Ocular Tolerability and *In Vivo* Bioavailability of Poly(ethylene glycol) (PEG)-Coated Polyethyl-2-Cyanoacrylate Nanosphere-Encapsulated Acyclovir

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ABSTRACT: Acyclovir-loaded polyethyl-2-cyanoacrylate (PECA) nanospheres were prepared by an emulsion polymerization process in the micellar phase and characterized. The influence of the presence of nonionic surfactant as well as other substances [i.e., 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD) and poly(ethylene glycol) (PEG)], on formulation parameters and loading capacity was investigated. In particular, the presence of PEG resulted in an increase of mean size and size distribution. To obtain PEG-coated PECA nanospheres with a mean size of <200 nm, Pluronic F68 at concentrations $>1.5\%$ (w/v) should be used during preparation. The presence of PEG also resulted in a change in zeta potential, from -25.9 mV for uncoated nanospheres to -12.2 mV for PEG-coated PECA nanospheres. The presence of HP-β-CyD elicited an increase of nanosphere size and size distribution, but zeta potential was not influenced. *In vitro* drug release from nanospheres was determined in both phosphate buffer (pH 7.4) and plasma. The presence of HP-β-CyD and PEG did not influence the acyclovir release rate in plasma. In the case of release in phosphate buffer, PEG-coated nanospheres showed a slower release. Ocular tolerability of PEG-coated PECA nanospheres was evaluated by the *in vivo* Draize test. This colloidal carrier was well tolerated, eliciting no particular inflammation at the level of the various ocular structures. *In vivo* ocular bioavailability was evaluated by instilling 50 μL of the acyclovir-loaded nanospheres only once in the conjunctival sac of rabbit eyes. At various time intervals, aqueous humour acyclovir content was determined by high-performance liquid chromatography. Acyclovir-loaded PEG-coated PECA nanospheres were compared with an aqueous solution of the drug and a physical mixture of acyclovir nanospheres. The acyclovir-loaded PEG-coated PECA nanospheres showed a significant ($p<0.001$) increase of drug levels (25-fold) in aqueous humor compared with the free drug or the physical mixture. This finding is probably due to an improved ocular mucoadhesion of PEG-coated PECA nanospheres.
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INTRODUCTION

Acyclovir is a highly specific inhibitor of herpes virus replication.[1] *In vitro* studies demonstrated

that concentrations of 0.02–0.2 and of 0.2–0.4 μg/mL reduce viral plaque formation by 50%for types I and II herpes simplex virus, respectively.[2] Varicella-Zoster and Epstein-Barr viruses are less sensitive with respect to acyclovir. These viruses can be inhibited only at concentrations of 1.2 and 1.6 μg/mL, respectively.[3]

The topical application of acyclovir is widely used for the treatment of various herpes simplex infections.[4] Some ocular pathologies may be treated with acyclovir,[5] which inhibits the viral DNA polymerase after phosphorylation by viral thymidine kinase. Herpes simplex keratitis represents a severe disease with about half a million cases per year in the United States alone.[6] Therapy for this disease is based on the topical application of antiviral agents to inhibit virus growth.[5]

Various acyclovir formulations are not able to ensure, following the oral, topical, or parenteral administration route, suitable drug levels in the target sites, probably because of their low water and lipid bilayer[7] solubility. Furthermore, because of poor water solubility, acyclovir has to be formulated as an ointment, which causes transient vision disturbance for ~30 min, thus reducing compliance. Encapsulation of chemotherapeutic agents in colloidal delivery systems can improve the biological activity both *in vitro* and *in vivo*; that is, reducing side effects, increasing the cellular and bacterial penetration, and ensuring a more suitable biodistribution.[8,9] Recently, liposomes have been proposed as therapeutically effective ophthalmic drug delivery devices for acyclovir.[7] Polymeric colloidal delivery systems seem to be promising in ocular therapy.

