Polyester Nanocapsules as New Topical Ocular Delivery Systems for Cyclosporin A

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Purpose. Nanocapsules composed of an oily core (Migliol 840) (MG) surrounded by a poly-ε-caprolactone (PECL) coat were evaluated as potential vehicles for the topical ocular administration of cyclosporin A (CyA).

Methods. A 2^3 experimental factorial design was applied to optimize the coating of the oily nanodroplets by a solvent displacement tecnique and to encapsulate a high dose of CyA. The variables investigated were: volume of oil (MG), amount of polymer (PECL), and volume of the organic solvent (acetone) used to dissolve the polymer.

Results. Nanocapsules had a mean size in the range of 210–270 nm, a negative zeta potential (between −55 and −60 mV) and a maximum loading capacity of 50% (CyA/PECL ratio). These highly loaded nanocapsules displayed a thick spongeous polymer coating around the oily nanodroplets. The corneal levels of CyA were up to 5 times higher for the encapsulated CyA than for the oily solution of CyA. In addition, these levels remained significantly higher than those of the control group (oily solution) for up to 3 days. Furthermore, the area-under-the-curve (AUC) values were significantly increased for the encapsulated CyA (319.98) with respect to the oily control (74.34).

Conclusions. The CyA-loaded nanocapsules are shown to be interesting vehicles for the improvement of the ocular penetration of CyA.

KEY WORDS: nanocapsules; polyepsiloncaprolactone; cyclosporin A; ophthalmic administration; corneal penetration.

INTRODUCTION

Over the last decade, biodegradable colloidal systems have been the subject of various studies in the view of their application as new topical ocular drug delivery vehicles. Wood et al. (1) were the first group to show that poly(alkylcyanoacrylate) PACA nanoparticles were able to adhere to the corneal and conjunctival surfaces, thus being promising vehicles for ocular drug delivery. Later, several researchers revealed the interest of PACA colloidal systems for the enhancement of the corneal penetration of hydrophilic and lipophilic drugs (2–3). However, in spite of these initial promising results, the potential of these new systems become limited following the observation that PACA nanoparticles penetrate into the outer layers of the corneal epithelium causing a disruption of the cell membranes (4). As an alternative to the PACA colloidal systems, recent studies have shown the utility of the poly-ε-caprolactone (PECL) nanocapsules as topical ocular drug delivery systems (3–5). In addition, we have shown, by confocal fluorescent microscopy, that PECL nanocapsules are specifically taken up by the corneal epithelium cells without damaging the cell membrane (6). Therefore, these nanocapsules could be considered as very promising carriers for the transport of drugs to the inner of the eye.

A current challenge in ocular drug delivery is to enhance the permeation of macromolecules, such as peptides and proteins, across the cornea. Among the different options, the use of penetration enhancers has probably received the most attention. However, the potential of these enhancers is still very limited by their local toxicity (7). In the present work, we have investigated the PECL nanocapsules as a different approach to increase the transport of macromolecular drugs across the cornea. For this purpose we selected the inmunossupressive agent CyA because of its major impact on the treatment of a variety of ophthalmic disorders and the lack of an adequate form of administration (8–9). In fact, the systemic administration of CyA is associated with very important systemic side effects (10), whereas the results obtained following topical application are quite variable and controversial (11). Therefore, the design of a delivery system which would transport CyA across the cornea would theoretically be of great benefit in reducing the dose and the local side effects.

MATERIALS AND METHODS

Chemicals and Animals

The polymer poly-ε-caprolactone (PECL) (MW: 40,000) was purchased from Aldrich Chemie (Germany). The oil Migliol® 840 and Poloxamer 188 (Synperonic® F68) were generously supplied by Lemmel (Spain) and from ICI (Spain) respectively. Lecithin (soybean L-α-lecithin 40% phosphatidylcholine) was supplied by Sigma Quimica (Spain). Cyclosporin A (CyA) was obtained as a gift from Sandoz (Basel, Switzerland). [Mebmt-b-^{3} H] Cyclosporin A (specific activity of 229 MBq/mg) was purchased from Amersham International (Buckinghamshire, U.K.). Tissue solubilizer (BTS-450) and liquid scintillation cocktails (Ready Organic® and Ready Safe®) were purchased from Beckman (Fullerton, CA, U.S.A.). All other chemicals were reagent grade chemicals.

