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Polyhydroxyethylaspartamide-based micelles for ocular drug delivery

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ABSTRACT

In this paper three copolymers of polyhydroxyethylaspartamide (PHEA), bearing in the side chains polyethylene glycol (PEG) and/or hexadecylamine (C_{16}) (PHEA-PEG, PHEA-PEG-C_{16} and PHEA-C_{16} respectively) have been studied as potential colloidal drug carriers for ocular drug delivery. The physical characterization of all three PHEA derivatives, using the Langmuir trough (LT) and micellar affinity capillary electrophoresis (MACE) techniques allowed to assume that whereas alone PHEA backbone is an inert polymer with respect to the interactions with lipid membranes and drug complexation, when PHEA chains are grafted with long alkyl chains like C_{16} or in combination C_{16} chains and hydrophilic chains like PEG, copolymers with lipid membrane interaction ability and drug complexation capability are obtained.

In vitro permeability studies performed on primary cultured rabbit conjunctival and corneal epithelia cells, using PHEA-C_{16} and PHEA-PEG-C_{16} as micelle carriers for netilmicin sulphate, dexamethasone alcohol and dexamethasone phosphate, demonstrated that in all cases drug loaded PHEA-C_{16} and PHEA-PEG-C_{16} micelles provide a drug permeation across ocular epithelia greater than simple drug solutions or suspensions. In particular PHEA-PEG-C_{16} acts as the best permeability enhancer in our experimental model. In vivo bioavailability studies conducted with PHEA-PEG-C_{16} micelles loaded with dexamethasone alcohol, confirmed that this system also provides a drug bioavailability greater in comparison with that obtained with water suspension of the same drug after ocular administration to rabbits.

1. Introduction

Ocular drug delivery is one of the most challenging topic faced by pharmaceutical researchers because of the difficulties met in eye drug administration, due to the specific anatomy, physiology and biochemistry of this organ, that make it practically inaccessible to extraneous compounds (Dey and Mitra, 2005; Koevary, 2003; Felt et al., 1999). For these reasons many efforts have been done to identify new drug delivery systems able to enhance ocular drug permeation and to control drug delivery release rate, keeping however high attention to high ocular tolerability and patient compliance (Felt et al., 1999; Duvvuri et al., 2003; Meqi and Deshpande, 2002).

In this contest stricking advantages can be given by nanotechnology whose use in last 10 years has been becoming more and more wide in pharmaceutical field with a global market expected of $3 billion within next 5 years (Sahoo et al., 2008; Del Amo and Urtii, 2008). In effect, although if with peculiarities concerning anterior or posterior eye segments, pharmaceutical nanotechnologies including nanosuspensions, solid lipid nanoparticles, liposomes and polymeric micelles can allow to overcome some of the inconveniences of conventional ocular drug delivery and, in many cases, improve water solubility of poorly soluble drugs and their chemical stability (Kayser et al., 2005). Among pharmaceutical nanotechnologies, to date not many investigations have been performed on ocular drug delivery by polymeric micelles, despite their increasing interest in drug parenteral administrations (Del Amo and Urtii, 2008).

Polymeric micelles are self-assembling colloidal systems obtained by assembling of block or graft amphiphilic copolymers (Matsumura, 2008; Torchilin, 2001). These systems seems to be very promising in ocular drug delivery for many peculiarities, including their high kinetic and thermodynamic stability, able to give a slow drug release and the ability to act as absorption promoters that can improve drug permeability across ocular epithelia (Torchilin, 2001; Harada and Kataoka, 2006).

From several years the ability to act as parenteral drug delivery systems of polyhydroxyethylaspartamide (PHEA) copolymers based micelles have been investigated in our laboratory. PHEA is a synthetic polymer having protein-like structure, obtained by the reaction of ethanolamine with polysuccinimide (PSI), itself prepared by thermal polycondensation of D,L-aspartic acid (Giammona et al., 1987). PHEA has good biopharmaceutical properties as drug carrier material such as high water solubility, multifunctionality, biocompatibility, low cost of production (Caliceti et al., 2001; Mendichi et al., 2003; Cavallaro et al., 2003, 2004).

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In a previous paper we reported the synthesis and biopharmaceutical characterization of amphiphilic graft derivatives of PHEA by introducing hydrophilic PEG of 2000–5000 Da and/or hydrophobic hexadecylalkyl groups (C_{16}) obtaining PHEA-PEG-C_{16} or PHEA-C_{16} copolymers (Caliceti et al., 2001). The synthesised graft amphiphilic copolymers have demonstrated to form polymeric aggregates in aqueous medium with size ranging from 10 to 30 nm and aggregation number between 8 and 20 (Mendichi et al., 2003) besides an high power to solubilize and incorporate hydrophobic drugs (Cavallaro et al., 2003, 2004) and the ability to influence drug distribution after parenteral administration (Caliceti et al., 2001).

In this paper the possibility to use polymeric micelles based on PHEA-PEG-C_{16} or PHEA-C_{16} copolymers for ocular drug delivery has been investigated. In particular interfacial properties of PHEA derivatives were evaluated by Langmuir trough technique and the extent of the interactions between micelles and a model drug (such as dexamethasone sodium phosphate) was estimated by the micellar affinity capillary electrophoresis (MACE). In vitro permeability studies across the ocular epithelia were performed by using PHEA-C_{16} and PHEA-PEG-C_{16} micelles containing hydrophobic drugs such as dexamethasone alcohol and hydrophilic drugs such as dexamethasone sodium phosphate and netilmicin; finally in vivo ocular bioavailability studies of dexamethasone loaded PHEA-PEG-C_{16} micelles were performed on rabbits.

2. Experimental

2.1. Chemicals

PHEA and derivatives were synthesised according to a previously reported procedure (Giammona et al., 1987; Caliceti et al., 2001).

Phosphate buffer saline (PBS) at physiological osmolality and pH 7.3 for LT experiments, anhydrous disodium phosphate and sodium phosphate monobasic for capillary electrophoresis were obtained from Merck. Dipalmitoyl phosphatidylcholine (DPPC) from Sigma and chloroform from Fluka. Dexamethasone sodium phosphate was supplied by Aventis; dexamethasone alcohol, netilmicin were obtained from SIFI SpA, Italy.

Dulbecco's modified Eagle medium (DMEM) with Ham's nutrient mixture F12 (1:1) were purchased from Sigma–Aldrich (Italy). 10% Fetal calf serum (FCS) and trypsin from BIOCHROM-Seromed, 10 ng/ml human recombinant epidermal growth factor (rHuEGF) from Intergen-BioSpa; 2 μg/ml hydrocortisone, 5 μg/ml bovine insulin, 0.1 μg/ml cholera toxin, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptopmycin, 50 μg/ml gentamicin and collagen I from rat tail from Sigma–Aldrich. Dispase II from Boeringher–Mannheim.

2.2. Apparatus

^{1}H NMR spectra were recorded in D_2O (Aldrich) using a Bruker Avance II 300 spectrometer operating at 300 MHz.

FT-IR spectra were performed by using Perkin Elmer 1720.