Polyalkyl-2-cyanoacrylate (PACA) nanoparticles, because of their biodegradability and ability to entrap a variety of drugs, have been proposed as drug delivery systems.[10] This delivery device has been widely studied as a possible drug carrier for intravenous[11] and oral administration.[12] More recently, PACA nanoparticles can function as vehicles for use in treating ophthalmic pathologies because improved ocular penetration and prolonged therapeutic response have been obtained for some drugs.[13]

In the case of PACA nanoparticles, appropriate modifications of the physicochemical parameters (i.e., size[14] and surface characteristics[15] can influence nanoparticle biodistribution and colloidal carrier cell recognition. To modify the surface of colloidal carriers, poly(ethylene glycol) (PEG) has been proposed.[16,17] PEG was attached to polymeric particles by adsorption or covalent binding. In particular, poly-*dl*-lactic acid nanoparticles, covalently coupled with PEG, presented higher colloidal stability and a reduced reticuloendothelial system (RES) uptake.[18] More recently, sterically stabilized poly(isobutylcyanoacrylate) nanoparticles have been developed by chemical coupling with PEG.[19]

The present paper describes the preparation and characterization of acyclovir-loaded polyethyl-2-cyanoacrylate (PECA) nanospheres obtained by micellar polymerization in the presence of PEG 6000 or 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD), as well as the presence of a nonionic surfactant necessary for the micelle formation. The presence of these two hydrophilic macromolecules may lead both to an increase of the drug solubility and to a more suitable interaction between the drug and the PECA network, eliciting a possible improvement of the carrier capacity.[20] These hydrophilic macromolecules may also function as protector colloids improving both physical properties and storage stability of the PECA nanoparticle colloidal carriers. Furthermore, the presence of PEG along the surface of PECA nanospheres can positively influence the potential of an ocular application (e.g., higher mucoadhesion and/or improved drug permeation) of this colloidal drug carrier.

In this paper, the ocular tolerability and humor aqueous bioavailability in the rabbit eye of acyclovir-loaded PECA nanospheres was also investigated.

EXPERIMENTAL SECTION

Materials

Ethyl-2-cyanoacrilate, used as the monomer for the polymerization, was obtained from Sigma Chemicals Company (St. Louis, MO). Pluronic F68, PEG 6000, and HP-β-CyD were supplied by Fluka (Buchs, Switzerland). Acyclovir was purchased from Sigma. Ketamine · HCl was from Parke Davis, Milan, Italy. Plasma was obtained from healthy voluntary blood donors. Double-distilled water was used throughout. All other chemicals were of analytical grade (Carlo Erba, Milan, Italy).

Nanopshere Preparation

Acyclovir-loaded PECA nanospheres were prepared by micellar polymerization[21] of ethyl-2-

cyanoacrylate in the presence of Pluronic F68 as a nonionic surfactant. Various amounts of the surfactants were used both in the absence and in the presence of PEG 6000 or HP-β-CyD (0.5 g). The polymerization medium consisted of a 0.01 M HCl solution (pH 2) in an ethanol/water mixture (50% v/v) containing suitable amounts of Pluronic F68. Acyclovir (165 mg) was dissolved in the polymerization medium before the addition of the monomer. Ethyl-2-cyanoacrylate monomer (1.6 mL) was slowly added (75 μL/min) under mechanical stirring (~1000 rpm) to 50 mL of the polymerization medium filtered through a 0.2-μm membrane filter (Sartorius, Göttingen, Germany). After the monomer polymerization was completed (3.5 h), the milky colloidal suspensions were neutralized with 0.1 M NaOH and centrifuged (model J2-21 Beckman, Fullerton, CA) at $31{,}500 \times g$ (Beckman JA-20.1 rotor, 16,500 rpm) to separate unloaded acyclovir. The sediments were suspended in ethanol/water (50% v/v) and centrifuged again to exclude drug residue. Finally, the nanoparticles were resuspended in double-distilled water or in 0.9% (w/v) NaCl solution.

Particle Size Determination

The mean size of PECA nanospheres was measured by photon correlation spectroscopic (PCS) analysis.[22] Samples were analyzed with a Brookhaven SM-200 spectrometer (Brookhaven Instruments Corporation, Holtsville, NY) at 20 ± 0.1°C (Haake D8-G, Berlin, Germany). The translational diffusion coefficient (D_T) was measured with a Brookhaven BI-2030AT 128-channel correlator. The light source was a 30 mW He-Ne laser (Spectra Physics, Mountain View, CA). Measurements were recorded in the angular interval from 20° to 140°. Mean D_T values were the average of three experimental determinations. A third-order cumulant fitting correlation function was performed.