Male albino New Zealand rabbits weighing between 2.5 and 3.0 kg were used in the *in vivo* study. The rabbits were fed a regular diet with no restrictions on the amount of food or water consumed.

Preparation of PECL Nanocapsules

Nanocapsules were prepared by the interfacial deposition technique as previously reported (12). The manufacturing process was carried out according to a 3^2 factorial experimental design combined with a multiple regression analysis. The three independent variables investigated were the amount of polymer, the volume of the oil and the volume of acetone (Table I). Briefly, different amounts of polymer (200 or 400 mg) and 100 mg of lecithin were dissolved in variable volumes of acetone (25 or 35 ml). Then, a variable volume of Migliol oil (0.5 or

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Table I. Particle size Distribution Parameters (mean particle size and polydispersity) and ζ Potential of the Formulations Designed

Formulation	Polymer (mg)	Oil (ml)	Acetone (ml)	Mean Particle Size ± S.D.* (nm) (polydispersity)	ζ Potential ± S.D.* (mV)
1	200	0,5	25	238.8 ± 1.95 (0.083)	−50.41 ± 0.80
2	200	0,5	35	212.7 ± 1.80 (0.153)	−46.84 ± 0.90
3	200	1	25	257.5 ± 4.61 (0.153)	−54.99 ± 1.02
4	200	1	35	257.4 ± 0.35 (0.178)	−51.98 ± 0.84
5	400	0,5	25	240.2 ± 1.00 (0.113)	−51.10 ± 0.67
6	400	0,5	35	228.1 ± 1.70 (0.099)	−50.46 ± 0.95
7	400	1	25	280.3 ± 0.95 (0.152)	−53.83 ± 0.80
8	400	1	35	253.2 ± 2.80 (0.127)	−53.61 ± 1.01

* Standard Deviation, n = 6.

1 ml) was added to the acetonic solution. This organic solution was poured, under moderate magnetic stirring, into 50 ml of aqueous phase containing 125 mg of Poloxamer 188. The acetone was finally removed under reduced pressure and the colloidal aqueous suspension was then concentrated to the desired final volume (10 ml). For the encapsulation of CyA, a variable amount of this drug (100 or 200 mg) was dissolved in the oily-acetonic phase. The [^{3}H] CyA-loaded nanocapsules were prepared by adding tracer amounts of [^{3}H] CyA to the oily solution of CyA to attain an activity value of 5 μCi / ml or 80 μCi/ml, depending on their in vitro or in vivo application.

Physicochemical Characterization of the Nanocapsules

The morphological examination of nanocapsules was performed using a transmission electron microscope (TEM, Philips CM12) following negative staining with sodium phosphotungstate solution (0,2%).

The particle size and electrophoretic mobility distributions were determined by photon correlation spectroscopy (PCS) and laser Doppler anemometry (LDA), respectively. The zeta potential values were calculated from the mean electrophoretic mobility values using the Smoluchowski's equation.

Evaluation of the Encapsulation Efficiency

The amount of CyA encapsulated into the nanocapsules was calculated by the difference between the total amount used to prepare the nanocapsules and the amount of CyA present in the aqueous phase, following separation of nanocapsules by an ultrafiltration- centrifugation technique (Ultrafree®-MC 30,000 MW, Millipore) 3000 × g, 15 min. The amount of [^{3}H]CyA was determined by liquid scintillation counting (LS 6000 LL, Beckman Instruments, Fullerton, CA, USA) using Ready Safe® as a scintillation cocktail.

