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Research paper

The influence of the use of viscosifying agents as dispersion media on the drug release properties from PLGA nanoparticles

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Abstract

Poly(lactide-*co*-glycolide) nanoparticles incorporating ciprofloxacin HCl were prepared by means of a W/O/W emulsification solvent evaporation method. The physicochemical properties of these particles were evaluated by measuring particle size, zeta potential and drug loading efficiency. Gamma-sterilised nanoparticles were dispersed in different isoviscous polymer solutions, commonly used as vehicles in eye drops. The influence of γ-irradiation of the viscosifying agents on the drug release properties of the dispersed nanoparticles was evaluated with respect to release in mannitol solution. The viscosity of the polymer solutions prepared was measured by flow rheometry and thereby the influence of temperature and sterilisation by autoclaving on viscosity was examined. Before and after freeze-drying and subsequent sterilisation by gamma-irradiation, the polymer solutions were also characterised by dynamic stress sweep and dynamic frequency sweep oscillation measurements to deduce possible structural changes. A possible relationship between the differences in ciprofloxacin release from the nanoparticles suspended in the various media and the network structure or rheological behaviour of the polymers was investigated.

Keywords: PLGA; Nanoparticles; Ciprofloxacin; Drug release; Rheology

1. Introduction

Eye drops are conventional dosage forms for topically administered ophthalmic drugs. The bioavailability of ophthalmic drugs in aqueous solutions is low due to a rapid elimination after instillation, because of reflex blinking, tear drainage and the barrier function of the cornea. Formulations can be optimised in order to increase the bioavailability of the drugs applied. Alternative dosage forms can also be chosen, for example instead of viscous eye drops, dispersed systems like biodegradable nanoparticles with prolonged release properties can be employed [1,2]. Nanoparticles are frequently prepared using poly(lactic acid) (PLA) or its copolymer with glycolic acid (PLGA), due to the biocompatibility and biodegradability of these materials [3–5].

Nanoparticles have the advantage of offering a sustained release of the drug, which is dispersed in the matrix, in order to obtain the required tear levels and therapeutic effects [4,5]. A combination of mucoadhesive polymers and nanoparticles can also be employed to prolong the residence time of the particles in the precorneal area [1,6]. Moreover, endocytosis through the corneal epithelium depends on the particle size and size distribution of the particles: nanoparticles have been found in corneal cells, but no presence of microspheres was reported [7,8].

The advantages of dispersed systems should be combined with a dosage form that is well accepted by patients and provides good comfort. As nanoparticles can be easily eliminated from the precorneal area, it is interesting to co-administrate them with viscous, viscoelastic or bioadhesive polymer solutions to prolong their residence time at the ocular surface, resulting in an increased drug bioavailability [1,2,9]. In addition, mixtures of polymers can be used to obtain a formulation with the required properties [10,11].

In the present study, the different polymers selected were hydroxyethylcellulose (HEC), poly(vinylalcohol) (PVA), poloxamer and carbomers (Carbopol®). HEC is a cellulose ether that has been widely used as viscolyser, while higher concentrations employed increased the ocular bioavailability of drugs. HEC is reported to show better tolerance than hydroxyproplymethycellulose (HPMC) or hydroxypropylcellulose (HPC) [5,12]. Aqueous PVA solutions have similar applications. The bioavailability of the drug was enhanced

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when the contact time between the eye drops and the ocular surface was increased [5]. Peppas and Mongia mentioned the possibility of controlling the mucoadhesive and drug release properties of PVA hydrogels after a process of freezing and thawing for several cycles [13]. Poloxamer undergoes reversible thermal gelation and sol–gel phase transitions with inverse temperature dependence [14,15]. It exhibits minor irritation toxicity, which makes it suitable for ocular use [15]. The possibility of ophthalmic use of poloxamer 407 as an in situ gelling system has been demonstrated by Miller and Donovan and Edsman et al. [15,16]. El-Kamel described an ocular delivery system consisting of a combination of poloxamer 407 and various viscosity-enhancing agents, like methylcellulose (MC) or HPMC [11].

Different kinds of Carbopol® were used in ocular formulations [5]. Polymers of the poly(acrylic acid) type have been suggested to possess very good bioadhesive properties [5,17,18]. Carbopol® 974P NF is the benzene-free alternative for Carbopol® 934P NF and can be employed in controlled release formulations. Carbopol® 980 NF is the most efficient thickener of all Carbopol® polymers on the market and is less heavily cross-linked than Carbopol® 974. Carbopol® 1342 NF contains a long alkyl chain acrylate, which increases its resistance to dissolved ions (BF Goodrich, 1994).

The aim of present study is to evaluate and compare formulations based on bioadhesive poly(acrylic acid) polymers or viscosity-enhancing polymers like PVA, HEC and poloxamer and to determine the influences of temperature, sterilisation by autoclaving and gamma-irradiation and freeze-drying on the viscoelastic properties of the polymers investigated.

The drug release properties of freeze-dried and gamma-sterilised nanoparticles dispersed in various polymer solutions were compared. The rheological characterisation of the different viscosifying agents used involved flow measurements, whereby the flow curves of the polymers were registered at different temperatures and before and after sterilisation by autoclaving. The resulting curves were fitted into mathematical models. Oscillatory rheological measurements allowed characterisation of the behaviour of the polymers as viscous, viscoelastic or elastic. Different parameters can be calculated to characterise viscoelastic behaviour [18]. This classification served to study the influence of the rheological behaviour of viscolysers and bioadhesive polymers on the drug release from the gamma-sterilised nanoparticles dispersed in reconstituted isoviscous polymer solutions.