Electrophoretic mobility and zeta potential distribution were measured with the Zetamaster (Malvern Instruments Ltd., Sparing Lane South, Worcs, England) particle electrophoresis analyzer setup equipped with a 5 mW HeNe laser (633 nm). Zeta limits ranged from −120 to 120 V. Strobing parameters were set as follows: strobe delay, −1.00; on time, 200.00; off time, 1.00. A Smoluchowsky constant $F(K_a)$ of 1.5 was used to achieve zeta potential values from electrophoretic mobility.

Determination of Drug Content

After the separation of the untrapped drug, PECA nanosphere suspensions were freeze-dried. To determine the amount of acyclovir entrapped within PECA nanospheres, 40 mg of the freeze-dried material were solubilized in 10 mL of acetonitrile/methanol (8:2, v/v). The organic solution (1 mL) was diluted with 0.1% w/v phosphoric acid up to a final volume of 10 mL. This solution was filtered through 0.2-mm poly(tetrafluoroethylene) (PTFE) membrane filters (Spartan-3; Schleicher & Schuell, Keene, NH) and submitted to high-performance liquid chromatography (HPLC) analysis. The chromatographic apparatus consisted of a Varian LC Star 9012 solvent delivery system (Varian Associates Inc., Walnut Creek, CA) equipped with a Rheodyne injector 7125 (fitted with a 10-mL loop; Rheodyne, Cotati, CA), a Kontron model 432 variable wavelength ultraviolet–visible (UV–vis) detector (Kontron Instruments, Zurich, Switzerland), and a Hewlett Packard 3394 integrator (Avondale, PA). The HPLC analysis was carried out on a Bondpack C_{18} reversed-phase column (5 mm, 250 × 4.6 mm i.d.; Waters). The mobile phase was 0.01 M trifluoroacetic acid(TFA)/methanol (93/7, v/v). The elution flow rate was set at 1 mL min^{-1}. Drug detection was performed at a wavelength of 254 nm. Acyclovir determination was carried out with a calibration curve previously obtained with 0.1% (w/v) phosphoric acid acyclovir solutions at known concentrations. The straight-line equation was $y = 2399.6x - 240.07$, where y is the peak area $\times 10^{-3}$ and x is the drug concentration (μg mL^{-1}). The linear regression value was $r = 0.99962$. The linearity of the method was valid within the interval 1–20 μg mL^{-1}. Other nanosphere components (i.e., PECA, Pluronic F68, PEG, HP-β-CyD and salts) showed no interference with the chromatographic method. Results are expressed as the percentage of the drug amount in 100 mg of dried material.

Acyclovir Release from Nanospheres

Drug release was assayed at prefixed time intervals. Various suspensions containing 10 mg of acyclovir-loaded nanoparticles in 10 mL of isotonic phosphate buffer solution (pH 7.4) at 37°C (sink condition) were prepared. A sample was withdrawn (1 mL) from each suspension maintained under continuous stirring and then filtered through 0.2-μm PTFE membrane filters. The

acyclovir release in the supernatant was estimated by HPLC. Each experiment was repeated three times.

Acyclovir release was also investigated in human plasma at 37°C. Release experiments were carried out by adding 15 mg of nanospheres to samples of preheated plasma (5 mL). Suspensions were prepared for a prefixed time interval. Samples were thermostated at 37°C under continuous stirring. At appropriate intervals, samples were centrifuged at 12,000 rpm, and 0.8 mL of supernatant was withdrawn. For deproteinization, 3.2 mL of 10% TFA aqueous solution were added to the plasma samples. After immediate mixing and centrifugation for 5 min at 12,000 rpm, 10 mL of clear supernatant was analyzed by HPLC. Every experiment was in triplicate.

Nanosphere Ocular Tolerability

The potential ocular irritancy and/or damaging effects of the formulations were evaluated according to a modified Draize test.[23] A slit lamp (model 4179T Sbis, Florence, Italy) was used. The congestion, swelling, and discharge of the conjunctiva were graded on scales 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. Fifty microliters of formulation were topically administered every 30 min for 6 h (12 treatments) in the right eye. At the end of the treatment, two observations, at 10 min and 6 h, were carried out to evaluate the ocular tissues. Corneal integrity was evaluated by methylene blue staining. Methylene blue was chosen because it does not rapidly diffuse through the stroma and hence provides a more accurate determination of the extent of epithelial damage.