UV spectroscopy was performed by Shimadzu UV160U spectrophotometer.

Weight average molecular weight of PHEA and of all its copolymers were measured by size exclusion chromatography (SEC) by using for PHEA-PEG copolymer 0.15 M NaCl water solution as mobile phase, 35 °C, 0.8 ml/min and two Ultrahydrogel columns from Waters (1000 and 250 Å of pore size) and for PHEA-C_{16} and PHEA-PEG-C_{16} by using dimethylacetamide (DMAc) as mobile phase, 80 °C, 0.6 ml/min and two mixed Jordigel GBR columns from Jordi (Bellingham, MA, USA).

Poly(ethylene oxide) standards (range 145–1.5 kDa) were used to obtain the calibration curve.

2.3. Langmuir trough (LT)

The experiments were carried out on a LT device KSV minitrough and Whilelmy Pt plate probe at $T = 20 \pm 1$ °C. Lipid monolayers were formed at the required surface pressure by dispensing measured volumes of DPPC chloroform solution on the aqueous surface from Hamilton high precision syringes. Dynamic area versus pressure experiments were performed by reducing the distance between Teflon barriers at a rate corresponding to a variation of 1.0 cm^2/min.

2.4. MACE

All the experiments were performed on a Hewlett Packard Model ^{3D}CE system with diode-array detector from 190 to 600 nm. The wavelength of the UV detector was set at 242 nm. A polyvinyl alcohol (PVA) coated capillary obtained from Agilent with 64.5 cm of total length (56 cm effective length) and 50 μm of internal diameter was used. The analysis was performed in the cathodic mode (detector at anode) applying a potential of −30 kV.

The capillary was rinsed with the running buffer for 60 min at the beginning of the analytical session and 5 min after each sample injection. Experiments were repeated at 25 and 37 °C.

Standard aqueous solution of dexamethasone sodium phosphate was prepared at 200 μg/ml and hydrodynamically injected into the apparatus at 50 mbar for 5 s.

Running buffer was a 50 mM phosphate buffer at pH 7.2 ± 0.1 to which different concentrations of the studied polymers in a range of 0.4–10 mg/ml were added.

Buffer solutions were filtered through a 0.22 μm filter and ultrasound degassed for 10 min before using. Experiments were carried out at two different temperatures (25 and 37 °C).

2.5. Bovine conjunctival epithelial cell (BCEC) culture protocol

BCEC methodology culture has been previously described (Civiale et al., 2003). Briefly, bovine eyes obtained from local slaughterhouses were used as the source of primary conjunctival and corneal epithelial cells. The whole bulbs were kept 30 min in 2% povidone solution in PBS to assure sterile conditions. Thereafter, fragments of bulbar conjunctiva were incubated in a dispase solution (2 mg/ml) for 1 h at 37 °C under orbital shaking, so achieving the separation of the epithelia from the stroma. The remaining tissue was cut in small pieces and incubated in trypsin–EDTA solution for 30 min, filtered and centrifuged at 1000 rpm for 5 min. Complete growth medium containing 10% FCS was added to the final pellet.

Conjunctival cells were plated at a density of 0.5×10^6 cells/cm^2 on Transwell® inserts (1.13 cm^2) coated with collagen in 12-well plates and bathed on the bottom-side with 1.5 ml of medium and 0.5 ml in the inside filter. The cells were allowed to adhere for 48 h at 37 °C in humidified atmosphere. Then the cells were washed with Hanks' balanced salt solution (HBSS). The medium was replaced daily until the cultures reached subconfluence. Plates were grown under immersion to subconfluence and then shifted to air-lifted conditions (medium only in the bottom-side of the insert) and fed daily. Conjunctival cells were used for permeation experiments 9 days after plating when transepithelial electrical resistance (TEER) was 4 ± 1 kΩ cm^2. TEER was also measured after each experiment to gauge the integrity of the cell layers.

2.6. Bovine corneal epithelial cells (BCoEC) culture protocol

To isolate and culture corneal ephitelial cells the above described procedure slightly modified was followed. Bovine eyes resided 20 min in the povidone solution and then the solution of dispase (2 mg/ml) was injected between the corneal epithelium and the stroma of each eye assuring the covering of the whole corneal sur-

face. The bovine eyes were left for 45 min in povidone solution and then rinsed with PBS and antibiotic. The epithelium was enzymatically separated from the stroma and it could be detached taking each eye with the cornea side up with a sterile forceps. The epithelium fragments were then left in a trypsin solution at 37 °C for 30 min. After centrifugation (5 min at 1000 rpm), the supernatant was discarded and replaced with a complete medium containing 10% FCS. The cells suspension was finally filtered through 40 μm filter (Falcon), plated and cultured analogously to conjunctival cells. Corneal cell growth permits to leave the cultures in air-lifted conditions.

TEER was measured after each experiment to gauge the integrity of the cell layers.

2.7. Preparation of micelle samples

For in vitro cell permeation experiments the micelle samples containing the tested drugs were prepared the same day of the experiment, mixing together the polymer and the drug in a homogeneous dry mixture and subsequently adding the correct amount of distilled water under stirring. The obtained solution was then sonicated and lyophilised as previously published (Cavallaro et al., 2004).

Two types of micelles were prepared for each drug such as dexamethasone alcohol (dexOH) dexamethasone sodium phosphate (dexNaP) and netilmicin sulphate (netSO):

(a) dexOH/PHEA-C_{16}, corresponding to 4.85% by weight of drug content at a polymer concentration of 161 μg/ml;
(b) dexOH/PHEA-PEG-C_{16}, corresponding to 3.77% by weight of drug content at a polymer concentration of 218 μg/ml;
(c) dexNaP/PHEA-C_{16}, corresponding to 50% by weight of drug content at a polymer concentration of 10.5 μg/ml;
(d) dexNaP/PHEA-PEG-C_{16}, corresponding to 50% by weight of drug content at a polymer concentration of 10.5 μg/ml.
(e) netSO/PHEA-C_{16}, corresponding to 50% by weight of drug content at a polymer concentration of 10.5 μg/ml;
(f) netSO/PHEA-PEG-C_{16}, corresponding to 50% by weight of drug content at a polymer concentration of 10.5 μg/ml.

Before using micelles were diluted in an adequate volume of modified Ringers' solution (GBR) to obtain the desired drug concentration. This solution contained 116 mM NaCl, 5.6 mM KCl, 0.8 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1.8 mM $CaCl_2{\cdot}2H_2O$, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, 15 mM HEPES and 0.075 mM bovine serum albumin (Civiale et al., 2004). The final drug concentrations in GBR were 0.02 mM for dexamethasone alcohol or phosphate and 6.3 mM for netilmicin sulphate (corresponding to 0.3% (w/v) of netilmicin base) (donor solutions).