2. Materials and methods

2.1. Materials

The poly(lactic-*co*-glycolic acid) or PLGA polymer employed was Resomer® RG 503 (Boehringer Ingelheim, Ingelheim am Rhein, Germany) with a molecular weight of 40,000 and a D,L-lactide/glycolide molar ratio of 52:48. Poly(vinylalcohol) or PVA (MW 30,000–70,000) was purchased from Sigma Chemicals Co. (St Louis, USA). Poloxamer 407 or Lutrol® F 127 from BASF (Ludwigshafen, Germany) was used. Natrosol 250G Pharm (hydroxyethylcellulose, HEC) was obtained from Aqualon (Heverlee, Belgium). Carbopol® 974P NF (CP 974), Carbopol® 980 NF (CP 980) and Carbopol® 1342 NF (CP 1342) were a gift from Noveon (Cleveland, USA). Ciprofloxacin HCl was supplied by Roig Farma (Barcelona, Spain). Dichloromethane was obtained from Sigma-Aldrich (Steinheim, Germany) and acetonitrile (HPLC grade) from Acros Organics (New Jersey, USA). Filtered (Porafil 0.20 Membranfilter, Düren, Germany) purified Milli Q water (Millipore, Mollsheim, France) was used throughout all the experiments. KCl and NaCl were obtained from Merck (Leuven, Belgium), $NaHCO_3$ from Merck (Darmstadt, Germany) and $CaCl_2$ and $MgCl_2$ from Sigma Chemicals Co. (St Louis, USA). These salts served for the preparation of simulated lachrymal fluid (SLF), 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 $MgCl_2{\cdot}6H_2O$, adjusted with 0.1 N HCl to a pH of 7.4 ± 0.1 [19].

2.2. Preparation methods

2.2.1. Preparation of ciprofloxacin-loaded PLGA nanoparticles

The nanoparticles were prepared by W/O/W emulsification solvent evaporation followed by high-pressure homogenisation [20]. Eight millilitres of an aqueous 2.50% w/v ciprofloxacin solution were emulsified by means of sonication for 1 min at 20 W (amplitude 50%) (Branson 450-D, 102-C with microtip, Branson, Danbury, USA) in an organic phase, which consisted of 2.00 g of PLGA dissolved in 20 ml dichloromethane. The resulting W/O emulsion was dispersed in 100 ml of the first outer water phase, a 1% w/v PVA stabiliser solution, and sonicated for 30 s at 15 W (amplitude 40%) 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 for 3 cycles. The emulsion was then diluted in the second outer phase, consisting of 0.3% w/v of PVA in water. The organic solvent was allowed to evaporate during 4 h at room temperature under agitation (700 rpm) with a magnetic stirrer (Variomag Electronicrührer Poly 15, H + P Labortechnik GmbH, Münich, Germany). The resulting nanosuspension was subsequently cooled down to −18 °C and freeze-dried (Leybold-Heraeus D8B, GT-2A, Köln, Germany). The freeze-dried nanoparticles were gamma-irradiated, employing Co^{60} as irradiation source (Gammir I-Sulzer irradiation unicell, IBA-Mediris, Sterigenics, Fleurus, Belgium) and received a dose of 25 kGy, considered as adequate for sterilising pharmaceutical products, according to the European Pharmacopoeia.

2.2.2. Preparation of the polymer solutions

To prepare polymer solutions with a viscosity in the range of 100 ± 20 mPa s at 32 °C, the required amount of the polymer powder was dispersed in an iso-osmotic 5.07% (w/v) mannitol solution and stirred at 70 °C for PVA and at 25 °C for HEC, poloxamer and Carbopol® using a magnetic stirrer until the powder was hydrated. Mannitol was chosen because it has no effect on the pH values of the solutions, and because upon the addition of sodium chloride or borate, the viscosities of Carbopol® solutions decrease dramatically [21]. Carbopol® was neutralised by adding the required amount of 1 M NaOH until pH 7.4 ± 0.1 was reached, in order to obtain the maximum viscosity. To ensure complete hydration, the cooled dispersions were stored at 6 °C for at least 12 h. Afterwards, the solutions were stored in vials (glass type I) and sterilised by autoclaving. Thirty millilitres of each sterilised polymer solution were freeze-dried (Leybold-Heraeus D8B, GT-2A, Köln, Germany). The freeze-dried polymers were gamma-irradiated using Co^{60} as irradiation source (Gammir I-Sulzer irradiation unicell, IBA-Mediris, Sterigenics, Fleurus, Belgium) and received a dose of 25 kGy. Freeze-dried or freeze-dried and gamma-sterilised polymer solutions were reconstituted by adding the proper amount of water.

2.2.3. Mixing of the nanoparticles with various polymers

Preparations with the same drug loading and amount of dispersed nanoparticles were employed throughout the study. A proper amount of freeze-dried, gamma-sterilised nanoparticles, accurately weighed, was added to a certain volume of the reconstituted polymer solution, in order to obtain a final concentration of 10 mg/ml nanoparticles. Agitation of the dispersion (500 rpm) was carried out with a magnetic stirrer (Variomag Electronicrührer Poly 15, H + P Labortechnik GmbH, Münich, Germany), in order to obtain a homogeneous dispersion.

2.3. Evaluation methods

2.3.1. Evaluation of the nanoparticles

2.3.1.1. Nanoparticle size and zeta potential analysis. The mean particle size Z_{ave} of the nanoparticles was determined by photon correlation spectroscopy (PCS) with a Zetasizer 3000 (Malvern Instruments, Malvern, UK). A portion of the freshly prepared suspension was diluted hundred times with filtered purified water. The Z_{ave} of each sample was determined four times and the average values were calculated.

The zeta potential of the nanoparticles was determined using electrophoretic light scattering (ELS). The freeze-dried nanoparticles were suspended either in aqueous mannitol solution (5.07% w/v) or in one of the iso-osmotic polymer solutions and diluted with water or SLF, which possessed a similar electrolyte composition compared to tear fluid. 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.

2.3.1.2. Drug loading determination. Twenty milligrams of freeze-dried gamma-sterilised nanoparticles, accurately weighed, were dispersed in 10.0 ml of purified water and sonicated 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 ciprofloxacin HCl concentration in the supernatant was determined by a validated HPLC method. The HPLC system consisted of a Gilson 321 pump (Gilson, Villiers-le-Bel, France), a UV–VIS 152 detector (Gilson, Villiers-le-Bel, France), a μ-Bondapack™ C_{18} 125 Å 10 μm column (Waters, Milford, USA) and an HP 3395 integrator (Hewlett-Packard Company, Palo Alto, USA). The mobile phase and flow rate used, corresponded to the method described in the monograph in the European Pharmacopoeia [22]. The concentration of ciprofloxacin HCl in the supernatant was detected at 278 nm and calculated using a calibration curve. The entrapment efficiency or EE was determined employing the following equation:

$$\text{EE}\ (\%) = (\text{actual drug loading/theoretical drug loading}) \times 100\%.$$

2.3.1.3. In vitro release tests. The in vitro release experiments were carried out 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 5.0 ml aqueous 5.07% w/v mannitol solution or one of the six viscolyser solutions with 50.0 mg dispersed nanoparticles. The acceptor compartment was filled with 18.0 ml SLF and was stirred magnetically at 200 rpm (Variomag Electronicrührer Poly 15, H + P Labortechnik GmbH, Münich, Germany). At predetermined time intervals, 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. The tests were carried out three times and mean drug release percentages were calculated. To perform the statistical analysis of the data, the Statistica® software (Statsoft, Tulsa, USA) was used.

