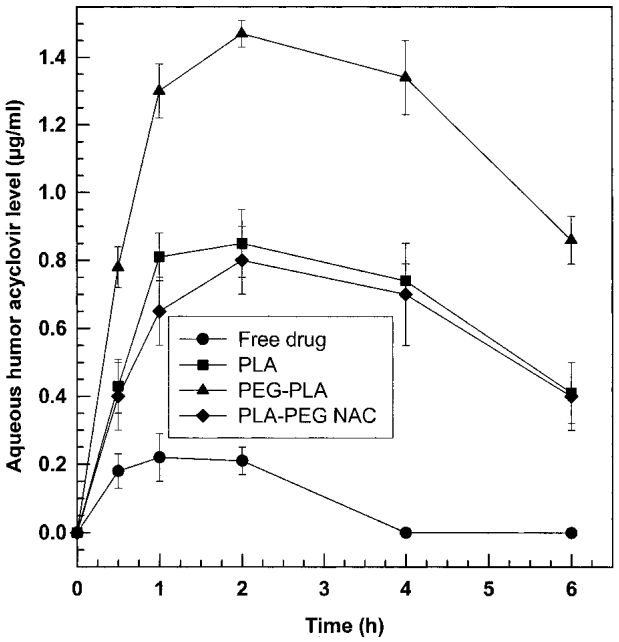

Fig. 4. Acyclovir levels in the aqueous humor after instillation of the nanoparticle and acyclovir free suspension in normal rabbit eyes and in *N*-acetylcysteine (NAC)-pretreated eyes. PLA nanospheres were made up of RES 203 (MW16,000) and prepared in the presence of Tween 80 (0.5% w/v) as nonionic surfactant. In the case of PEG-coated nanospheres, DSPE-MPEG was added to the preparation medium. Each point is the average of six different experiments ± the standard deviation. PEG-coated PLA nanosphere–acyclovir physical mixture determined no significant improvement of drug permeation compared to free acyclovir (data not reported). Acyclovir concentration in various ocular formulations was 1% (w/v).

The *in vitro* release data seem to be in agreement with *in vivo* pharmacokinetic profiles, considering that PLA nanosphere ocular formulations showed an *in vitro* release of acyclovir of ~50% at 2 h and a T_{max} at 2 h in the *in vivo* experiments, respectively. Ocular pharmacokinetics of nanospheres showed a controlled drug release, which could be elicited by the colloidal carrier mucoadhesion on the cell surface, thus allowing a long ocular permanence and a prolonged release in comparison with the drug suspension. This increase of acyclovir bioavailability provided by PEG-coated PLA nanospheres could be caused by an improved mucoadhesion and by an enhancer effect (34–36). The possible penetration enhancer effect seems to be a nanosphere-mediated phenomenon because a simple PEG-coated PLA nanosphere–acyclovir mixture determined no significant improvement of drug penetration compared to free acyclovir (data not reported). As previously demonstrated (37), the presence of polyoxyethylene moieties on the nanosphere surface could elicit an increased permeability of cellular membranes at the level of the cell–nanosphere contact point, thus allowing a specific penetration enhancer effect for drug molecules entrapped within the colloidal carrier.

After pretreatment with NAC, a significant ($p < 0.001$) AUC drop (48%) was observed in the group treated with pegylated nanospheres. This finding can be attributed to the absence of PEG–mucin force of interaction after the NAC treatment. The significant difference in acyclovir ocular bioavailability between coated and uncoated PLA nanospheres could be correlated to the different interactions between the nanosphere surface and the corneal epithelium, thus showing that the carrier interaction with ocular structures is an important factor influencing the ocular pharmacokinetics of the drug.

Endocytosis has been reported as a potential pathway of interaction between nanoparticles and ocular epithelial cells (10,11). This entrance pathway does not seem to have a fundamental role in the improvement of ocular bioavailability of acyclovir-loaded PEG-coated PLA nanospheres with respect to uncoated ones. The eye pretreatment with *N*-acetylcysteine is able to remove the mucus from the ocular surface, but it does not alter the ocular epithelial cell metabolism and physiology. Therefore, after mucus removal from the ocular surface, a higher C_{max} value (Table IV) should be observed for pegylated nanospheres than uncoated ones if endocytosis had a certain role in the enhancement of ocular bioavailability for PEG-coated PLA nanospheres. Although we have no direct evidence, endocytosis probably occurs in a similar way for both PEG-coated and uncoated PLA nanospheres and can partially contribute to the higher acyclovir C_{max} values than that observed for the free drug suspension.

Colloidal properties of PLA nanospheres may also facilitate paracellular transport or passage through the cornea, thus leading to a greater drug transport into the ocular tissues.

CONCLUSIONS

These findings demonstrate that acyclovir can be entrapped in a polymeric colloidal drug delivery system made

Table IV. Key Parameters Describing the Aqueous Humor Pharmacokinetics of Acyclovir after a Single Instillation (50 µl) of Various Formulations in the Rabbit Eye

Key parameters	Free drug	PLA[a]	PEG-PLA[a]	PEG-PLA[a] with NAC[b]
Total time when acyclovir was still detectable (h)	2	>6	>6	>6
Maximum concentration of drug (µg/mL)[c]	0.2 ± 0.1	0.9 ± 0.1*	1.5 ± 0.1*†	0.8 ± 0.1*‡
Time when C_{max} was detected (h)	1	2	2	2
$AUC_{0 \rightarrow 6}$ (µg · mL^{-1} · h ± SD)[c]	33.7 ± 10.3	235.6 ± 19.2*	424.1 ± 24.1*†	221.2 ± 40.5*‡

[a] PLA nanospheres were made up of RES 203 (MW 16,000) and prepared in the presence of Tween 80 (0.5% w/v) as nonionic surfactant. In the case of PEG-coated nanospheres, DSPE-MPEG was added to the preparation medium. Acyclovir concentration in various ocular formulations was 1% (w/v).

[b] The rabbit eyes were pretreated before the administration of the formulation with an *N*-acetylcysteine solution (0.1 M) every 5 min for 55 min.

[c] Statistical analysis: *$p < 0.001$ vs. free drug formulation, †$p < 0.001$ vs. PLA nanospheres, ‡$p < 0.001$ vs. PEG-coated PLA nanospheres.

up of PLA, which can provide a sustained ocular drug release and an increase of the acyclovir levels in aqueous humor. In particular, PEG-coated PLA nanospheres were much more efficient in improving the ocular bioavailability of acyclovir. The biologic results on acyclovir bioavailability, as well as ocular carrier tolerability, prompted us to use PLA nanospheres as a potential ophthalmic dosage delivery system for the treatment of ocular viral infections, thus allowing a better compliance and an increased intraocular level of the antiviral agent.

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