Acyclovir Ocular Bioavailability

Male New Zealand albino rabbits (Charles River, Calco, Italy), 1.8–2.2 kg, free of ocular inflammation or gross abnormalities were used. Animal procedures conformed to the ARVO (Association for Research in Vision and Ophthalmology) resolution on the use of animals in research. Aqueous acyclovir levels were monitored 30, 60, 120, 240, and 360 min after a single instillation of 50 μL of each formulation into the conjunctival sac. Before paracentesis, the rabbits were anesthetized by an intravenous injection of 25 mg/kg of ketamine · HCl. Aqueous humor (150 μL) was withdrawn through the limbus, with a syringe with a 26G needle, and stored at −20°C. The aqueous samples were treated with a solution of 2% $ZnSO_4 \cdot 7H_2O$ that was mixed by vortex and centrifuged. The supernatant was filtered through a 0.2-μm PTFE membrane and then analyzed by HPLC. Chromatography was performed with a Hewlett Packard model 1100 equipped with a diode array detection (DAD) operating at 254 nm. A Hypersil ODS reversed-phase column (150 ± 4.6 mm i.d.; Alltech, Milan, Italy) thermostated at 27°C was used. The mobile phase was 0.7 M sodium acetate (pH 6.0) with a flow rate of 1 mL min^{-1}. Aqueous acyclovir levels were calculated from linear regression of external standards of acyclovir, relating peak area and concentration. Standards in a range 1.0–55.5 μg/mL were used for the calibration curve. The straight-line equation was $y = -1.0013x + 0.00046$ (y = drug concentration; x = peak area) with an $r^2 = 0.9995$. The method was reproducible, with a relative standard deviation of 1.80%. The sensitivity was 0.61 μg/mL. No interfering peaks were observed in the blank aqueous humor chromatograms.

RESULTS AND DISCUSSION

PECA nanospheres are formed by a micellar polymerization process, which is very rapid and can be influenced by the environmental conditions of the polymerization medium; that is, pH value, type and concentration of the nonionic surfactant, the presence of electrolytes and temperature.[21, 24, 25] PECA nanospheres, during the growing phase, are extremely plastic and able to provide aggregation phenomena. For this reason, the presence of a nonionic surfactant is of significant importance to influence the colloidal stability, particle size, and polymeric chain molecular weight.[26]

The presence in the polymerization medium of substances that are able to give nucleophilic attack (e.g., some drugs, macromolecules) can influence the PECA nanosphere formation and the physicochemical properties of the polymeric network.[9, 27] For this reason, the influence of macromolecules, such as PEG 6000 and HP-β-CyD, on some important parameters of the colloidal system, such as particle size distribution, loading capacity, and *in vitro* drug release were investigated. These macromolecules, in addition to the presence of Pluronic F68, can contribute in different ways to modify the hydrophilic/hydrophobic characteristics of the acyclovir-loaded nanosphere surface, a requisite that can play an

Table 1. Preparation Conditions, Dimensional Analysis, and Zeta Potential of Various PECA Nanosphere Formulations[a,b,c]

Batch	Formulation Conditions			Size (nm)	PI[d]	Zeta Potential (mV)[e]
	Pluronic F68 (g)	PEG-6000 (mg)	HP-β-CyD (mg)			
NS1	0.25	—	—	220 ± 15	0.12	−25.9 ± 1.8
NS2	1.50	—	—	165 ± 25	0.14	−24.7 ± 2.3
NS3	0.25	500	—	450 ± 30	0.25	−12.7 ± 2.5
NS4	1.50	500	—	190 ± 25	0.11	−12.2 ± 1.9
NS5	1.50	—	500	345 ± 20	0.83	−24.1 ± 2.2

[a] Each value is the average of three different experiments ± standard deviation.
[b] Nanosphere suspensions were separated from the untrapped acyclovir and submitted to PCS analysis after suitable dilution with filtered (0.2-μm membrane filter) phosphate buffer (pH 7.4).
[c] PECA nanospheres prepared in the absence of acyclovir presented no significant variation of the surface and dimensional properties (data not reported).
[d] Polydispersity index.
[e] Zeta potential values were obtained from electrophoretic mobility (velocity divided by electric field; $\mu m\ s^{-1}\ V^{-1}\ cm^{-1}$) by the Smoluchowsky constant $F(K_a) = 1.5$.