2.3.2. Rheological characterisation

The rheological analyses were performed using a controlled-stress rheometer (Carri-Med CSL^2 100, TA Instruments, Brussels, Belgium) equipped with a double concentric cylinder geometry (height of 20.5 mm).

2.3.2.1. Flow measurements. Shear rate ramps have been performed to determine the apparent viscosity of the different polymer solutions as a function of the shear rate applied. After a preshear procedure of 30 s at a shear

rate of 30 s^{-1}, allowing the preparation to be spread homogeneously, the test samples were equilibrated for 10 min at 25 ± 0.1 °C, the temperature at which the in vitro release experiments were performed or at 32 ± 0.1 °C, the temperature at the ocular surface [23]. The shear rate was then increased linearly from 1 to 100 s^{-1} within a period of 3 min and decreased linearly from 100 to 1 s^{-1} within the same time interval. The flow measurements were performed two times and mean values were calculated.

For non-Newtonian, viscoelastic, shear-thinning fluids, different mathematical models can be applied to describe the behaviour of the polymer solution. A Bingham plastic is a non-Newtonian liquid characterised by the shear stress being a linear function of the shear rate applied, but with a critical stress which needs to be exceeded before flow is detected (Eq. (1)).

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

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

A non-Newtonian liquid with a yield stress, whereby shear stress is not a linear function of shear rate applied, can be analysed with the Herschel-Bulkley model (Eq. (2)).

$$\sigma = \sigma_y + k\gamma^n \quad (2)$$

with k is the consistency index (Pa.s) and n is the shear rate index, ranging from $n = 1$ for Newtonian systems to $n = 0$ for non-Newtonian systems.

The viscosity of the solutions was measured in order to study the influence of temperature and the autoclaving process on the rheological behaviour of the polymers.

2.3.2.2. Oscillatory rheometry. The oscillation technique measures both the viscous and the elastic properties of a sample simultaneously. Two types of oscillation measurements were performed at 25 ± 0.1 °C. Oscillatory shear rheological experiments are performed to characterise the polymer network. During a dynamic stress sweep (DSS), oscillation stress was increased logarithmically from 10^{-3} to 10 Pa at a constant frequency of 1 rad/s. Two viscoelastic parameters, G', the in-phase, storage or elastic modulus and G'', the out-of-phase, loss or viscous modulus, were recorded. From the generated double logarithmic plot of G' and G'' versus the stress applied, the linear viscoelastic region (LVER) was detected, where the polymer network stayed intact. A stress value situated in the LVER (0.01 Pa) was chosen to perform a frequency ramp (DFS), whereby small stress oscillations were performed at a constant stress amplitude at a range of increasing oscillatory angular frequencies (ω) from 0.1 to 10 rad/s. During these experiments, the kind of network structure present in the viscosifying agent solution could be revealed. In DFS measurements of gels, with a network of secondary bonds, a constant value of G' and G'' is detected over the whole frequency range, with the value of G' exceeding that of G''. The slope values of G' and G'' versus frequency (log–log plot) are close to zero. In the case of solutions with physical entanglements, G'' values are larger than G' values and the polymer network behaves as a viscous system rather than as an elastic one, because when the radial frequency increases, G' values equal G'' values at the cross-over frequency while at still higher frequencies G' and G'' values are less frequency dependent and a high frequency plateau develops with a G' value larger than the G'' value. At low frequencies, the slopes of the curve when G' and G'' are plotted versus frequency (log–log plot) have limiting slopes with values equal to 2 and 1, respectively [18].

DSS procedures were performed three times on each polymer solution, and dynamic frequency sweeps were performed twice, after which mean values of the slope of the curve ($\log G'/\log \omega$) and standard deviations were calculated.

3. Results and discussion

3.1. Characterisation of the nanoparticles

3.1.1. Physical characterisation: particle size and zeta potential measurements

The average size of the nanoparticles, obtained before freeze-drying, was 209.5 nm with a standard deviation of 2.5 nm. The zeta potential values of these nanoparticles in the different dispersion media are shown in Table 1. The dispersions of the freeze-dried, gamma-sterilised nanoparticles in the polymer solutions or in aqueous mannitol 5.07% w/v solution were either diluted with water, to evaluate the in vitro charge or with SLF, to simulate the effects occurring during application. From these results, one can deduce that the polymer solution in which the nanoparticles were dispersed had a marked influence on the zeta potential values when the dispersion was diluted with water. Probably, a certain degree of adsorption of the polymers

Table 1
Mean zeta potential values with standard deviations (mV) of nanoparticles dispersed in mannitol solution (5. 07% w/v) or in different polymer solutions and diluted with water or SLF ($n = 3$)

Conc. polymer	Mannitol (% w/v)	PVA 13.20	HEC 2.30	Pol 15.10	CP 974 0.08	CP 980 0.045	CP 1342 0.0175
Water	−9.4 (2.6)	−1.0 (1.1)	−0.7 (0.6)	−2.3 (0.2)	+3.2 (2.0)	−19.0 (5.0)	−22.0 (5.9)
SLF	−5.9 (1.7)	−5.9 (1.3)	−3.8 (1.1)	−5.4 (0.9)	−13.3 (4.6)	−6.9 (1.8)	−6.5 (0.9)

present occurred at the nanoparticles surface. A study of Vandervoort and Ludwig also showed the influence of the polymer selected as stabiliser during particle preparation, on the zeta potential of the nanoparticles obtained: when Carbopol® or poloxamer were used as stabilisers, the particles possessed more pronounced negative zeta potential values than when HEC or MC were employed [20]. The same trend is also observed in the present study. However, it is remarkable to note the positive zeta potential value of the nanoparticles dispersed in aqueous CP 974 compared to the extreme negative values in the case of the two other kinds of CP. Although, the major difference between CP 974 and CP 980 is the degree of cross-linking: CP 980 is a slightly cross-linked polymer while CP 974 is more heavily cross-linked. Carbopol® 1342 contains a lipophilic modification (BF Goodrich, 1994). Differences in network structure influenced adsorption on the nanoparticles and consequently the zeta potential of the PLGA particles. When SLF was used as a diluting vehicle, the influence was less pronounced than in water. The nanoparticles with a positive zeta potential in water became, however, the most negative when dispersed in SLF. In SLF, all zeta potential values were similar: the relative high salt concentration caused the equalising of the zeta potential values, because ions of the medium neutralised partly charges on polymers and dispersed particles by compressing the electrical double layer which surrounds them, thereby making their surface charges similar.