Evaluation of the Coating Efficiency. Stability after Ultracentrifugation

To investigate the protective effect of the PECL coat of nanocapsules against coalescence of the oil core (separation of the oil as a bulk phase) , 5 ml of nanocapsules suspensions were ultracentrifuged at 120,000 × g for 30 min at 4°C. Upon centrifugation the nanocapsules suspension separates into transparent layers of free oil and water and an opaque layer consisting of a concentrate of nanocapsules (cream). The analysis of these phases provides information on the stability of the suspensions.

In Vitro Release Studies

Ten μl of the suspension of nanocapsules containing 100 μg of CyA and traces of [^{3}H]CyA were diluted in 10 ml of phosphate buffer pH 7.4 and maintained under constant agitation at 37°C. At predetermined time intervals, 300 μl of release medium were filtered through an Ultrafree® MC unit (30,000 MW, Millipore, USA) and centrifuged (3,000 × g, 5 min). CyA released was determined by liquid scintillation counting as described previously.

In Vivo Studies

Ten μl of the suspension of nanocapsules or the same volume of an oily control solution containing 10 mg/ml of CyA and traces of [^{3}H]CyA were administered to the cul-de-sac of the right eye of fully-awake New Zealand rabbits. Four additional instillations were given at 15 minute intervals. At different times after instillation, rabbits were sacrificed with an intravenous injection of sodium pentobarbital. The eyes were rinsed with normal saline, then the corneas were dissected and then digested at 35°C in 1 ml of tissue solubilizer. Afterwards, 30 ml of acetic acid and 10 ml of Ready Organic® scintillation cocktail were added to the samples. Finally, the samples were allowed to stand for at least 24 h in the dark to minimize chemiluminescence.

RESULTS AND DISCUSSION

We have formerly shown that PECL nanocapsules are interesting vehicles for the ocular administration of drugs with a moderate lipophilicity (3). Based on this previous experience, the first step in the present work was to apply this technology for the development of a new aqueous ophthalmic formulation of the peptide CyA. Bearing in mind this application and because of the hydrophobic character of CyA (solubility in water at 20°C: 32.9 ± 6.7 μg/ml) an important objective was to encapsulate a high amount of the peptide in the nanocapsules in order to achieve a concentration of 10 mg/ml of CyA in the final suspension (recommended concentration for in vivo administration) (8–9). First, we prepared the nanocapsules by the standard procedure (12) and observed an important amount of CyA crystals. This phenomenon was explained by the diffusion of a certain amount of CyA from the oily solution to the external aqueous medium, during the spontaneous emulsification process, followed by its precipitation as free crystals. This crystallization process was not observed when we substantially reduced the concentration of CyA in the preparation.

To optimize the encapsulation of CyA within PECL nanocapsules our approach was to investigate the feasibility of producing nanocapsules using quantities of polymer and oil higher

than indicated in the standard procedure (100 mg of PECL and 0.5 ml of oil). For this purpose we used a 2^3 experimental factorial design in which the variables investigated were the amount of polymer, the volume of the oil Migliol® 840 and volume of the organic solvent acetone. Table I compares the physicochemical parameters (particle size distribution and zeta potential) for the series of formulations designed. Results indicate that particle size and zeta potential are slightly influenced by the variables under investigation, the limit values being observed for formulations 2 and 7. Thus, formulation 2, which contains low amounts of polymer and oil and was prepared with a high volume of acetone (35 ml), had the minimum size (212.7 nm) and the lowest zeta potential value (−46.84 mV) whereas formulation 7, which contains the maximum amount of oil and polymer and was prepared with a low volume of acetone, had the largest size (280.3 nm) and the highest zeta potential value (−53 mV). Since these data were obtained from the factorial design, it was possible to interpret them statistically by an analysis of variance combined with a multiple regression analysis. From this analysis it was concluded that the amount of polymer and oil had a significant influence on the size of the nanocapsules. In addition, the interaction of these variables with the volume of the organic solvent was statistically significant. The simultaneous effect of two variables on the particle size are illustrated in the surface response diagrams displayed in Fig. 1. On the other hand, the zeta potential of the nanocapsules was significantly influenced by the amount of polymer and by the interactions between the three variables under investigation. The increased zeta potential observed for preparations containing a low amount of polymer or a high volume of oil can be explained in terms of the coating efficiency of the oil by the polymer. In fact, we found (results not shown) that the negative character of the zeta potential is due to the presence of lecithin and free acids in the oil. Since the same amount of lecithin was incorporated in the formulations, the differences in the zeta potential should be attributed to the oil.