2.8. Drug permeability studies

The cell layers were first washed with HBSS (with or without Ca^{2+}), then incubated for 15 min with a modified Ringers' solution (GBR). The reference solutions were constituted of 0.02 mM of dexamethasone alcohol or phosphate in GBR and 0.8% DMSO (to enhance dexamethasone alcohol solubility) at pH 7.4. This medium was not toxic to the cells. The netilmicin reference solution was constituted of 6.3 mM of netilmicin sulphate in GBR at pH 7.4. Concerning permeability studies, a methodology already described in the literature (Civiale et al., 2004) was used. Briefly, culture cells were incubated with donor and reference solutions for 120 min at 25 or 37 °C under orbital shaking at 50 rpm. Acceptor compartments were sampled (500 μl) at 30, 60, 90 and 120 min, by replacing with fresh buffer without drug to ensure sink conditions. All micelle samples were tested with at least five replicate multilayers.

Permeated dexamethasone alcohol or phosphate concentrations were quantified by HPLC (Hewlett-Packard 1100 system) using a Hypersil ODS 5 μm, 4,6/125 mm column with a pre-column H5ODS-10CS; the UV detection wavelength was 242 nm (detector HP 1100 UV) and the mobile phase was MeOH/KH_2PO_4 0.05 M (6.8 g/l), 53%/47% pH 6, flow = 1 ml/min, injection volume: 100 μl, the retention time of dexamethasone alcohol was 12.5 min and of dexamethasone sodium phosphate was 5.9 min.

The permeated netilmicin concentration was determined by the same HPLC system using a LUNA C18, 5 μm, 4,6/250 mm column with a pre-column H5ODS-10CS; the UV detection wavelength was 330 nm (detector HP 1100 DAD) and the mobile phase was CH_3CN/KH_2PO_4 0.05 M (pH 7.3), 30%/70%, flow = 1 ml/min, injection volume: 100 μl, the retention time of netilmicin was 8.3 min.

The regression lines (peak area vs. obtained concentration) determined from the analyses of serial standards were generated to quantify the dexamethasone alcohol, dexamethasone phosphate and netilmicin sulphate concentrations in the acceptor chamber. Cumulative corrections were made for the previously removed samples in determining the total permeated amount.

2.9. Data analysis

The apparent permeability coefficients (P_{app}, cm/s) were calculated from the linear portion of a plot of the cumulative amount of drug appearing in the receiver fluid versus time.

P_{app} values were calculated according to the following equation:

$$P_{app} = \frac{dQ/dt}{C_0 A}$$

dQ/dt: observed flux in nM/s for drug. A: surface of the filter membrane (1.13 cm^2); C_0: the initial drug concentration in the donor fluid.

Statistical tests were used to determine the level of significance between the data, namely an analysis of variance, one way ANOVA plus the Bonferronis' multiple comparison Test or Dunnetts' multiple comparison test (P value) models.

2.10. In vivo studies

2.10.1. Animals

Male albino rabbits, weighing 1.8–2.5 kg, used throughout the study were purchased from Charles River (Italy). Prior to experiments, the rabbits were housed in standard cages and allowed free access to food and water. The experiments done on rabbits were conform to the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23) and the ARVO Resolution on the Use of Animals in Research.

In vivo studies were carried out treating rabbits with a single administration of 50 μl of PHEA-PEG-C_{16} micelle solution containing dexamethasone alcohol (0.1% w/v) (prepared as previously reported) in the conjunctival lower sac. The vehicle was a phosphate buffer isotonic and at pH 7.3. After euthanasia, samples of aqueous humour were taken in a time course of 30–180 min. The fluid was purified from proteins by precipitation with methanol and after centrifugation the clear supernatant was analysed by HPLC according to protocol above reported for drug permeability studies.

3. Results and discussion

PHEA copolymers were synthesized by reaction of a starting polysuccinimide (PSI) with PEG_{5000}-NH_2 obtaining PSI-PEG_{5000}. Subsequently, PHEA-PEG-C_{16} copolymer was prepared by reaction of PSI-PEG_{5000} with hexadecylamine and subsequent reaction with ethanolamine. PHEA-C_{16} was prepared by the reaction of PSI with

Table 1
Molecular characterization data of the PHEA copolymers.

Polymer	Degree of derivatization[a] (% moles/polymer repeating units)		Weight average molecular weight (kDa)	Polydispersity index[d]
	C_{16}	PEG_{5000}		
PHEA	–	–	42.0[b]	1.71
PHEA-PEG	–	1.8	33.0[b]	1.61
PHEA-C_{16}	8.0	–	32.0[c]	1.78
PHEA-PEG-C_{16}	7.7	2.5	36.6[c]	1.84

[a] Determined by ^{1}H NMR comparing the integral of a peak attributable to linked derivatizing groups and the integral of a peak assigned to starting polymeric backbone by the following ratio: DD = (polyethyleneglycol groups or hexadecyl groups/polymer repeating units) × 100 mol.
[b] Determined by size exclusion chromatography (SEC) using 0.15 M NaCl water solution as mobile phase, 35 °C, 0.8 ml/min and two Ultrahydrogel columns from Waters (1000 and 250 Å of pore size).
[c] Determined by size exclusion chromatography (SEC) using DMAc as mobile phase, 80 °C, 0.6 ml/min and two mixed Jordigel GBR columns from Jordi (Bellingham, MA, USA).
[d] Polydispersity index is equal to Mw/Mn.

hexadecylamine and subsequent reaction with ethanolamine, following the procedure elsewhere reported (Giammona et al., 1987; Caliceti et al., 2001). The self-assembling ability of PHEA-PEG-C_{16} copolymers and PHEA-C_{16} into micelles has been already showed in our previous investigations, together with the ability of these micelles to increase the water solubility of poorly soluble drug and drug bioavailability after intravenous administration (Caliceti et al., 2001; Mendichi et al., 2003; Cavallaro et al., 2003, 2004). In Table 1 all data concerning the physico-chemical characterisation of PHEA copolymers used in this study are reported.

As it can be seen, molecular weight of copolymers were minor than PHEA, despite the attachment of PEG and C_{16} chains; this because, under the used experimental synthesis conditions, a certain degradation in the polymeric backbone occurs (Caliceti et al., 2001; Mendichi et al., 2003; Cavallaro et al., 2003, 2004). Moreover in order to better compare the behaviour of PHEA-PEG-C_{16} or PHEA-C_{16} based micelles, as ocular drug delivery, similar hydrophobic derivatization degrees were considered for both copolymers. Finally PHEA-PEG copolymer, without hydrophobic chains, was also considered in our studies in order to evidence the role of hydrophobic portion in the self-assembling properties of PHEA copolymers. PHEA-C_{16} and PHEA-PEG-C_{16} copolymers showed a good loading capacity towards the hydrophobic drug used in this study (i.e. dexametasone-OH), similar to that reported for others hydrophobic drugs (Cavallaro et al., 2004). Moreover, the obtained micelles showed dimension ranging from 19 to 23 nm in the case of PHEA-PEG-C_{16} and 10–13 nm in the case of PHEA-C_{16} copolymer. These data are in good agreement with those already published (Cavallaro et al., 2004).

