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International Journal of Pharmaceutics 355 (2008) 307–313
INTERNATIONAL JOURNAL OF PHARMACEUTICS
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Pharmaceutical Nanotechnology

Diclofenac sodium delivery to the eye: *In vitro* evaluation of novel solid lipid nanoparticle formulation using human cornea construct

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Received 23 October 2007; received in revised form 5 December 2007; accepted 6 December 2007
Available online 17 December 2007

Abstract

Solid lipid nanoparticles (SLNs) were prepared with a combination of homolipid from goat (goat fat) and phospholipid, and evaluated for diclofenac sodium (DNa) delivery to the eye using bio-engineered human cornea, produced from immortalized human corneal endothelial cells (HENC), stromal fibroblasts and epithelial cells CEPI 17 CL 4. Encapsulation efficiency was high and sustained release of DNa and high permeation through the bio-engineered cornea were achieved. Results obtained in this work showed that permeation of DNa through the cornea construct was improved by formulation as SLN modified with phospholipid.

Keywords: Diclofenac sodium; Solid lipid nanoparticles; Surface modification; Human cornea construct; Drug permeation; Ocular drug delivery

1. Introduction

Solid lipid nanoparticles (SLNs) consist of solid lipids in nanosized range dispersed in aqueous medium. SLN combine the advantages and avoid the disadvantages of other colloidal carrier systems such as liposomes and polymer nanoparticles, and are regarded as their alternative (Müller et al., 2000; Müller and Ruge, 1998; Gulati et al., 1998). When optimised, SLN exhibit high physical stability, protection of incorporated labile actives against degradation and excellent *in vivo* tolerability (Wissing, 2002; Jenning et al., 2000; Müller et al., 2002). However, these systems generally exhibit a low drug pay-load capacity and drug expulsion during storage due to the transition of highly ordered lipid particles (Wissing et al., 2004). These disadvantages can be remedied by using structured lipid matrices in SLN formulation and surface modification of the particle (Attama et al., 2006; Sastry, 2000).

Delivery of drugs to the tear film is routinely done with eye drops, which are well accepted and for most patients easy to use. However, attainment of an optimal drug concentration at the site of action is a major problem. Poor bioavailability of drugs from ocular dosage form is mainly due to the pre-corneal loss factors which include tear dynamics, non-productive absorption, transient residence time in the cul-de-sac, and relative impermeability of the corneal epithelial membrane (Pijls et al., 2005; Kaur et al., 2004). Development of an alternative to solution-type eye drop that would provide sustained delivery of a drug is a major challenge. Many sustained ocular drug delivery devices have been studied (Ghate and Edelhauser, 2006; Liu et al., 2007; Vandervoort and Ludwig, 2007). Recently, Friedrich et al. (2005) studied drug release and permeation from nanosuspensions based on solidified reverse micellar solution with a view to assessing their applicability in ocular drug delivery. In this study, SLN formulations as sustained ocular drug delivery systems are described. A novel lipid matrix consisting of 30% (w/w) of phospholipids (Phospholipon 90G®) in goat fat (templated homolipid, Attama and Müller-Goymann, 2007) was used to formulate SLN. SLN formulations are adhesive (Müller-Goymann, 2004), and could prolong the residence time of the dosage form in the eye and increase bioavailability.

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doi:10.1016/j.ijpharm.2007.12.007

Cornea, a multilayered tissue is the main route for intra-ocular absorption. *In vitro* permeability studies are usually performed using isolated corneal tissues from slaughtered or laboratory animals, but this could lead to difficulties due to anatomical and physiological differences between the animal and human eye. Bio-engineering human corneal tissues from donor cornea cells as has been reported, would eliminate species-related problems and avoid animal experiments (Reichl et al., 2003). Corneal equivalents have been observed to show tight junctions and desmosomes in the flattened apical cell layer (Toropainen et al., 2001), and resembles intact cornea with morphologically identifiable desmosomes, tight junctions, microvilli and cell layers with apical flat cells.

In this work, a homolipid from *Capra hircus* (goat fat) templated with Phospholipon 90G® was used in the formulation of SLN-containing DNa. The phospholipid directly modifies the surface and crystal characteristics of SLN. The formulated SLN were further studied for delivery of the active pharmaceutical ingredient to the eye using bio-engineered human cornea as the drug transport barrier. Goat fat containing phospholipid has earlier been evaluated for its suitability in the formulation of lipid nanoparticles, where lipid nanoparticles with good stability and crystal characteristics, which would favour drug loading were obtained (Attama and Müller-Goymann, 2007). This work is a continuation of the evaluation of this emerging homolipid for drug delivery applications.