3.1.2. Drug loading of the nanoparticles and release of ciprofloxacin HCl

The EE of the gamma-irradiated nanoparticles prepared was 61%, which corresponded to an absolute drug loading of 25 μg ciprofloxacin HCl/mg nanoparticles. In order to eliminate the gamma-sterilisation of PLGA nanospheres as a factor determining drug release, all nanoparticles were gamma-irradiated before dispersion in polymer solutions [24]. The mean drug release patterns of ciprofloxacin from the nanoparticles, dispersed in four different vehicles before

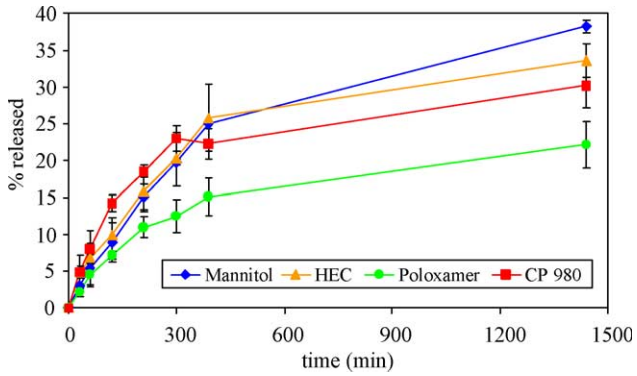

Fig. 1. Release of ciprofloxacin from nanoparticles dispersed in solutions of mannitol or viscosifying agents, before gamma-sterilisation (mean, $n = 3$). Diamonds stand for nanoparticles dispersed in mannitol solution (reference solution), triangles for dispersion in HEC, circles for dispersion in poloxamer and squares for dispersion in Carbopol® 980.

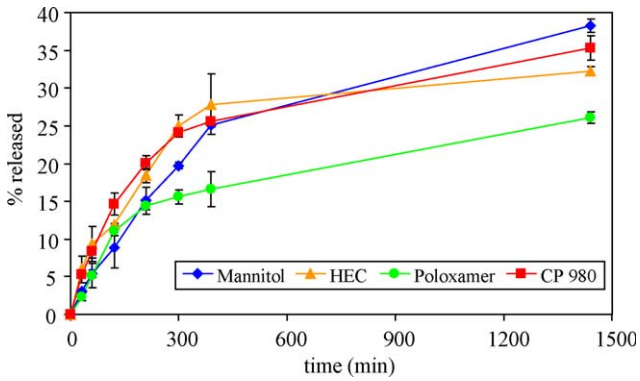

Fig. 2. Release of ciprofloxacin from nanoparticles dispersed in solutions of mannitol or viscosifying agents, reconstituted after freeze-drying and gamma-sterilisation (mean, $n = 3$). Diamonds stand for nanoparticles dispersed in mannitol solution (reference solution), triangles for dispersion in HEC, circles for dispersion in poloxamer and squares for dispersion in Carbopol® 980.

gamma-irradiation of the viscosifying agents, are shown in Fig. 1, while the drug release profiles of the nanoparticles, dispersed in polymer solutions which were reconstituted after freeze-drying and gamma-sterilisation, are given in Fig. 2. The drug releases after 1, 5 and 24 h expressed as % of the drug loading and the area under the curve ($AUC_{0\rightarrow 24\ h}$) are summarised for all polymer solutions and for an aqueous 5.07% w/v mannitol solution in Table 2. Before gamma-irradiation of the polymers, drug release kinetics in HEC and in CP 1342 solutions were comparable and slightly slower than in mannitol, the non-viscous reference solution. PVA and CP 980 caused a small decrease of the drug release and the release kinetics were the slowest when the nanoparticles were dispersed in CP 974 or in poloxamer. When the nanoparticles were dispersed in freeze-dried, irradiated and subsequently reconstituted polymer solutions, the release patterns shifted towards higher release rates. Drug release kinetics in mannitol, PVA, HEC, CP 974, 980 and 1342 solutions became comparable, in contrast to the release kinetics in poloxamer which were still the slowest.

Since differences in release patterns between the different polymers were obvious, viscosity measurements and rheological characterisation of the polymer solutions is performed to try to explain the origin of this influence.

3.2. Rheological characterisation

3.2.1. Flow measurements

In a preliminary study, polymer solutions with different concentrations were prepared to determine which concentration was appropriate to obtain a viscosity of 100 ± 20 mPa s at 32 °C. The results of the flow measurements are summarised in Tables 3a and 3b, showing the influence of temperature (25 or 32 °C) and sterilisation (before or after autoclaving) on the viscosity of the samples. The best fittings for the analysis of PVA, HEC and poloxamer on the one hand and Carbopol® solutions on

Table 2
Drug release (%) and total AUC (% × hours) of nanoparticles dispersed in polymer solutions or in mannitol ($n = 3$)

Time (h)	Mannitol	PVA	HEC	Pol	CP 974	CP 980	CP 1342
(A) Before γ-irradiation of the freeze-dried polymer solutions							
1	5.44	3.43	6.85	4.51	4.63	8.08	7.55
5	19.73	13.70	20.22	12.48	14.54	23.03	25.59
24	38.27	38.87	33.58	22.14	26.03	30.22	29.35
$AUC_{0\rightarrow 24\ h}$	641.97	565.07	612.89	385.95	454.52	564.84	610.64
(B) After γ-irradiation and reconstitution of the freeze-dried polymer solutions							
1	5.44	3.85	9.33	5.14	5.01	8.34	7.94
5	19.73	14.91	25.00	15.59	20.21	24.17	26.96
24	38.27	39.82	32.25	26.11	32.57	35.3	31.78
$AUC_{0\rightarrow 24\ h}$	641.97	601.86	636.79	449.91	597.04	645.27	680.79

$AUC_{0\rightarrow 24\ h}$, area under the curve.

the other hand were obtained with the Bingham and the Herschel-Bulkley model, respectively. The chosen mathematical models gave a good representation of the real behaviour of the polymer solutions, as can be derived from the determination coefficients in Tables 3a and 3b, which were in all cases larger than 0.998. This indicated a good prediction by the models employed, compared to the registered flow curves of the polymer considered. PVA, HEC and poloxamer solutions can thus be considered as Newtonian systems, except for the fact of possessing a yield stress. Carbopol® solutions on the contrary can be seen as non-Newtonian, since the *n*-value or rate index in Table 3b did not approach one. Sterilisation of the polymer solutions by autoclaving influenced the viscosities obtained. The viscosities of all dispersions after autoclaving and measured at 32 °C, appeared to be in a range between 90 and 120 mPa s.