To further investigate the coating efficiency of the oily nanodroplets by the PECL coat, the nanocapsules were exposed to ultracentrifugation to promote a phase separation process. Three different phases were expected: (a) a cream indicating a good stabilization of the oily nanodroplets; (b) a layer of free oil indicating that nanocapsules leak their oil content during centrifugation and therefore they are not perfectly stabilized; (c) a sediment evidencing that the polymer was not deposited around the oily nanodroplets but formed nanoparticles separately. Table II, which summarizes the phases we observed following ultracentrifugation, indicates that formulations 1, 2 and 8 were well coated and were conveniently stabilized. On the other hand, formulations 5 and 6 were composed mainly of nanoparticles, as it was evidenced by the important sediment.

Based on the previous results, formulations 1 and 8 were selected for the encapsulation of CyA. As shown in table III, the CyA loading efficiency of these nanocapsules is extremely high (more than 90% of CyA was encapsulated) giving a final ratio drug/polymer of 50/100. These results are very encouraging since, to the best of our knowledge, this is the highest drug/polymer ratio reported for a nanoencapsulated drug system. In table III it can be also seen that the encapsulation of CyA has no significant effect on the size and zeta potential of the nanospheres (compared with values in table I). In order to analyze the inner structure of the CyA-loaded nanocapsules

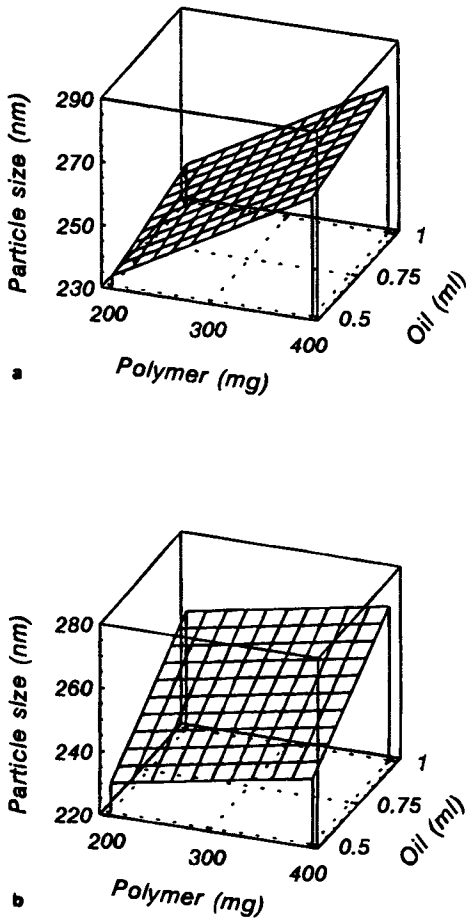

Fig. 1. Three- dimensional response surface showing the effect of oil volume and polymer amount on mean particle size of nanocapsules suspensions prepared with a) 25 ml and b) 35 ml of acetone. Equations of the response surfaces corresponding to mean particle size for nanocapsules prepares with: a) 25 ml: Particle size: 190 + 0.172 A + 17.175 B, b) 35 ml: Particle size: 190 + 0.057 A + 55.291 B where A and B represent the amount of polymer and the volume of oil respectively.

and investigate the presence of free crystals samples were characterized by TEM. Micrographs in Fig 2 show that nanocapsules prepared with 200 mg of PECL have a small oily core surrounded by a thick and porous polymer coating. This spongeous-like coating structure is very different to the one observed for conventional nanocapsules (100 mg of polymer) and could be attributed to the immediate precipitation of a high amount of PECL around the oil. This thick coating could also be seen as a barrier that prevents the migration of the CyA molecules to the external aqueous medium, corroborated by the absence of free crystals of CyA.