2. Materials and methods

2.1. Materials

Phospholipon 90G® (Phospholipid GmbH, Germany), diclofenac sodium (DNa, Synopharm, Germany), thimerosal (Synochem, Germany), sorbitol and polysorbate 80 (Tween 80®) (Across Organics, Germany) were used as procured from their manufacturers without further purification. All other reagents and solvents were HPLC grade and were used as such without further purification. Goat fat was obtained from a batch processed according to earlier procedure (Attama et al., 2003). Bidistilled water was used for nanoparticle preparation. The lipid matrix (templated homolipid) used in SLN formulation corresponds to 30% (w/w) of Phospholipon 90G® in goat fat and was prepared by fusion. The cell cultures used were provided by Nestec (Nestec Ltd., Nestlé Research Centre, Lausanne, Switzerland) and Cornea Bank of MHH, Hannover.

2.2. Formulation of SLN

SLN contained 5.0% (w/w) of lipid matrix, 1.0% (w/w) of polysorbate 80, 0.0, 0.5, 0.75 or 1.0% (w/w) of DNa (respectively GF-P90G-SLN, GF-P90G-SLN 0.5%, GF-P90G-SLN 0.75% and GF-P90G-SLN 1%), 4.0% (w/w) of sorbitol, 0.005% (w/w) of thimerosal and enough double-distilled water to make 100.0% (w/w), and were formulated by hot homogenisation using high-pressure homogeniser (EmulsiFlex-C5, Avestin, Canada) after formulation of primary emulsion with Ultra-Turrax mixer (T25 basic, Ika Staufen, Germany) at 19,000 rpm for 5 min (Attama and Müller-Goymann, 2007). Prior to SLN formulation, DNa was incorporated into the lipid matrix during fusion of phospholipid with goat fat. SLN containing no phospholipid but with 1.0% (w/w) Tween 80® and 1.0% (w/w) DNa only (GF-SLN 1%), and that containing only goat fat without the drug and phospholipid (GF-SLN) were prepared for comparison.

2.3. Particle size and zeta potential measurements

The particle size of the SLN was determined by phase angle light scattering (PALS) using Zetasizer Nano-Series (Nano-ZS, Malvern Instruments, England). Samples were diluted with double-distilled filtered water before measurement. The zeta potentials of the formulated SLN were also determined using the same instrument. For the zeta potential measurements, each sample was diluted with bidistilled water and the electrophoretic mobility determined at 25 °C and dispersant dielectric constant of 78.5. The obtained electrophoretic mobility values were used to calculate the zeta potentials using the software DTS Version 4.1 (Malvern, England) as previously described (Attama et al., 2007). In each case, the experiment was carried out four times.

2.4. Wide angle X-ray diffraction (WAXD)

WAXD was used to investigate the crystalline character of the formulated SLN. Wide angle X-ray studies were done on the formulated SLN as earlier described (Attama and Müller-Goymann, 2007) using an X-ray generator (PW3040/60 X'Pert PRO, PANalytical, Netherlands) connected to a copper anode, which delivered X-ray of wavelength, $\lambda = 0.1542$ nm at a voltage of 40 kV and an anode current of 25 mA. The SLN samples were filled into capillaries and WAXD measurements were taken with a goniometer (MPD-System, PANalytical, Netherlands). The interlayer spacing d, was calculated from the scattering angle θ, using Bragg's equation.

2.5. Differential scanning calorimetry (DSC)

The degree of crystallinity of the lipid nanoparticles was determined as described before (Attama and Müller-Goymann, 2007) using a calorimeter (DSC 220C) and a disc station (5200H, Seiko, Tokyo, Japan). About 5 mg of each SLN was weighed into an aluminium pan, sealed hermetically, and the thermal behaviour determined in the range of 10–125 °C at a heating rate of 5 °C min^{-1}. Baselines were corrected using empty pans. Transition temperatures where possible, were determined from the endothermic peak minima.