Table 3a
Flow data of PVA, HEC and poloxamer solutions in 5.07% w/v mannitol, fitted with the Bingham model, stress = $\sigma_y + \eta_p \times$ rate ($n = 2$)

Polymer		σ_y	η_p	R^2
PVA	Before; 32 °C	0.096	0.113	0.99993
	After; 32 °C	0.076	0.110	0.99998
	After; 25 °C	0.024	0.127	0.99999
HEC	Before; 32 °C	0.421	0.132	0.99923
	After; 32 °C	0.235	0.102	0.99964
	After; 25 °C	0.188	0.101	0.99973
POL	Before; 32 °C	0.201	0.101	0.99963
	After; 32 °C	0.062	0.090	0.99997
	After; 25 °C	0.006	0.074	0.99998

σ_y, yield stress (Pa); η_p, plastic viscosity (Pa.s); R^2, determination coefficient; Before, before autoclaving; After, after autoclaving of the polymer solutions.

Table 3b
Flow data of Carbopol® solutions in 5.07% w/v mannitol, fitted with the Herschel-Bulkley model, stress = $\sigma_y + \eta_p \times \text{rate}^n$ ($n = 2$)

Polymer		σ_y	η_p	n	R^2
CP 974	Before; 32 °C	0.002	0.107	0.709	0.99953
	After; 32 °C	0.024	0.120	0.689	0.99852
	After; 25 °C	0.000	0.141	0.679	0.99988
CP 980	Before; 32 °C	0.093	0.088	0.664	0.99921
	After; 32 °C	0.112	0.099	0.659	0.99892
	After; 25 °C	0.014	0.143	0.688	0.99970
CP 1342	Before; 32 °C	0.071	0.083	0.662	0.99938
	After; 32 °C	0.086	0.096	0.661	0.99903
	After; 25 °C	0.097	0.110	0.656	0.99914

σ_y, yield stress (Pa); η_p, consistency (Pa.s)^n; n, rate index; R^2, determination coefficient; Before, before autoclaving; After, after autoclaving of the polymer solutions.

Autoclaving (121 °C, 2 bar) caused a viscosity decrease for PVA, HEC and poloxamer. On the contrary, an increase of the viscosity for the three different CP could be due to a better hydration of the macromolecules because gelification, not completed after the neutralisation, continued due to heating during the sterilisation and the cooling down process [21].

At 25 °C, the temperature at which the in vitro release experiments were performed, the viscosity of PVA, CP 974 and CP 1342 solutions was about 15% higher, of CP 980 45% and in the case of poloxamer, the viscosity was 15% lower compared to the values measured at 32 °C. This temperature change had no influence on the viscosity of the HEC-solution. In the case of poloxamer, the decrease expresses a phase transition from gel to sol, known as the low temperature sol–gel boundary [14]. Lenaerts et al. described a biexponential relation between poloxamer concentration and temperature [25]. At 23 °C and above, the rheological behaviour of poloxamer would change from Newtonian to non-Newtonian, due to sol–gel transition in a temperature interval between 23 and 26 °C [26]. Also in more concentrated solutions (>20%), a shift from Newtonian to pseudoplastic and plastic behaviour occurs [25].

Consequently, viscosity changes will occur when preparations at room temperature are instilled in the *cul de sac* of the eye.

3.2.2. Oscillation rheology

DSS measurements were conducted on all viscosifying agents examined in order to investigate the possible

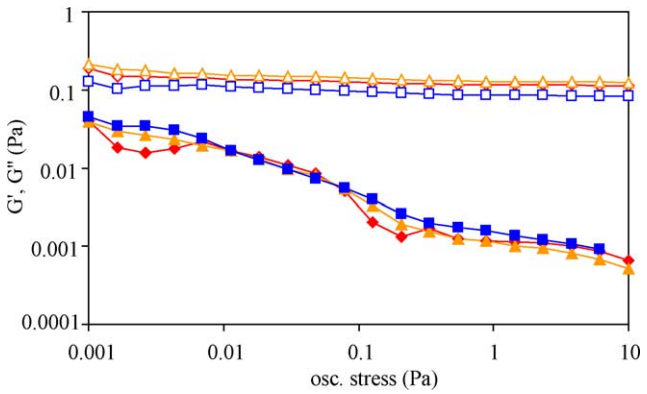

Fig. 3. Dynamic Stress Sweep curves of PVA before (diamonds) and after freeze-drying (triangles) and after gamma-sterilisation (squares). Closed symbols stand for G' (elastic modulus) and open symbols for G'' (viscous modulus).

formation of a network structure. The behaviour of the polymer solutions before and after freeze-drying and after reconstitution of the freeze-dried and subsequent gamma-irradiated polymers was evaluated. The DSS curves (see Figs. 3–8) show the different rheological behaviour of the polymers investigated.

The viscoelastic properties of PVA and poloxamer solutions can be derived from Figs. 3 and 4, respectively, which show the G' (elastic) and G'' (viscous) values as a function of the stress applied. Fig. 5 summarises the DSS measurements of the viscous and elastic moduli of the solutions prepared with freeze-dried, gamma-irradiated PVA, HEC and poloxamer.