important role in bioadhesion and/or mucoadhesion of the colloidal carrier at the level of the ocular epithelium.[28]

The polymerization medium consisted of 50% (v/v) ethanol, which increased the solubility of acyclovir. Table 1 shows the formulation parameters and the size analysis. The presence of macromolecules and the concentration of Pluronic F68 influenced the mean nanosphere size. In particular, the presence of PEG 6000 and HP-β-CyD elicited an increase in size and polydispersity index of PECA nanospheres. PEG 6000 doubled the mean size of acyclovir-loaded PECA nanospheres prepared in the presence of 250 mg of Pluronic F68.

Recent studies demonstrated that the presence of PEG in the polymerization environment of PACA nanospheres allowed the formation of sterically stabilized colloidal systems.[29] The PEG-coated acyclovir-loaded PECA nanoparticles were prepared at a 2.0 ± 0.1 pH value. To obtain a suitable colloidal stability of PECA nanospheres at this pH value, the presence in the polymerization medium of another steric stabilizer is necessary, as reported by Lourenco et al.[29] In fact, the polymerization medium pH is a fundamental parameter during the preparation of PEG-coated PACA nanospheres.[19] In the absence of other stabilizers, when the polymerization is carried out at pH > 1.5, colloidal nanospheres are not obtained, just polymer aggregates. To obtain a good colloidal stability with a narrow size distribution and a high nanosphere yield it is necessary to carry out the polymerization at a pH between 0.75 and 1.5. Our findings showed that the formation of PEG-coated PECA colloidal nanospheres was still possible at a pH > 1.5 by adding to the polymerization medium another steric stabilizer, such as Pluronic F68, in addition to the presence of PEG 6000.

Nonionic surfactant concentration noticeably influenced the mean PECA nanosphere size: the higher the concentration the smaller the colloidal size. By increasing the Pluronic F68 concentration up to 1.5 g, it was still possible to achieve PECA nanospheres with a mean size < 200 nm (Table 1). In the case of the Pluronic series used as nonionic surfactant, concentrations ≥ 3% (w/v) determine the formation of nanospheres with a colloidal size of ~165 nm, whereas an increase of mean size is achieved at lower concentrations.[21] These findings can be related to the critical micelle concentration (cmc) value of Pluronic F68. A 2–3% (w/v) concentration is higher than the cmc of Pluronic F68, allowing the polymerization within a certain number of micelles. Thus, the higher the Pluronic F68 concentration, the greater the number of micelles in the polymerization medium, and hence the smaller nanosphere size.[30]

As shown in Table 1, zeta potential was influenced by the presence of PEG 6000. The presence of the PEG macromolecules along the surface of the PECA nanospheres resulted in a reduction of the colloidal system zeta potential (−12.7 mV) with respect to nanospheres prepared in the absence of PEG 6000, which showed a zeta potential of −25.9 mV. The presence of HP-β-CyD only slightly influenced the PECA nanosphere zeta potential, but causing a marked

Table 2. Entrapment capacity of PECA Nanosphere Colloidal Suspensions Containing Ayclovir Prepared in the Absence and in the Presence of Both PEG 6000 and HP-β-CyD at Different Concentrations of Nonionic Surfactant Pluronic F68[a,b,c]

Batch	Loading Capacity (% w/w)[d]
NS1	2.18 ± 0.23
NS2	2.24 ± 0.18
NS3	2.27 ± 0.21
NS4	1.98 ± 0.19
NS5	1.73 ± 0.15

[a]Each value is the average of three different experiments ± standard deviation.
[b]Nanosphere suspensions were separated from the untrapped acyclovir and analyzed for drug content by HPLC.
[c]Preparation parameters of various batches are summarized in Table 1.
[d]Loading capacity is expressed as percentage of drug amount in 100 mg of freeze dried PECA colloidal suspension.

increase of the nanosphere mean size and polydispersity index (Table 1).