2.6. HPLC methodology

HPLC determination of DNa concentration was done using a Waters 515, 717plus, 486 HPLC system (Waters, Eschborn, Germany) at 276 nm, with data analysis by Waters Millenium 32 Chromatography Manager Software. The column used was Hypersil® ODS 5 μm, 250 mm × 4 mm column (Grom, Rothenburg-Hailfingen, Germany) with this solvent system: acetonitrile/distilled water/acetic acid (40:60:2). The standard

HPLC method used for DNa was as described previously (Baydoun and Müller-Goymann, 2003). An injection volume of 20 μl and a flow rate of 1.1 ml/min were used, and DNa could be detected at a retention time of 8.2 min and a wavelength of 276 nm. Linear correlation between peak area and DNa concentration ($r^2 = 0.9998$) was obtained within the concentration range of 1–25 μg/ml, with a limit of quantification of 1.27 μg/ml.

2.7. Drug encapsulation efficiency (DEE%)

The percentage of drug entrapped by the SLN was determined indirectly after centrifugation in a membrane concentrator (Vivaspin 6 MCO 5000, Vivascience AG, Hannover, Germany) for 90 min at 9000 × *g* and 5 °C, in a Beckman Coulter Allegra™ 64R centrifuge (Beckman Coulter, Palo Alto, CA, USA). The drug concentration in the aqueous continuous phase was determined by HPLC. The drug content of the nanoparticles was thereafter calculated considering the initial amount of drug loaded. This determination was done three times and at least 2 weeks after preparation to allow for complete crystallization of the nanoparticles. This is consonance with an earlier finding from crystallization studies of the unloaded nanoparticles that there was no significant increase in crystallinity of the SLN after 1 month of storage (Attama and Müller-Goymann, 2007). However, the fact that drug expulsion from previously encapsulated state is possible has to be kept in mind, thus pretending a higher drug encapsulation than actually existing, although there was no evidence of drug crystals within the continuous phase of the nanosuspension.

2.8. In vitro drug release studies

In vitro release studies were carried out in a modified Franz diffusion cell (FD-C) (Franz, 1975) over 6 h at a temperature of 37 ± 1 °C. The diffusion barrier was a siliconised Spectrapore® membrane MWCO 6000–8000 (Spectrum Laboratories Inc., The Netherlands). In each case, a finite dose of the SLN was introduced into the donor compartment and the open ends of the apparatus sealed with Parafilm® to prevent evaporation. Isotonic phosphate buffer (pH 7.4) was used as the acceptor and was stirred with a magnetic stirrer at 600 rpm during the experiment. Aliquots of 1 ml of the acceptor phase were withdrawn at intervals and the withdrawn samples analysed for drug content by HPLC. Sink condition was maintained throughout the release period. Data obtained in triplicate were analysed graphically.

2.9. Bio-engineering of human cornea construct (HCC) and transport studies

HCC was constructed step by step in Transwell® cell culture inserts (Corning Incorporated, NY, USA) as reported (Reichl et al., 2003, 2005) using immortalized human corneal endothelial cells (HENC), stromal fibroblasts and epithelial cells (CEPI 17 CL 4). HENC (2×10^5 cells) were seeded onto a polycarbonate filter covered with a layer of type 1 collagen acid extracted from rat tail, and grown to confluence within 7 days in medium F99. A type 1 collagen gel matrix containing 6×10^4 stromal fibroblasts was then cast on top of the confluent endothelial cell layer. Endothelial stroma equivalent was cultivated approximately 4 days submerged in Dulbecco's modified Eagle's medium (DMEM).

Epithelial cells (CEPI 17 CL 4, 1.5×10^5 cells) were seeded onto the contracted collagen lattice and grown in medium DMEM/F12 for additional 7 days submerged to confluence. After epithelium became confluent, tissue construct was lifted to the air–liquid interface for an additional 10 days and cultivated in medium DMEM/F12 with a reduced serum content of 2%. Within 10 days, a multilayered epithelium was formed. The cultures were maintained in a humidified incubator at 37 °C with 5% CO_2 and medium was also replaced three times a week.

Drug transport studies through bio-engineered HCC were carried out using a modified Franz diffusion cell. The modified FD-C consists of a donor compartment and an acceptor compartment. The donor compartment was filled with the SLN formulation from each batch, whereas the acceptor compartment contained phosphate buffer (pH 7.4). The barrier (HCC) was sandwiched on top of a polycarbonate membrane between the two compartments. Sampling was done at predetermined time intervals up to 6 h from the acceptor compartment and volume of acceptor was kept constant throughout the experiment with fresh buffer. The whole apparatus was kept at 37 °C in a water bath while the acceptor solution was stirred with a magnetic stirring bar. Permeation studies were performed in triplicates.