The solutions prepared with PVA and HEC were mainly viscous systems because the values of G'' were larger than those of G'. The very small length of the LVER in the plots of HEC and PVA solutions show the absence of interacting chains. Freeze-drying and gamma-irradiation had no influence on the viscoelastic behaviour of PVA or HEC. Hassan et al. mentioned the formation of physical

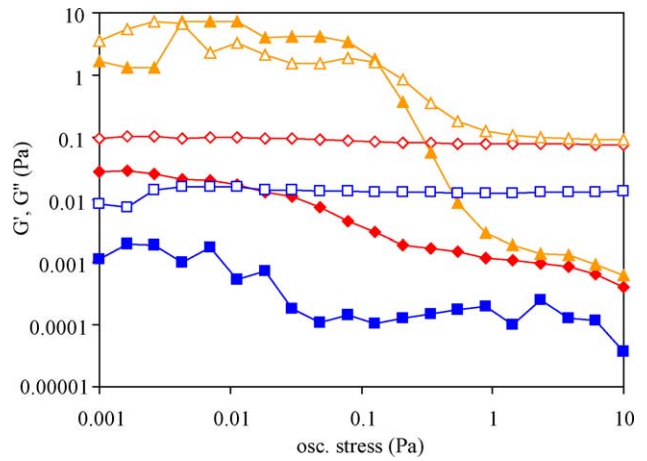

Fig. 4. Dynamic stress sweep of poloxamer before (diamonds) and after freeze-drying (triangles) and after gamma-sterilisation (squares). Closed symbols stand for G' (elastic modulus) and open symbols for G'' (viscous modulus).

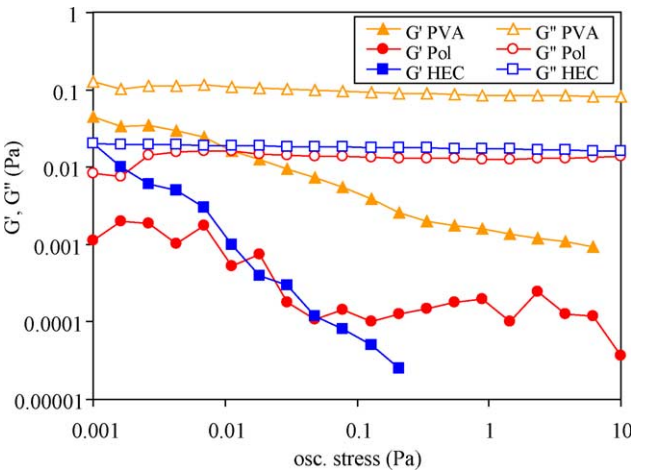

Fig. 5. Dynamic stress sweep curves of PVA, HEC and poloxamer after freeze-drying, gamma-sterilisation and reconstitution. Closed symbols stand for G' (elastic modulus) and open symbols for G'' (viscous modulus).

cross-links in PVA, but after repeated freezing/thawing cycles [27]. Divergent reports have been published about gamma-irradiation of PVA. Zainuddin et al. reported formation of radicals in irradiated PVA powder or its aqueous solution and subsequent cross-linking, while Zhang and Yu investigated the degradation of PVA aqueous solutions by gamma-irradiation with a reduction of the average degree of polymerisation as a consequence, indicating chain scission [28,29]. In the case of HEC, similar effects due to irradiation have been reported, namely cross-linking after irradiation of concentrated aqueous HEC or MC solutions, while polymers exposed to gamma-rays in solid state and in aqueous solution of low concentration underwent degradation. [30].

Poloxamer solutions were characterised by small G'- and large G''-values, pointing to a mainly viscous polymer solution containing a network stabilised by physical entanglements (see Fig. 4). Lenaerts et al. explained the behaviour of poloxamer solutions by the existence of a network consisting of weak interactions, probably due to hydrogen bonds [25]. For all preparations, the value of G' being larger or equal to G'' in the low stress region indicated that secondary bonds were formed between the polymer molecules. Since both viscous and elastic moduli were present, poloxamer solutions or gels can be considered as viscoelastic.

From Fig. 5, which shows all polymers with mainly viscous properties, it can be observed that the main difference between poloxamer on the one hand and PVA and HEC on the other hand was the length of the LVER, which was longer in the case of poloxamer, indicating that this polymer has more pronounced elastic properties than PVA or HEC.

The influence of freeze-drying and gamma-sterilisation on the viscoelastic behaviour of a CP 980 solution can be derived from Fig. 6. The viscoelastic properties of all Carbopol® solutions are summarised in Fig. 7 (before

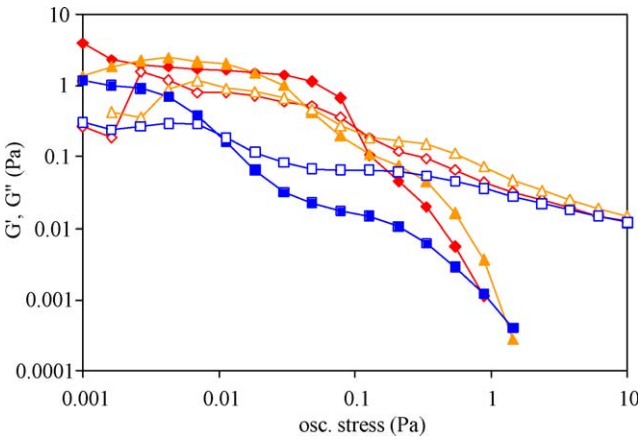

Fig. 6. Dynamic stress sweep curves of CP 980 before (diamonds) and after freeze-drying (triangles) and after gamma-sterilisation (squares). Closed symbols stand for G' (elastic modulus) and open symbols for G'' (viscous modulus).

freeze-drying of the polymers) and Fig. 8 (after reconstitution of the freeze-dried gamma-sterilised polymers). From Figs. 7 and 8, it can be concluded that all Carbopol® solutions had mainly elastic properties and consisted of cross-linked networks with secondary bonds (elastic modulus G' larger than viscous modulus G'') [18]. The stress sweep curves in Fig. 7 demonstrated that the solution prepared with non-freeze-dried CP 980 behaved most elastically, in contrast to those prepared with CP 974 and CP 1342 which had the least and intermediate elastic behaviour, respectively. The changes in elasticity allowed differentiation between the behaviour of the different CP-solutions. According to Zhu et al. and Jabbari and Nozari, gamma-irradiation of polyacrylic acid aqueous solutions can cause formations of free radicals, followed by cross-linking, recombination or chain scission [31,32]. The G' value of CP 974 increased after freeze-drying, while gamma-irradiation had no influence (graph not shown). In