Formulation 1 was finally selected for in vitro release and in vivo evaluation. The criteria for this selection was the acute ocular tolerance of the nanocapsules (results not shown). In this sense we found that, despite the good tolerance of all formulations, formulation 1, containing a CyA/PECL ratio of 200/100, causes less irritation than formulation 8.

The in vitro release studies showed that CyA is rapidly released from PECL nanocapsules upon a high dilution in a buffer solution at 37°C (75 % released in 5 min). However, the

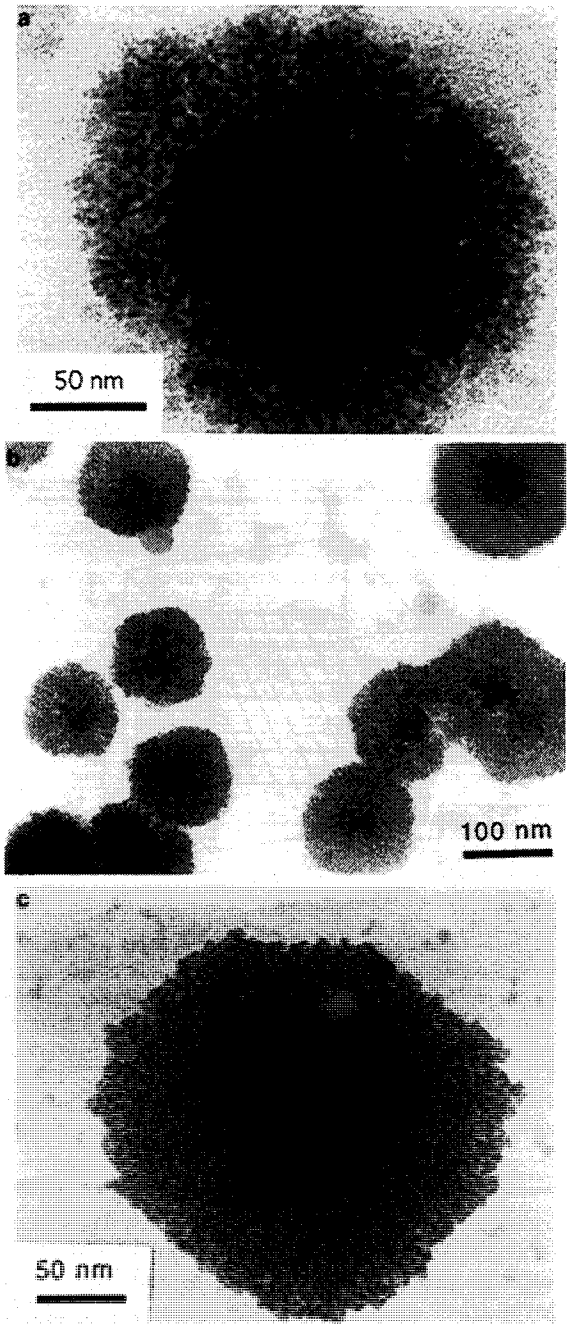

Fig. 2. CyA concentrations achieved in cornea after instillation of CyA-loaded nanocapsules (□) and oily control solution (■). Shown are mean values ± S.D. (n = 3) (* $P < 0.05$, ** $P < 0.01$). Pharmacokinetic parameters:

	AUC (mg h g^{-1})	Ke × 10^{-2} (h^{-1})
Oil	74.34 ± 3.53	2.33 ± 0.28
Nanocapsules	319.98 ± 11.05	3.06 ± 0.16

release rate was slowed down when the volume of the release medium was reduced. This behavior is well in accordance with the one previously observed for CyA-loaded PACA nanocapsules (13) and indicates that the PECL coating does not affect the release process, however, the partition of CyA between the oil and the external aqueous medium is the key factor governing the release.