The permeation parameters of DNa from the SLN were calculated by plotting the amounts of drug permeated through HCC (μg/cm^2) versus time (min). The steady-state flux (J) values across HCC were evaluated from the linear ascents of the permeation graphs by means of the relationship:

$$J(\mu g/(cm^2 s)) = \frac{dQ}{A\,dt} \quad (1)$$

where Q indicates the quantity of substance crossing HCC, A is the area of HCC exposed, and t is the time of exposure. The permeation coefficient P in each case was calculated from the following equation:

$$P(cm/s) = \frac{J}{C_0} \quad (2)$$

where C_0 represents the initial drug concentration in the donor compartment.

2.10. Data and statistical analysis

All experiments were performed in replicates for validity of statistical analysis. Results were expressed as mean ± S.D. ANOVA and Student's *t*-tests were performed on the data sets generated using Origin for Windows®. Differences were considered significant for p-values <0.05.

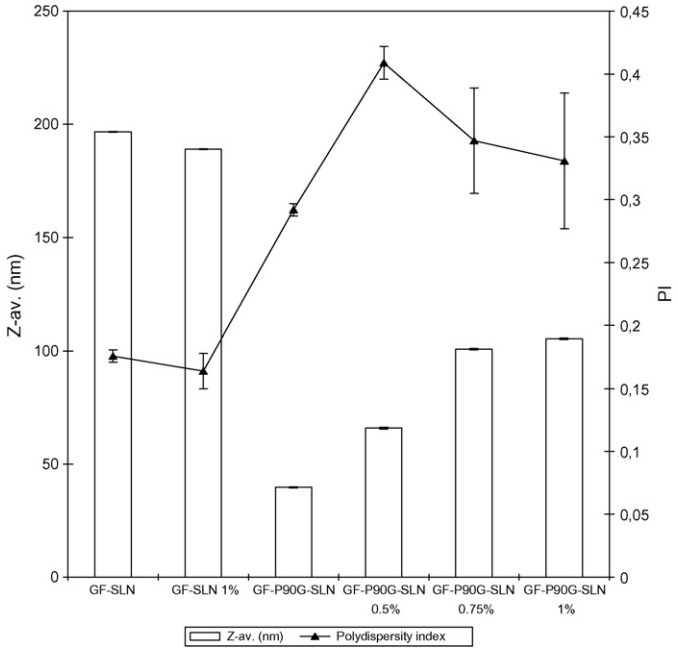

Fig. 1. Particle size analysis of the SLN (mean ± S.D., $n = 4$).

3. Results and discussion

3.1. Particle size and zeta potential

Particle sizes of the SLN determined after 4 weeks of preparation as presented in Fig. 1 shows very small particles. There was significant increase ($p < 0.05$) in particle size with increase in drug load but polydispersity indices were slightly reduced with increase in drug loading. Particle size, particle size distribution and stability are a major issue considered by formulation scientists when formulating dispersed systems especially those intended for parenteral or ocular administration. For ocular administration, irritation and tear wash out may occur on administration of large-sized particles, since smaller particles are better tolerated (Kaur et al., 2004). Very small particles as nanoparticles possess adhesive properties (Müller-Goymann, 2004), which could prolong the residence time of the drug in the cul-de-sac, prevent tear wash out (due to tear dynamics), and increase ocular bioavailability. The zeta potentials of the drug-loaded SLN presented in Table 1 indicate very stable particles as all the SLN possessed significantly higher ($p < 0.05$) absolute zeta potential values compared with SLN without drug. Presence of the drug increased the zeta potential values as the drug-loaded SLN possessed absolute zeta potential values which were significantly ($p < 0.05$) higher than the value recorded for the unloaded SLN. SLN prepared with only phospholipid without the mobile surfactant had the lowest absolute zeta potential value. This means it is unstable despite its very low particle size and would aggregate easily on storage. The ranking order for stability using the zeta potential as a guide is GF-SLN 1% > GF-P90G-SLN 0.75% > GF-P90G-SLN 1% ≈ GF-P90G-SLN 0.5% > GF-SLN > GF-P90G-SLN. Although the SLN formulated with goat fat and the mobile surfactant alone without the phospholipid and containing 1.0% DNa possessed the highest absolute zeta potential, it possessed higher crystallinity, lower drug load and higher particle size than other drug-containing SLN. These factors coupled with the tendency to agglomerate eliminates it from being the best SLN in this study.