PECA nanospheres were prepared in the presence of HP-β-CyD to improve acyclovir solubility in the polymerization medium, thus trying to increase the drug loading capacity of the colloidal system. As reported in Table 2, the drug entrapment was not influenced by the preparation conditions (i.e., presence of PEG 6000 or HP-β-CyD, or Pluronic F68 concentration). Probably, the acyclovir loading capacity is dependent on the drug physicochemical characteristics and drug–polymeric matrix affinity and interaction.[31]

The acyclovir release profile from PECA nanospheres was studied *in vitro* both in phosphate buffer solution (pH 7.4) and in human plasma. In each experiment, acyclovir release as free drug was determined by HPLC analysis. Acyclovir release at pH 7.4 from various PECA nanospheres obtained following different preparation conditions is shown in Figure 1. All PECA nanosphere systems provided a biphasic acyclovir release profile, characterized by an initial phase of rapid drug leakage followed by a more gradual drug release. Probably, in the acyclovir-loaded nanoparticles, part of the drug is simply adsorbed at the surface of the colloidal particle rather than homogeneously dispersed and/or solubilized in the PECA polymeric network. According to previous investigations,[32] this particular drug disposition within the colloidal carrier can justify the initial rapid release due to acyclovir desorption.

The presence of PEG 6000 on the PECA nanosphere surface also influenced the release

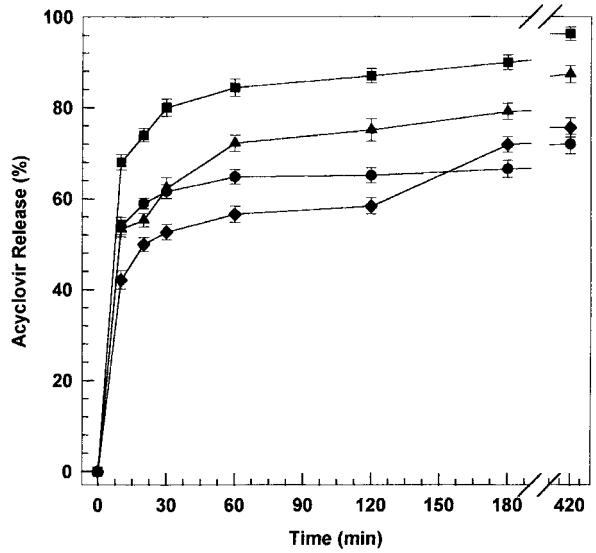

Figure 1. Acyclovir release from various PECA nanosphere colloidal systems suspended in isotonic phosphate buffer (pH 7.4). Release experiments were carried out at 37 ± 0.2°C, immediately after sample preparation. Untrapped drug was removed by centrifugation. Each point represents the mean value of three different experiments ± standard deviation. Key: (■) NS1; (◆) NS3; (●) NS4; (▲) NS5. Batch NS2 has a release profile similar to NS1 (data not reported). Various formulation parameters of various batches are summarized in Table 1.

profile of acyclovir, as well as colloidal mean size, polydispersity index, and zeta potential. In fact, as shown in Figure 1, PEG-coated PECA nanospheres provided a slower acyclovir release. After a 7-h release, PECA nanospheres prepared in the absence of PEG 6000 released ~95% acyclovir, whereas PEG-coated PECA nanospheres showed a ~70% drug release.

Acyclovir release from PECA nanospheres in human plasma is shown in Figure 2. Also in these conditions, PECA nanosphere colloidal systems prepared in the presence of PEG-6000 released acyclovir more slowly than uncoated-PECA nanospheres. After 7 h of release, a 100% drug release was observed for all PECA nanosphere systems. These findings can be due to the presence of estherases, which catalyze PECA polymeric network degradation,[12] determining a leveling effect on the acyclovir release rate. Therefore, the drug release for all acyclovir-loaded PECA nanospheres is modulated both by simple desorption/diffusion through the polymeric matrix and by bioerosion of the colloidal systems.