3.1. Evaluation of interfacial properties of PHEA derivatives by Langmuir trough technique

The LT monolayer technique is a fully in vitro approach for the simulation of cellular membrane interactions with extracellular matrix. It was taken for a preliminary screening before working with living cells and observing biological parameters in situ. In fact, a phospholipid monolayer spread at the air/aqueous interface reproduces the biological membrane with which the molecules interact from an aqueous solution (Maget-Dana, 1999). Phospholipid monolayers as model membranes display homogeneity, stability and planar geometry: the LT monolayer technique is therefore a suitable model to study the interactions of a hydrosoluble polymer dissolved in the extracellular medium with the membrane surface of the target cell. Besides this technique is quite similar to that used to study drug molecular transport across biomembrane model (Maget-Dana, 1999; Castelli et al., 2004).

The experiments on LT need the proper choice of a number of parameters including nature and packing of the lipid molecules, composition of the subphase and temperature.

The interaction is measured maintaining constant the film area and measuring the surface pressure shifts on adding the penetrating compound to the subphase. The surface pressure is defined as the difference between the surface tension of the air/subphase interface (γ_o) and the surface tension of the insoluble monolayer (γ_i).

$$\Pi_\tau = (\gamma_o - \gamma_\tau) \quad (1)$$

Herein, the insoluble monolayer is a phospholipid monolayer and the penetrating compound is PHEA or one of its derivatives. The parameters that are taken into account are:

1. the initial pressure of the lipid film, Π^i which reflects the packing of the lipids in the monolayer;
2. the concentration, C, of the polymer in the subphase;
3. the maximum change, $\Delta\Pi^{lip}$ in surface pressure of the lipid film upon interaction with the polymer dissolved in the subphase.

The results are usually reported by plotting the interaction pressure $\Delta\Pi^{lip}$ versus Π^i.

The lipid film pressure at which the polymer no longer penetrates ($\Delta\Pi^{lip} = 0$) is defined as exclusion pressure, $\Delta\Pi^{ex}$, that is indicative of the extent of the lipid/surface active polymer interactions.

Surface tension at PBS air interface was measured for the PHEA copolymers and results are shown in Fig. 1. The fall of surface tension caused by the inlet of molecules at the air/aqueous interface, is indicative of the surface activity of the polymers. The grafted copolymers steeply decrease the surface tension of the solution until a minimum surface tension is reached. More precisely, PHEA-PEG surface tension stabilises at concentration lower than 10 μg/ml while PHEA-C_{16} and PHEA-PEG-C_{16} reaches the plateau at considerably higher concentration (350 μg/ml). The difference between PBS surface tension (73 mN/m) and the plateau value of polymer solutions surface tension is the limiting surface pressure. Surface pressures are 17 and 19 mN/m for PHEA-C_{16} and PHEA-PEG-C_{16} and approximately 10 mN/m for PHEA-PEG. PHEA showed little surface activity and a limiting surface pressure of 3 mN/m was reached.

It can be speculated that self-assembling aggregate formation occurs in correspondence of the plateau region or earlier; therefore from the surface tension data a region in which there are polymer aggregates in solution may be clearly identified in correspondence of the plateau region of concentrations.

One additional information is provided by the comparison of the surface tension after a compression–expansion cycle of the air/aqueous interface where the polymer is adsorbed. In Fig. 2 are reported the curves of PHEA derivatives. The hysteresis is due to the compression–expansion rate that is faster than the polymer relaxation times. In the case of PHEA-PEG polymer partitioning between the bulk and the surface is a fully reversible process and the com-

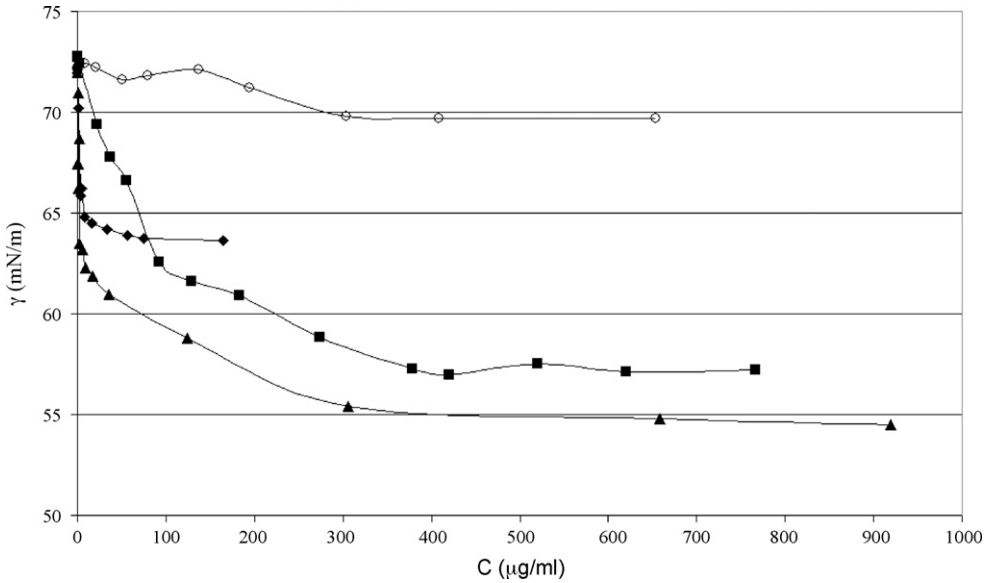

Fig. 1. Surface tension of PHEA derivatives as function of their concentration in PBS. Symbols are: (circles) PHEA; (rhombic) PHEA-PEG; (triangles) PHEA-PEG-C16; and (squares) PHEA-C16.

pression of the layer does not involve surface pressure variations. Conversely, the increase of surface pressure following compression is a consequence of the irreversible adsorption of PHEA-C_{16} and PHEA-PEG-C_{16} at the air/aqueous interface. Adsorption of the highly hydrophobic fatty acid moieties at the interface is a plausible interpretation of the observed irreversibility.

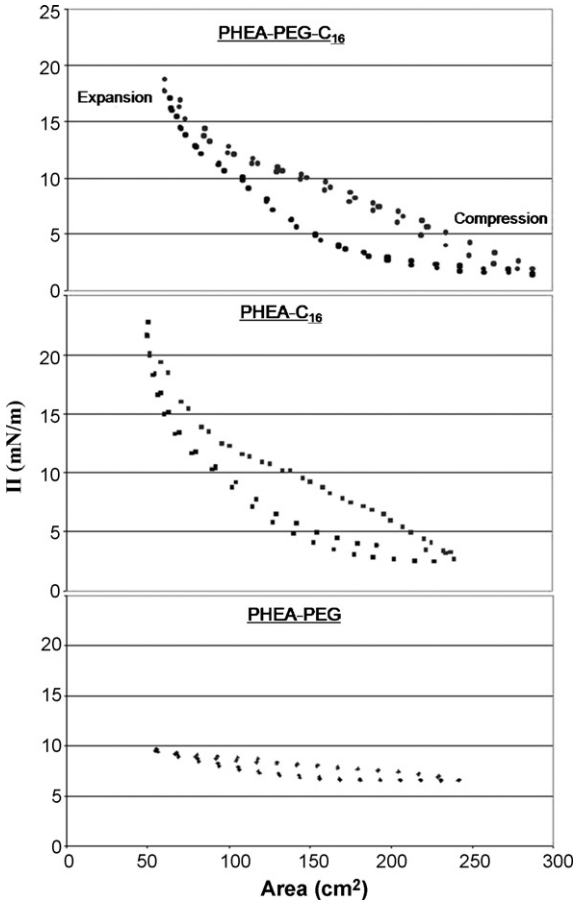

Fig. 2. Compression–expansion curves of PHEA derivatives. From the top PHEA-PEG-C_{16} and PHEA-C_{16} increase surface pressure after compression, PHEA-PEG surface pressure changes slightly with compression. PHEA (not shown) does not present surface pressure variations.