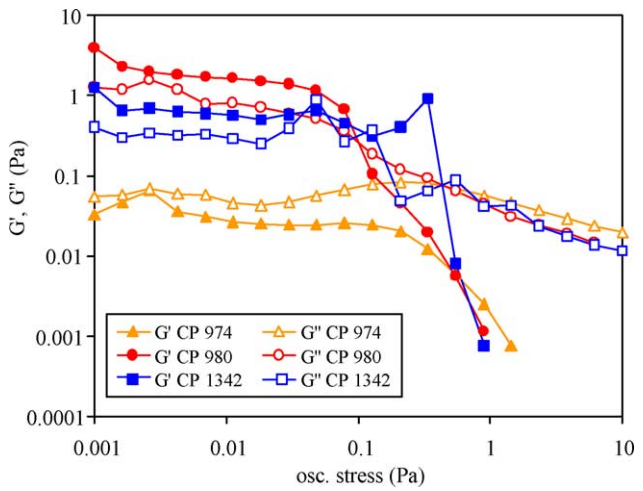

Fig. 7. Dynamic stress sweep curves of Carbopol® before gamma-irradiation. Closed symbols stand for G' (elastic modulus) and open symbols for G'' (viscous modulus).

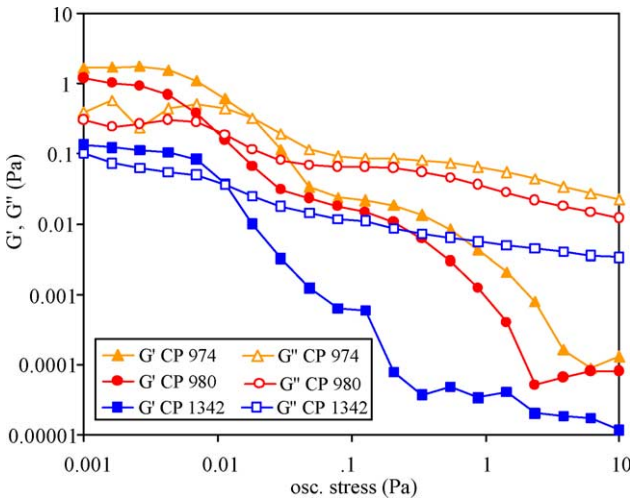

Fig. 8. Dynamic stress sweep curves of Carbopol® after freeze-drying, gamma-sterilisation and reconstitution. Closed symbols stand for G' (elastic modulus) and open symbols for G'' (viscous modulus).

the case of CP 980, the behaviour was opposite as can be seen from Fig. 6: there was no influence of freeze-drying, but the elastic modulus decreased after gamma-sterilisation. In the case of CP 1342, like for poloxamer, freeze-drying and gamma-irradiation led, respectively, to an increase and a decrease in elasticity, compared to the elasticity before freeze-drying (graph not shown). According to the data obtained from the manufacturer, gamma-irradiation may sometimes result in an increase of the viscosity of Carbopol® solutions or gels, but Deshpande and Shirolkar described gamma-irradiation (2.5 Mrad) of ophthalmic sustained release formulations of pilocarpine and showed that this dose may be used for sterilisation of Carbopol® gels without significant impact [33]. CP 980 seemed to be more resistant to freeze-drying followed by gamma-irradiation treatments than CP 1342.

After the selection of an oscillation stress value from the LVER, dynamic frequency sweeps (DFS) were recorded only for Carbopol®, since the LVER obtained in the stress sweep measurements of the non-elastic polymers was too small (see Fig. 5).

In DFS curves of CP, the G' value was larger than the G'' value and both moduli were independent of the frequency (graphs not shown). The slope values of the curves of G' or G'' versus frequency (log–log plots) are given in Table 4. It can be concluded that all three kinds of CP behaved as (weak) gels, since the values approximated zero. From the frequency sweep data, one can deduce that secondary bond formation in CP 974, 980 and 1342 solutions was present, because the values of the elastic moduli G' were larger than the viscous moduli G'', and both were independent of the frequency. Before freeze-drying, elasticity of CP 980 was larger than that of CP 1342, while CP 974 had the lowest elasticity (see Fig. 7). After freeze-drying, CP 974 behaved most elastically and this behaviour did not change after gamma-irradiation. The elasticity of CP 974 increased after

Table 4
Mean values of slopes of DFS (dynamic frequency sweep) curves (log G' or G'' versus log ω) of Carbopol® solutions ($n = 2$)

	Before freeze-drying		After freeze-drying		After γ-sterilisation	
	Slope (G')	Slope (G'')	Slope (G')	Slope (G'')	Slope (G')	Slope (G'')
CP 974	0.03	0.05	0.10	0.14	0.07	0.09
CP 980	0.37	0.24	0.04	0.04	0.01	0.03
CP 1342	0.04	0.06	0.03	0.06	0.01	0.02

freeze-drying but decreased to the start value of 0.30 Pa after gamma-irradiation. In the case of CP 980 and CP 1342, elasticity before and after freeze-drying was comparable and larger than after gamma-sterilisation. Thus, sterilisation procedures resulted in a decrease of elasticity of the polymer network.

3.3. Influence of rheological properties on drug release patterns

A possible correlation between the drug release rates and the rheological behaviour of the various polymers examined was investigated. Eventual differences are only due to the polymers, since the nanoparticles used have all undergone the same treatment and are thus not a variable factor.

From Fig. 1 and Table 2, it can be deduced that before gamma-irradiation of the polymers, nanoparticles dispersed in HEC and PVA solutions, the most viscous polymers, and CP 1342 and CP 980 solutions, the most elastic polymers, showed the highest ciprofloxacin release rates. Thus, the rheological behaviour (viscous or elastic) of some polymers tested did not seem to influence the release significantly. Only in poloxamer and CP 974 the slowest release rates were measured.

The *P*-values of the Newman-Keuls test, presented in Table 5, indicate that release in poloxamer solution was significantly different from all polymer solutions used and from the reference mannitol solution. The $AUC_{0\rightarrow 24\ h}$ value for poloxamer was significantly different from HEC, CP 980, CP 1342 and mannitol solutions, but not from CP 974 and PVA solutions. From the rheological study, it can be derived that poloxamer is viscoelastic with an elastic modulus G' being smaller than the viscous modulus G'', so the viscous properties are more pronounced than the elastic. This rheological behaviour differs from the elastic Carbopols® ($G' > G''$) and from the viscous PVA and HEC solutions, which show no elastic properties at all. For poloxamer 15–30% aqueous solutions, Lenaerts et al. stated that the created hydrogels showed a high porosity which had practically no influence on the diffusion of some model-drugs [25]. In our study, however, poloxamer had a marked influence on the drug release patterns. But another explanation for the slow release rate measured using a diffusion cell could be the solubilisation of ciprofloxacin in the micellar poloxamer solution. Therefore, the solubility of ciprofloxacin was determined in mannitol 5.07% (w/v), in water and in poloxamer 15.10% (w/v) solution, but no significant difference was measured.