The presence of HP-β-CyD resulted in no variation of acyclovir release in human plasma

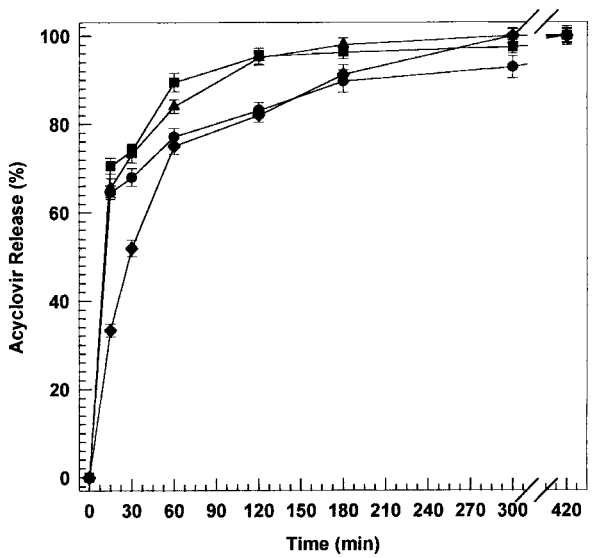

Figure 2. Acyclovir release from various PECA nanosphere colloidal systems suspended in human plasma. Release experiments were carried out at 37 ± 0.2°C, immediately after sample preparation. Untrapped drug was removed by centrifugation. Each point represents the mean value of three different experiments ± standard deviation. Key: (■) NS1; (◆) NS3; (●) NS4; (▲) NS5. Batch NS2 has a release profile similar to NS1 (data not reported).Various formulation parameters of various batches are summarized in Table 1.

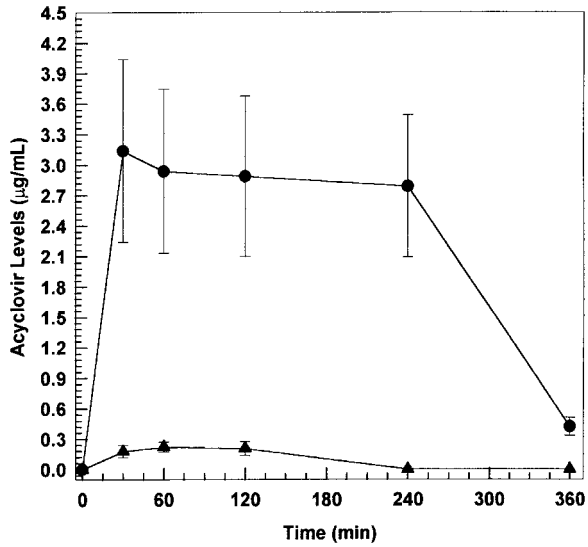

Figure 3. Acyclovir levels in the aqueous humor after single instillation (50 μL) of drug-loaded PEG-coated PECA nanosphere colloidal suspension (●) or free drug suspension (▲) in the rabbit eye. Each value is the average of six different experiments ± standard deviation. Acyclovir-loaded nanospheres showed a significantly higher ($p < 0.001$) levels of acycloir in aqueous humour than free drug. PEG-coated PECA nanosphere-acyclovir physical mixture resulted in no significative improvement of drug permeation compared with free acyclovir (data not reported).

(Figure 2), whereas the drug release was slightly slower in phosphate buffer (pH 7.4) compared with PECA nanospheres prepared in the absence of HP-β-CyD.

Colloidal drug delivery systems have been reported to be promising ocular delivery devices.[7,13,33] In fact, the colloidal properties of various systems can achieve a better interaction with the corneal epithelium (in terms of paracellular transport and/or passage), thus leading to a greater drug transport into the ocular tissues.[34] Considering that particular attention has been recently focused on polymeric carriers containing long linear chains of PEG for pharmaceutical and biomedical applications,[35, 36] PEG-coated PECA nanospheres have been investigated as potential ophthalmic drug delivery systems.

To evaluate *in vivo* bioavailability of both free and entrapped acyclovir, the drug-loaded PECA nanospheres (NS4) coated with hydrophilic polymer PEG 6000 were compared with both a formulation of empty nanospheres physically blended with acyclovir and an aqueous dispersion of acyclovir. All three formulations tested contained the same drug concentration. Because the aqueous T_{max} (peak-time) of most topically administered drugs is ~30 min[37] and because our *in vitro* studies showed a T_{max} at 30 min, the aqueous acyclovir levels of the various formulations were monitored from 30 min up to 6 h after a single formulation instillation (50 μL).