The presence of C_{16} tails determines enhanced surface activity of the polymer and this observation was confirmed by studying the interactions between a DPPC monolayer spread on the water surface and the polymers dissolved in the bulk. The excess surface pressure due to the polymer partition at the interface was measured and related to the surface pressure exerted by DPPC on the aqueous subphase (see Fig. 3).

The maximum increase in surface pressure found for the three PHEA derivatives, extrapolating Π_o to zero corresponds to their limiting surface pressure at the air/aqueous interface. Thus, the occurrence of specific interactions membrane-polymer can be ruled out. Anyway, in the case of PHEA-C_{16} and PHEA-PEG-C_{16}, there are variations of Π ($\Delta\Pi$) even at high Π_o indicating the ability of polymer to adsorb at the monolayer/air interface. With the present experiments it may be concluded that PHEA does not show binding with the monolayer, showing that it is an inert entity and that in the case of PHEA copolymers likely interactions occur between the C_{16} chains and the non-polar moiety of the lipid layer, without specific interactions with polymer/membrane. This finding evidences the different behaviour of PHEA and PHEA copolymers in comparison with other polypeptides, that have antibiotic activity and specifically interact with model phospholipid membranes (Nagy et al., 1998) and support their compatibility with biological substrates like ocular tissues.

3.2. *Evaluation of drug carrier properties of PHEA derivatives by capillary electrophoresis*

The interactions of PHEA derivatives with a tester drug, dexamethasone sodium phosphate, was studied by capillary electrophoresis, that is commonly identified as an analytical technique suitable for the separation of chemical substances including ions, drug metabolites, peptides, proteins, oligonucleotides and DNA in aqueous systems and in the presence of complex biological matrices. One peculiar feature of capillary electrophoresis is its capability of separating compounds through the formation of complexes between compound and micelles into the capillary. This characteristic may be taken as an indicator of the extent of interactions between the compound and the micelles. This area of research is

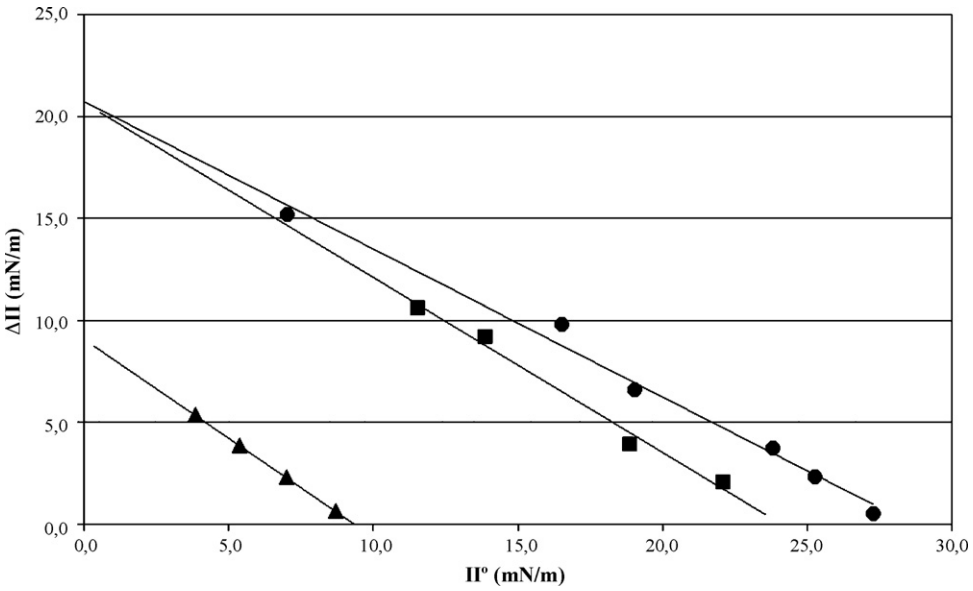

Fig. 3. PHEA derivatives penetration into DPPC monolayer. Symbols are: (triangle) PHEA-PEG; (squares) PHEA-C_{16} and (circles) PHEA-PEG-C_{16}. PHEA does not induce pressure changes of the phospholipid layer.

named micellar affinity capillary electrophoresis (MACE) (Heegard et al., 1998). In our case, the configuration is such that the micelle is stationary with respect to the electrophoretic flow and that the electrosmotic flow (EOF) is eliminated. The EOF suppression is achieved by using a capillary coated with polyvinyl alcohol (PVA) that renders uncharged capillary walls.

For all the experiments of this study, the alone drug solution was injected in the capillary, while running buffer contained the polymer. In this way interactions between the drug and polymer molecules occurred within the capillary.

Considering that the micelle concentration is in great excess compared to the compound, a 1:1 stoichiometric binding of the complex was assumed. The complexation reaction, occurring between a compound and micelles within a capillary, is:

$$D + P \overset{k}{\rightleftarrows} DP$$

where D = compound; P = micelle; DP = compound–micelle complex.

The partition coefficient (k), is defined as:

$$k = \frac{[DP]}{[D_f][P_f]} \tag{2}$$

where [DP] = concentration of the compound–micelle complex; $[P_f]$ = concentration of the free micelle; $[D_f]$ = concentration of the free compound.

If the micelle concentration is much too greater than the compound concentration:

$$[P_f] \cong Cp$$

where C_p = overall micelle concentration.

Therefore, the mobility of the system (μ_i) is calculated by the following formula:

$$\mu_i = \frac{[D_f]}{[D_f]+[DP]}\mu_f + \frac{[DP]}{[D_f]+[DP]}\mu_c \tag{3}$$

where μ_f = mobility of compound; μ_c = mobility of the compound–micelle complex.

In our case, the micelle is introduced in the capillary as the stationary phase, so that $\mu_c = 0$, and therefore Eq. (3) becomes:

$$\mu_i = \frac{[D_f]}{[D_f]+[DP]}\mu_f \tag{4}$$

Following Eq. (2):

$$\frac{[DP]}{[D_f]^n} = kC_p$$

$$\frac{\mu_f}{\mu_i} = kC_p + 1 \tag{5}$$

In general, the electrophoretic mobility is defined as the ratio between the velocity of migration of the compound and the applied electric field.

In the CE system the mobility is given by the following equation:

$$\mu = \frac{L_T L_E}{Vt} \tag{6}$$

where L_T = total length of capillary; L_E = effective length of capillary; V = applied voltage and t = time of retention of the compound.