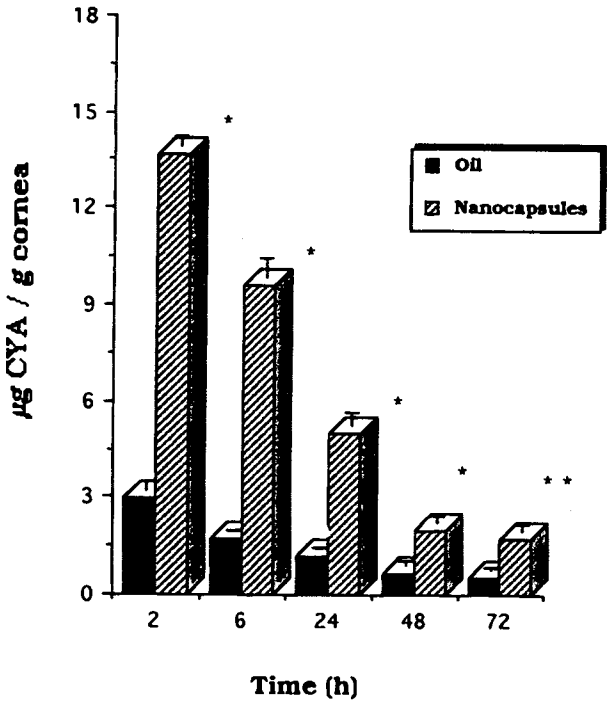

Fig. 3. Electron transmission microphotography of a) free, b) and c) CyA-loaded nanocapsules after negative staining.

Finally, the corneal penetration of CyA encapsulated in PECL nanocapsules was evaluated and compared to the penetration of CyA dissolved in the MG oil. As shown in Fig. 3, after 2 hours post-instillation of CyA-loaded nanocapsules, the concentration of CyA in cornea was 5 times higher than the one observed after the instillation of the oily solution of CyA. These differences were statistically significant (Student t test) for up to 72 hours. The values of the area-under-the-curve (AUC) corneal concentration vs. time were significantly increased for the encapsulated CyA (319.98) with respect to the oily control solution (74.34). In addition, we observed that the elimination constant of CyA from the cornea (first order transport kinetics) was similar for the nanoencapsules and the oil. In other words, the elimination rate of CyA from the cornea was not substantially modified by its encapsulation, however the total amount transported was highly increased. This favorable

Table II. Relative Amount of Cream, Oil Free and Sediment Produced after Ultracentrifugation of the Nanocapsules

Formulation	Cream	Free Oil	Sediment
1	++++*	—	—
2	++++	—	—
3	+++	++	—
4	++	+++	—
5	++	—	+++
6	++	—	+++
7	+++	+	++
8	++++	—	—

* (++++: high, +++: middle, ++: little, +: very little, — none) n = 4.

Table III. Mean Particle Size, ζ Potential and Percentage of CyA Encapsulated in Formulations 1 and 8

Formulation	CyA/PECL (mg)	Particle size ± S.D.* (nm) (polydispersity)	ζ Potential ± S.D.* (mV)	% CyA encapsulated
1	200/100	231.8 ± 4.01 (0.111)	−55.66 ± 0.06	99.36 ± 0.42
8	400/200	268.8 ± 10.6 (0.157)	−54.07 ± 0.34	99.13 ± 0.58

* Standard Deviation (n = 4).

penetration of CyA, when it was administered in the encapsulated form, could be explained by the previously observed fact that nanocapsules are taken up by the corneal epithelium cells (6) and therefore they can carry CyA through the cornea. Similar results were recently reported by Pleyer et al. (14) who studied the ocular penetration of CyA encapsulated in liposomes. However, it should be pointed out that the increase in the corneal penetration achieved using liposomes with respect to an oily solution was much lower than we found using nanocapsules. This could be explained by a different mechanism of interaction of liposomes and nanocapsules with the corneal epithelium. In fact, up until now, there is no clear evidence of the corneal penetration of the liposomes as there is for the nanocapsules.