The concentrations of acyclovir in the aqueous humor after a single instillation of the various formulations are reported in Figure 3. Acyclovir-loaded nanospheres (NS4) showed a significantly higher ($p < 0.001$) capability of making the drug permeate into aqueous humor compared with a free acyclovir suspension at all times. Key pharmacokinetic parameters describing the time profile of acyclovir levels within aqueous humor are reported in Table 3. Colloidal PECA nanosphere formulation (NS4) provided a noticeably sustained drug release in the aqueous humor compared with free acyclovir formulations. In particular, PEG-coated PECA nanospheres were able to ensure therapeutically effective[38] levels up to 360 min after ocular administration of the formulation. The aqueous $AUC_{0 \rightarrow 360}$ values were significantly ($p < 0.001$) greater for acyclovir-loaded PECA nanospheres than for the free drug,

Table 3. Key Parameters Describing the Aqueous Humor Pharmacokinetics of Acyclovir after Single Instillation (50 μL) of Each Formulation

Key Parameter	NS4[a]	Free Acyclovir
Time interval when acyclovir was still detectable (min)	360	120
Maximum concentration of drug (μg/mL)	3.14	0.22
Time when C_{max} is detected (min)	30	60
$AUC_{0\rightarrow360}$ (μg mL^{-1} min ± SD)	846.6 ± 22.3	33.7 ± 10.3

[a]Preparation parameters of PEG-coated PECA nanospheres are summarized in Table 1.

with a 25-fold increase. The *in vivo* data seem to be in agreement with data coming from *in vitro* (phosphate buffer and human plasma) acyclovir-loaded PECA nanosphere release profiles. In fact, the PECA nanosphere formulation, showing an *in vitro* burst effect, ensured a higher availability of the drug during the first hour. After 4 h from the administration of the ophthalmic formulation, acyclovir levels in the ocular aqueous humor drastically decreased, probably because of the enzymatic degradation of acyclovir at the level of the internal compartment of the eye, as well as because of the lacrimal drainage of the suspension. A longer pharmacokinetic beyond 360 min was meaningless because of the clearance of the suspension by the lacrimal fluid.

The ability of PEG-coated PECA nanospheres (NS4) to improve and prolong the corneal penetration of acyclovir can be attributed, according to literature,[39] to a longer and closer drug contact with the epithelial ocular surfaces. Proof of this hypothesis is the uptake of PACA fluorescent nanoparticles by corneal and conjunctival epithelial cells.[40] The longer ocular permanence of PEG-coated PECA nanospheres on epithelial cell surfaces can be explained in terms of colloidal drug carrier mucoadhesion. PEG chains can ensure an interpolymer interaction via hydrogen bonds,[35] with the mucin macromolecules present on the ophthalmic surface. The increased acyclovir penetration could be also due to a certain penetration enhancer effect of PEG. These PEG chains, which are present on the nanopshere surface, as are other hydrophilic macromolecules (e.g., chitosoan),[41] may induce a reversible opening of the tight junctions of the conjunctival tissue and hence allow paracellular transport of acyclovir across ophthalmic tissues.

For a polymeric drug delivery system to be proposed as an ophthalmic drug carrier it is important not only to assay the biopharmaceutical properties and biological and/or therapeutic effectiveness, but also the ocular tolerability. Therefore, *in vivo* ocular irritancy towards PECA nanospheres was carried out following a modified Draize test protocol.[23] *In vivo* ocular tolerability results showed no evidence of inflammation and/or discomfort in rabbit eyes. A negligible conjunctival hyperemia was observed 10 min after the end of the treatment, with no significant difference in the three formulations tested (data not reported). No sign of inflammation was observed 6 h after the end of the treatment with the three formulations. Therefore, the potential clinical interest of PEG-coated PECA nanoparticle formulations is also because of the absence of irritant activity *in vivo*.

In conclusion, our *in vivo* results reported here show that PEG-coated PECA nanospheres are able to increase acyclovir ocular bioavailability compared with the raw drug. The improved acyclovir bioavailability may be due to a better interaction of PEG-coated PECA nanoparticles with the corneal epithelium. PECA colloidal nanospheres can facilitate the paracellular transport and/or passage of acyclovir through the cornea, thus leading to a greater drug transport in ocular tissues. Therefore, PECA colloidal formulations may be useful in clinical practice for a better compliance and an improved outcome.

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