In our case V, L_T and L_E are kept constant, so the mobility ratio is reduced to:

$$\frac{\mu_f}{\mu_i} = \frac{t_i}{t_f} \tag{7}$$

where t_i and t_f are the compound retention times respectively in the presence and in the absence of micelles.

Consequently, k may be directly calculated from the experimental retention times of the compound versus the C_p:

$$\frac{t_i}{t_f} - 1 = kC_p \tag{8}$$

$$\frac{(t_i - t_f)}{t_f C_p} = k \tag{9}$$

From the shifts of dexamethasone sodium phosphate migration time the partition coefficient k was calculated according to Eq. (9). The migration times varied within a standard deviation that ranged between 1% and 4%. In order to calculate k, the experiments were repeated at different polymer concentrations (see Fig. 4A and B).

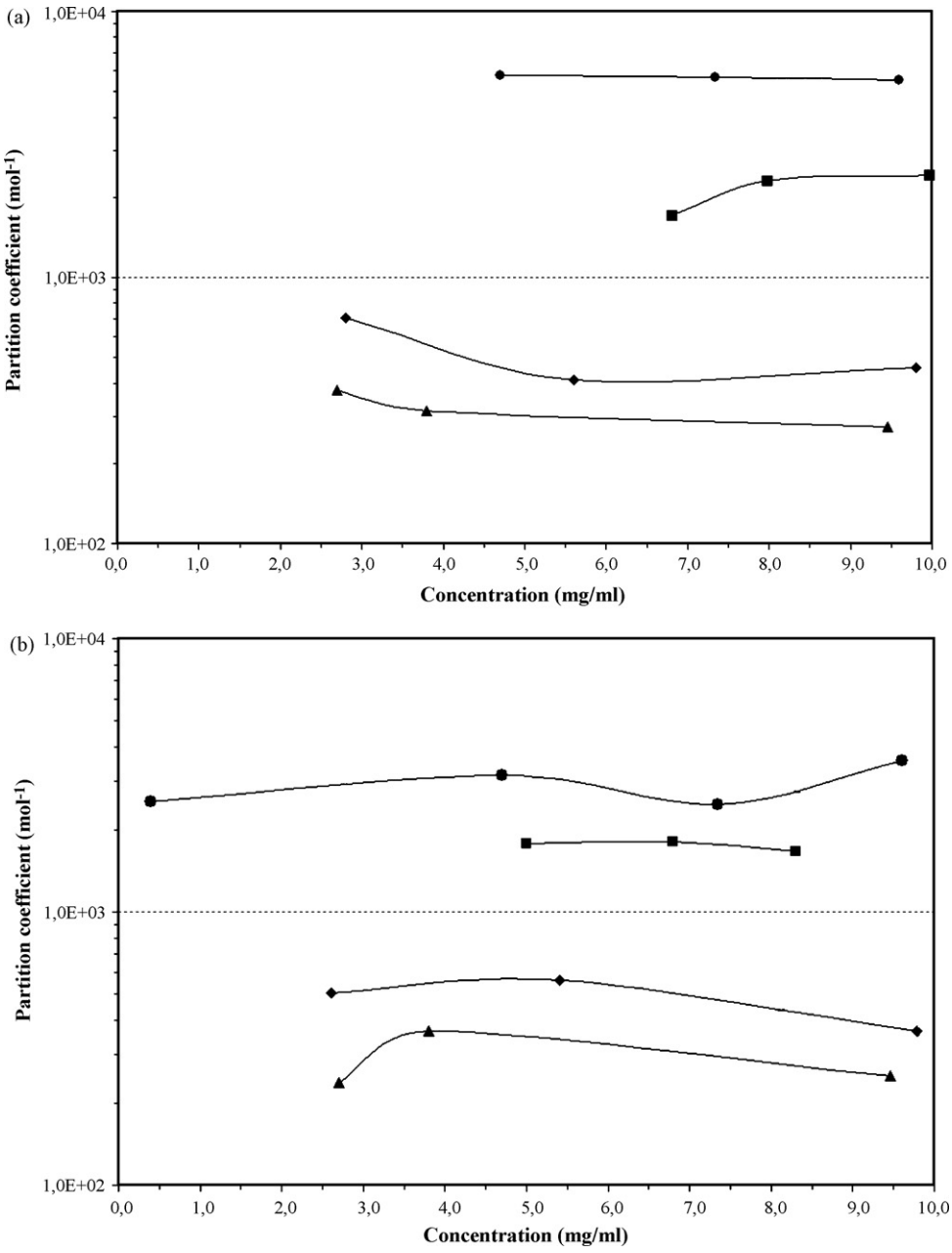

Fig. 4. (A) partitioning coefficient, *k*, vs. polymer concentration at 25 °C. Symbols are: (rhombic) PHEA; (triangle) PHEA-PEG; (squares) PHEA-C_{16} and (circles) PHEA-PEG-C_{16}. (B) partitioning coefficient, *k*, vs. polymer concentration at 37 °C. Symbols are: (rhombic) PHEA; (triangle) PHEA-PEG; (squares) PHEA-C_{16} and (circles) PHEA-PEG-C_{16}.

In Table 2 are reported the partition coefficient of the studied polymers. As can be seen, PHEA and PHEA-PEG partition coefficient at 25 °C are similar and five folds lower than the constants of PHEA-C_{16} that in turn is the half of PHEA-PEG-C_{16}. At 37 °C the retention capacity of the derivatives decreases. The differences, among the two temperatures, though not substantial, reflect the property of non-ionic self-aggregate polymers to dehydrate at physiological temperature. From the plot of *k* versus *C*p it was found some dependence of *k* on the polymer concentration (Fig. 4) indicating that the assumption of 1:1 stoichiometry may be inexact. In fact, it is possible that the number of dexamethasone sodium phosphate molecules hosted in each micelle may vary with the micelles concentration, as was also found in previous studies (Mrestani and Neubert, 2001).

From experiments concerning interactions at the interfaces and the ability of to form a complex with a compound, PHEA and PHEA-

Table 2
Partition coefficient by moles of dexamethasone sodium phosphate complex with PHEA derivatives.