Drug release in CP 974 solution differed from all other solutions employed, except from PVA solution (see Table 5), but the $AUC_{0\rightarrow 24\ h}$ value did not change significantly when compared to the non-viscous mannitol solution or to the other polymers employed, except for poloxamer. Since the zeta potential value also changed when CP 974 was used, this can be due to adsorption of the polymer at the nanoparticles' surface, which can also influence the drug release properties. Drug release and $AUC_{0\rightarrow 24\ h}$ values for CP 980 and 1342 were not significantly different from mannitol solution. Carbopol® 974 has, according to the manufacturer, a higher cross-linking density than CP 980. This could explain why a higher drug diffusion and release rate were measured when CP 980 instead of CP 974 was used as viscosifying agent.

Camber and Lundgren showed that sodium hyaluronate (HA), a pseudoplastic polymer used as an additive in ophthalmic formulations, did not affect the diffusion rate for

Table 5
P-values (Newman-Keuls test) of differences in drug release before gamma-irradiation

	Pol	CP 974	PVA	CP 980	CP 1342	HEC	Mannitol
Pol		0.0495	0.0037	0.0002	0.0002	0.0002	0.0002
CP 974	0.0495		NS	0.0344	0.0251	0.0159	0.0272
PVA	0.0037	NS		NS	NS	NS	NS
CP 980	0.0002	0.0344	NS		NS	NS	NS
CP 1342	0.0002	0.0251	NS	NS		NS	NS
HEC	0.0002	0.0159	NS	NS	NS		NS
Mannitol	0.0002	0.0272	NS	NS	NS	NS	

NS, not significant ($P > 0.05$).

low molecular weight compounds, since the relatively small volume occupied by the HA network in the solution allowed the low molecular weight drugs to diffuse independently of the concentration of HA [34]. This could explain why only insignificant differences in release rates and $AUC_{0\rightarrow 24\ h}$ values were observed between dispersions of nanoparticles in non-viscous mannitol solution versus the viscous polymer solutions PVA and HEC and the elastic CP 980 and 1342.

After dispersion of the nanoparticles in reconstituted PVA, HEC, CP 974, 980 and CP 1342 solutions (after freeze-drying and gamma-irradiation), similar drug release rates and $AUC_{0\rightarrow 24\ h}$ values as in mannitol solution were measured (see Fig. 2 and Table 2), while the reconstituted polymer solutions exhibited different rheological properties: CP being elastic while PVA and HEC exhibiting a viscous behaviour. Also under these experimental conditions the different rheological behaviour of some viscosifying agents did not play a significant role. Only a higher release due to decreased viscosity of the reconstituted polymer solutions was observed. According to the Newman-Keuls test, only the release of ciprofloxacin out of the nanoparticles dispersed in viscoelastic poloxamer was significantly slower and the $AUC_{0\rightarrow 24\ h}$ value lower compared to the other polymers tested. This can be explained by differences in rheological behaviour: poloxamer is viscoelastic with mainly viscous properties, while PVA and HEC are viscous and CP 974, 980 and 1342 elastic.

For poloxamer, the release percentages ($P = 0.043$), but not the $AUC_{0\rightarrow 24\ h}$ value, differed significantly after gamma-irradiation. The increased release percentages could be due to the decreased G'' modulus.

Drug release percentages ($P = 0.001$) and $AUC_{0\rightarrow 24\ h}$ value ($P = 0.044$) of CP 974 after gamma-irradiation were higher than before (ANOVA analysis). Freeze-drying and gamma-sterilisation had an influence on the polymer structure and consequently the rheological properties of CP 974 because both the viscous and the elastic modulus have increased in comparison with the moduli before freeze-drying. These changes could be responsible for the drug release increase.

No significant influence of gamma-irradiation on drug release rates and $AUC_{0\rightarrow 24\ h}$ values was seen for CP 980 or 1342, while both G' and G'' values decreased. The difference between the viscoelastic behaviour before and after irradiation was however less pronounced than with CP 974 (see Figs. 7 and 8).

Regarding HEC and PVA, little differences in release after freeze-drying and gamma-sterilisation were observed. This can be explained since no differences in their viscous behaviour occurred only a decrease of G'' value due to chain scission during gamma-sterilisation. The small influence of freeze-drying is in agreement with Hassan et al., who reported that drug release characteristics in PVA hydrogels, prepared with repeated freezing/thawing cycles, did not change, although differences in PVA dissolution amounts and rates were observed [35].

From the results obtained, it can be deduced that the choice of the viscosifying agent influences the drug release percentages and AUC value, but when extrapolating these results to an in vivo situation, one has to keep in mind that the viscosity behaviour of the polymers is different at the temperature of the eye surface (32 °C) compared to the instillation temperature (25 °C) and that blinking causes a viscosity decrease in non-Newtonian systems.

4. Conclusions

Ciprofloxacin-incorporated PLGA nanoparticles could be prepared with reproducible size and drug loading characteristics. By adding mannitol and by mixing with viscosifying agents, nanoparticles with various zeta potential values were obtained. Flow measurements demonstrated that the rheological behaviour of PVA, HEC and poloxamer solutions was Newtonian, only the three Carbopol® solutions behaved as non-Newtonian systems. From oscillation measurements, it could be derived that CP 974, CP 980 and CP 1342 were mainly elastic and consisted of physically cross-linked networks with secondary bonds. However, after freeze-drying and gamma-sterilisation, PVA, HEC, CP 974, CP 980 or CP 1342 caused similar and comparable drug release profiles from the nanoparticles. When the porosity of the weak gel network structure of Carbopols® was high, drug release was controlled only by the nanoparticle formulation itself. Only in the case of poloxamer, a prolonged slow release was observed, due to differences in viscoelastic behaviour. Thus, depending on the concentration of antibiotic required in the tear film the most adequate polymer should be selected.

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