CONCLUSIONS

PECL nanocapsules having a high polymer and oil (Miglio® 840) content were developed according to an experimental factorial design. These modified nanocapsules allow the encapsulation of a very high amount of CyA (50% CyA/polymer ratio). To the best of our knowledge this is the first report showing this enormous loading efficiency of a peptide in a colloidal drug carrier. The interest of the formulations developed for topical ocular administration was clearly demonstrated by the fact that nanocapsules promote the penetration of CyA to a very high degree. Therefore, this new formulation may open new prospects in the topical ocular applications of CyA and may help with the elucidation of its ocular therapeutic response. In addition, the results obtained for CyA suggest the utility of PECL nanocapsules as peptide ocular carriers.

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REFERENCES

1. R. W. Wood, V. H. K. Li, J. Kreuter and J. R. Robinson. Ocular disposition of poly-hexyl-2-cyano(3-^{14}C) acrylate nanoparticles in the albino rabbit. *Int. J. Pharm.* **23**:175–183 (1985).
2. C. Losa, P. Calvo, E. Castro, J. L. Vila-Jato and M. J. Alonso. Improvement of ocular penetration of amikacin sulphate by association to poly(butylcyanoacrylate) nanoparticles. *J. Pharm. Pharmacol.* **43**:548–552 (1991)
3. C. Losa, L. Marchal-Heussler, F. Orallo, J. L. Vila Jato and M. J. Alonso. Design of new formulations for topical ocular administration: polymeric nanocapsules containing metipranolol. *Pharm. Res.* **10**:80–87 (1993)
4. A. Zimmer, J. Kreuter and J. R. Robinson. Studies on the transport pathway of PBCA nanoparticles in ocular tissues. *J. Microencapsulation* **8**:497–504 (1991).
5. L. Marchal-Haussler, H. Fessi, J. P. Devissaguet, M. Hoffman and P. Maincent. Colloidal drug delivery systems for the eye. A comparison of the efficacy of three different polymers: polyisobutylcyanoacrylate, polylactic-coglycolic acid, poly-epsilon-caprolactone. *S.T.P. Pharm. Sci.* **2**:98–104 (1992).
6. P. Calvo, C. Thomas, M. J. Alonso, J. L. Vila Jato and J. R. Robinson. Study of the mechanism of interaction of poly(ε-caprolactone) nanocapsules with the cornea by confocal laser scanning microscopy. *Int. J. Pharm.* **103**:283–291 (1994).
7. Y. Rojanasakul, Mechanisms of ocular peptide absorption and its enhancement. In D. S. Hsieh (ed.), *Drug permeation enhancement*, Marcel Dekker, Inc., New York, 1994, pp. 397–410.
8. J. C. Zhao and X. Y. Jin. Local therapy of corneal allograft rejection with cyclosporine. *Am. J. Opthalmol.* **119**:189–194 (1995).
9. E. J. Holland, T. W. Olsen, J. M. Ketcham, C. Florine, J. H. Krachmer, J. J. Purcell, S. Lam, H. H. Tessler and J. Sugar. Topical cyclosporin A in the treatment of anterior segment inflammatory disease. *Cornea* **12**:413–419 (1993).
10. A. G. Palestine, R. B. Nussenblatt and C. C. Chan. Side effects of systemic cyclosporine in patients not undergoing transplantation. *Am. J. Med.* **77**:652–656 (1984).
11. M. W. Belin, C. S. Bouchard and T. M. Phillips. Update on topical cyclosporin A. *Cornea* **9**:184–195 (1990).
12. H. Fessi, F. Puisieux, J. P. Devissaguet, N. Ammoury and S. Benita. Nanocapsules formation by interfacial deposition following solvent displacement. *Int. J. Pharm.* **55**:R1–R4 (1989)
13. S. Bonduelle, C. Foucher, J. C. Leroux, F. Chouinard, C. Cadieux and V. Lenaerts. Association of cyclosporin to isohexylcyanoacrylate nanospheres and subsequent release in human plasma in vitro. *J. Microencapsulation* **9**:173–182 (1992).
14. U. Pleyer, B. Elkins, D. Rücker, L. Sabine, J. Grammer, J. Chou, K. H. Schmidt and B. J. Mondino. Ocular absorption of cyclosporin A from liposomes incorporated into collagen shields. *Curr. Eye Res.* **13**:177–181 (1994).