	Polymers			
	PHEA, *k* (mol^{-1})	PHEA-PEG, *k* (mol^{-1})	PHEA-C_{16}, *k* (mol^{-1})	PHEA-PEG-C_{16}, *k* (mol^{-1})
25 °C	520 ± 160	320 ± 50	2150 ± 380	5650 ± 120
37 °C	302 ± 65	285 ± 70	1750 ± 760	2940 ± 520

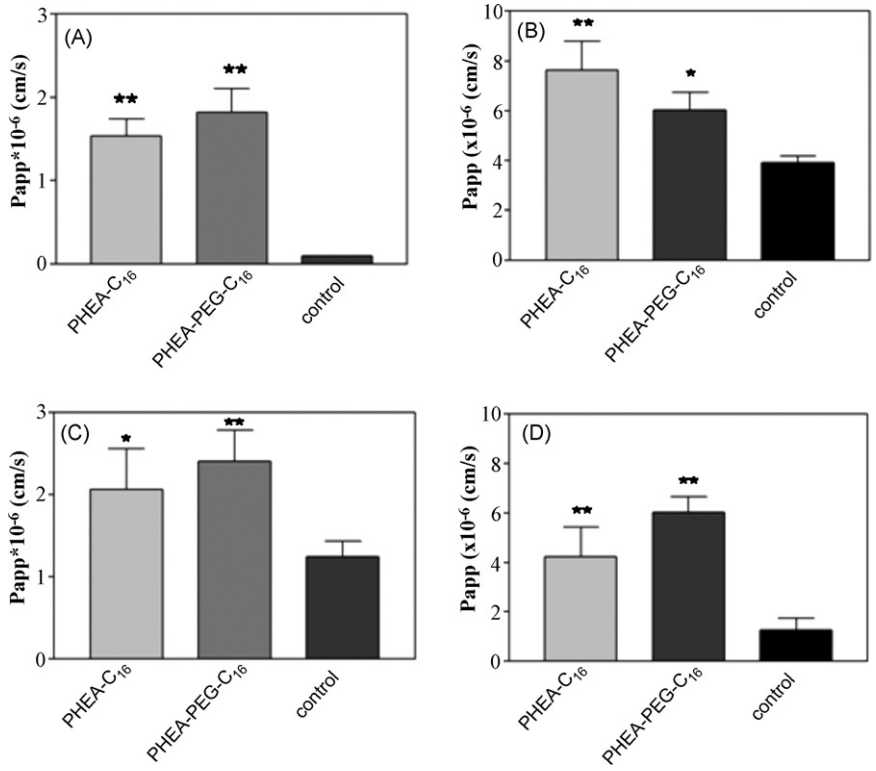

Fig. 5. Permeability of dexamethasone-NaP across BCEC (A); dexamethasone across BCEC (B); dexamethasone-NaP across BCoEC (C); dexamethasone across BCoEC (D) in the presence of PHEA-C_{16} and PHEA-PEG-C_{16}. Stars symbolise significant differences respect to control group: $^{**}P < 0.001$; $^{*}P < 0.01$.

PEG were found rather inactive for the purpose of drug delivering so that only PHEA-C_{16} and PHEA-PEG-C_{16} were retained for in vitro cellular permeation studies.

3.3. *In vitro cellular evaluation of drug permeation across the ocular epithelia*

In regards to the results obtained by capillary electrophoresis investigations we decided to study the potential permeability enhancer effect of two of the studied polymers, PHEA-C_{16} and PHEA-PEG-C_{16} in an in vitro permeability model. To study this potential enhancing effect on transepithelial passage across epithelial membrane, dexamethasone alcohol including its phosphate ester and the aminoglycoside netilmicin sulphate were chosen as model drugs. To this aim, we used a primary multilayer culture from bovine conjunctival (BCEC) and corneal (BCoEC) epithelial cells already developed in our laboratory that reproduces the physical (Trans Epithelial Resistance (TEER), polarity) and biological properties of the original tissue (Civiale et al., 2003; Paladino et al., 2004). In fact, a characteristic of the cellular cultures used is the constitution of a multilayered culture, that bear physical (TEER) and biological properties comparable to the actual ocular epithelium. In previous studies, primary cultured rabbit conjunctival and epithelial cells have been already used to evaluate permeability characteristics of a series of drugs (Saha et al., 1996a,b). These cultures showed morphological and tight junctions structures resembling to those characteristic of the intact tissue. On the other hand, the choice to use both conjunctival and corneal cells is justified by the evidence that in topical ophthalmic preparations, corneal absorption represents one major route of transport for most drugs (Hughes and Mitra, 1993), but at the same time conjunctival absorption is a non-negligible way for the drug penetration into the organ (Ahmed, 2003).

Even if it is reported in the literature that TEER from corneal epithelial cells is higher than TEER from conjunctival epithelium, in our experimental conditions, 7 days after plating, the obtained TEER from corneal cells was only $0.3 \pm 0.05\ k\Omega\ cm^2$ less than the conjunctival epithelium. This value is maintained for 14 days.

In our permeation study we observed that PHEA-C_{16} and PHEA-PEG-C_{16} are capable of increasing the permeation of all the tested drug across corneal and conjunctival epithelia. Moreover, a preliminary study showed that each moiety of these polymers separately or combined did not affect the TEER across the multilayered cultures (data not reported).

As reported in the graphs of Fig. 5, dexamethasone sodium phosphate (dexamethasone-NaP, graph A) is able to permeate across BCEC only when loaded into PHEA-PEG-C_{16} and PHEA-C_{16} micelles, while its permeability across BCoEC (graph C) is significantly increased by the two copolymers. On the other hand, it is worthy of note that dexamethasone-NaP was found fully hydrolysed to dexamethasone alcohol in both control experiments, while when loaded into PHEA-PEG-C_{16} and PHEA-C_{16} micelles it is partially protected by degradation (50% unaltered drug was found in the acceptor chamber).

In general, these data show a significant increase in permeability of dexamethasone when formulated with PHEA derivatives at a polymer concentration around 0.02% in the case of the micelles with dexamethasone alcohol and around 0.001% for the micelles prepared with dexamethasone phosphate. These differences of the polymer concentrations are justified by the great difference in the water solubility of dexamethasone alcohol with respect to dexamethasone phosphate. In fact, the first is quite insoluble in water, while its phosphate ester shows a water solubility of about 10 mg/ml.

The mechanism of steroid permeation is transcellular and the polymer aggregates may be able to intrude into the cell mem-

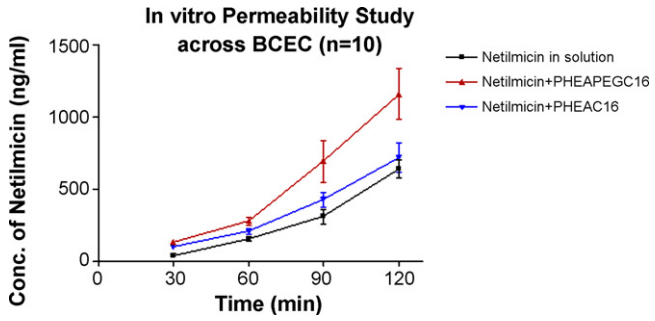

Fig. 6. Permeability of netilmicin sulphate across BCEC. Dunnetts' multiple comparison test (P value): netSO in solution vs. netSO + PHEA-PEG-C_{16} $P < 0.05$; netSO in solution vs. Net + PHEA-C_{16} $P > 0.05$; netSO + PHEA-PEG-C_{16} vs. netSO in solution $P < 0.05$; netSO + PHEA-PEG-C_{16} vs. netSO + PHEA-C_{16} $P > 0.05$.

branes and release the molecule thereafter. Otherwise the very hydrophilic dexamethasone sodium phosphate would not pass trough the hydrophobic epithelial cell membranes. Definitively, from a formulative point of view, PHEA-C_{16} and PHEA-PEG-C_{16} micelles with dexamethasone sodium phosphate provide a drug bioavailability greater than dexamethasone alcohol in comparison with the simple drug solution. From another point of view PHEA-C_{16} and PHEA-PEG-C_{16} micelles confer a partial water solubility to dexamethasone alcohol by micellization, and consequently acting as enhancer of permeability, permit its transcellular permeation across the ocular epithelia, providing so the possibility to formulate the hydrophobic dexamethasone in water solution for ocular administration.

A similar increase in the epithelia permeation was observed for netilmicin sulphate as well, as shown in Figs. 6 and 7, even if this drug has a molecular structure very different from the steroid one of dexamethasone.

In particular, the coefficients of permeability of netilmicin significantly increased when formulated with PHEA-PEG-C_{16} derivative (Student's t-test, $P < 0.05$) that definitively acts as the best enhancer of permeability across the ocular epithelia.

These results demonstrated that the enhancer permeability effect shown by the two copolymers is independent on the chemical structure or water solubility of the encapsulated drug but may be a consequent of the polymers ability to interact with biological membranes, apart from the drug, promoting drug penetration into the phospholipids bilayer. These considerations are in agreement with the LT results.

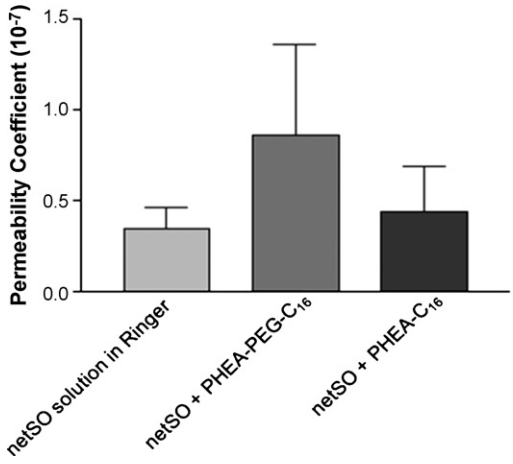

Fig. 7. Coefficient of permeability of netSO across BCoEC. Student's t-test: $P < 0.05$ between X and Y, and between Y and Z.

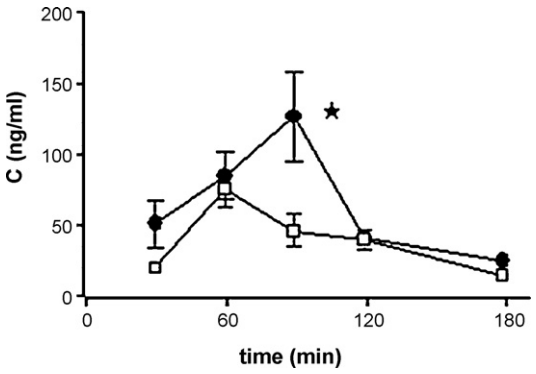

Fig. 8. Dexamethasone concentrations in aqueous humour after administration to rabbits. Circles are PHEA-PEG-C_{16} micelles; squares are dexamethasone suspension. Each data point corresponds to the mean dexamethasone concentration in ng/ml ± SEM determinated in the aqueous humour at each sample time. *Student's t-test, $P < 0.05$.

3.4. In vivo bioavailability studies

The in vivo studies were carried out to evaluate the real enhancing effect of PHEA-PEG-C_{16} on the transcorneal passage of dexamethasone alcohol when formulated into the micelles of this polymer, compared to its water suspension. Dexamethasone alcohol was selected as model drug for the in vivo study in reason of its low water solubility and high pharmacological activity in the treatment of common ocular diseases, while PHEA-PEG-C_{16} resulted the best permeability enhancer for transcorneal and conjunctival absorption.

As shown in Fig. 8, the area under the curve (AUC) obtained after ocular administration to rabbits of the PHEA-PEG-C_{16} micelles (9494 ng/(ml min)) is 40% higher than the AUC of control (5976 ng/(ml min)).

As expected, this result confirmed that PHEA-PEG-C_{16} acts not only as a solubilizing agent for dexamethasone alcohol but also provide a drug bioavailability in vivo greater compared to the water suspension of the some drug. To date, various research groups are working to find an effective drug delivery technology to circumvent the ocular barriers without causing significant patient discomfort or alteration to the protective ocular mechanisms. Only recently, promising results for ocular drug delivery in terms of improved bioavailability were reported in the literature also by using others colloidal drug delivery systems, such as nanocapsules (De Campos et al., 2003) and nanosuspensions (Pignatello et al., 2002). For the first, in fact, it was demonstrated that the nature of the coating effects the interaction of the system with the epithelial cells and their further transport across the corneal epithelium (De Campos et al., 2003); on the other hand, for nanoparticles suspensions prepared from polymeric resins and loaded with flubiprofen, the incorporation of the drug in the polymer system enhanced its active concentration in the aqueous humour, showing the ability of these systems to improve drug permeation (Pignatello et al., 2002). In our case, the use of polymeric micelles is an innovative approach that offers many advantages. Unimers preparation and micelle drug loading are simple processes; the ability of PHEA-PEG-C_{16} to act either as drug permeability enhancers or drug solubilizing agent pen the route to the possibility to develop pharmaceutical formulations of dexamethasone alcohol in water solution as micelle colloidal dispersion in a lower drug concentration, and so lowering the side effects and increasing the therapeutic index.

4. Conclusions

A family of copolymers derived from the chemical modification of PHEA were studied as potential colloidal drug carrier

systems to enhance drug permeability across ocular epithelia.

We carried out a biophysical characterization of all PHEA derivatives named PHEA-PEG, PHEA-C_{16} and PHEA-PEG-C_{16}, using the LT and MACE techniques in order to select the best candidate as carrier for ocular delivery. The obtained results were sufficient to assume that PHEA itself is an inert polymer with respect to the interactions with lipid membranes and drug complexation. On the contrary, when PHEA chains are grafted with long alkyl chains like C_{16} and/or hydrophilic chains like PEG, both interface and self-aggregating properties come into view.

In particular, the MACE study demonstrates that the presence of C_{16} moiety is more determinant to confer self-aggregating and phospholipids interaction properties than PEG. However, PEG chains may add some temperature dependence to the partitioning coefficient, typical of non-ionic polymers.

The above results let us to conclude that PHEA-C_{16} and PHEA-PEG-C_{16} could be the best candidates as carriers for ocular delivery, in fact, we carried out in vitro permeability studies across primary cultured rabbit conjunctival and corneal epithelia cells using PHEA-C_{16} and PHEA-PEG-C_{16} as micelle carriers for three tester drugs, netilmicin sulphate, dexamethasone alcohol and dexamethasone phosphate. These studies demonstrated that both PHEA-C_{16} and PHEA-PEG-C_{16} micelles loaded with each drug provide a drug permeation across ocular epithelia greater than simple drug solutions or suspensions. In particular PHEA-PEG-C_{16} acts as the best permeability enhancer in our experimental model. In vivo bioavailability studies conducted with PHEA-PEG-C_{16} micelles loaded with dexamethasone alcohol, confirmed that this system also provides a drug bioavailability greater in comparison with that obtained with the water suspension of the same drug after ocular administration to rabbits.

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