Table 1
Zeta potentials of the SLN

Batch of SLN	Zeta potential (mV ± S.D., $n = 4$)
GF-SLN	−29.2 ± 0.5
GF-SLN 1%	−49.1 ± 0.7
GF-P90G-SLN	−12.5 ± 2.3
GF-P90G-SLN 0.5%	−36.8 ± 0.8
GF-P90G-SLN 0.75%	−42.6 ± 1.2
GF-P90G-SLN 1%	−38.4 ± 1.7

3.2. Drug encapsulation efficiency

Encapsulation efficiencies above 90% were recorded for the SLN batches except the SLN prepared without phospholipid. This shows the lipid matrix, which contains phospholipid possessed spaces where the drug was localised and drug expulsion was almost non-existent compared with the SLN formulated without phospholipid. The high drug loading was also contributed by the behaviour of DNa in the presence of a phospholipid, as diclofenac shows thermotropic liquid crystalline behaviour (Müller-Goymann, 2004) and may participate in the microstructure of the system. The encapsulation efficiencies (mean ± S.D., $n = 3$) obtained for the drug-loaded SLN are presented in Table 1. There was no significant difference ($p < 0.05$) between the loading efficiencies of the SLN-containing phospholipid, but there was a significant difference ($p < 0.05$) in the loading efficiencies of SLN with and without phospholipid. This was due to complex formation between phospholipid and the drug, and the participation of drug in the structures formed at the particle surface.

3.3. Differential scanning calorimetry (DSC) analysis

DSC was used to underline the high drug encapsulation potency of the SLN. The higher the enthalpy of the transitions, the more crystalline the SLN and consequently, the more difficult it will be for any drug to be encapsulated. The DSC result is presented in Fig. 2. The result shows low crystalline state for the phospholipid-containing SLN, which helped the particles record high drug encapsulation efficiency compared with the SLN prepared without phospholipid, which showed a slight endothermic signal at about 50 °C, confirming their higher crystallinity.

3.4. Wide angle X-ray diffraction (WAXD) analysis

WAXD (Fig. 3) shows presence of low crystallinity for all the SLN in terms of a diffuse halo from about $2\theta = 20^\circ$ to 40°. A single interference of low intensity could be proven at about $2\theta = 19.3^\circ$ ($d = 4.60$ Å), indicating the SLN recrystallized in stable beta modification. This property complements DSC as it

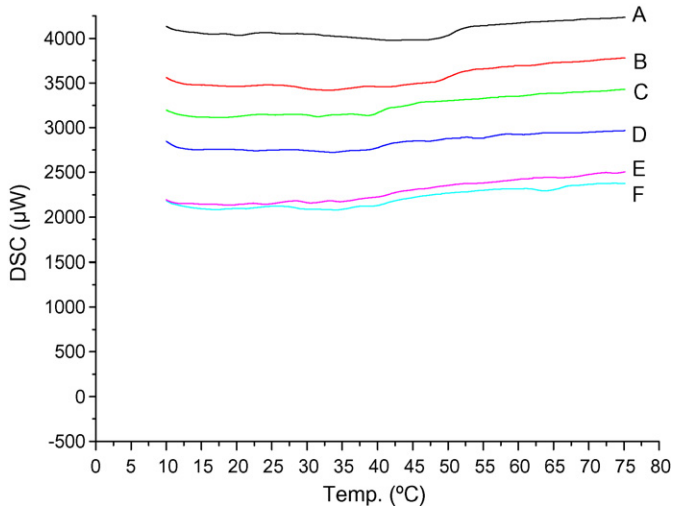

Fig. 2. DSC traces of the SLN: A = GF-SLN, B = GF-SLN 1%, C = GF-P90G-SLN, D = GF-P90G-SLN 0.5%, E = GF-P90G-SLN 0.75% and F = GF-P90G-SLN 1%.

gives information about the crystallinity of the nanoparticles. The SLN with higher intensity, which indicates higher crystallinity would produce greater sustained release effect all things being equal. As expected due to the very low drug concentrations below the detection limit of crystalline material which is around 5%, there was absence of characteristic reflections of the pure drug—DNa. Time-resolved WAXD analysis may give information on the dynamics of lipid crystallization with time. As such, increase in intensity of the lipid nanoparticles would indicate possible expulsion of incorporated drug on storage but the result of drug encapsulation efficiency showed high values, meaning most of the drug is fully or partly within the lipid particle. Although time-resolved WAXD analysis was not done here, previous studies with this lipid matrix showed absence of increase in crystallinity with time (Attama and Müller-Goymann, 2007). WAXD patterns of the SLN were obtained after 4 weeks of preparation.

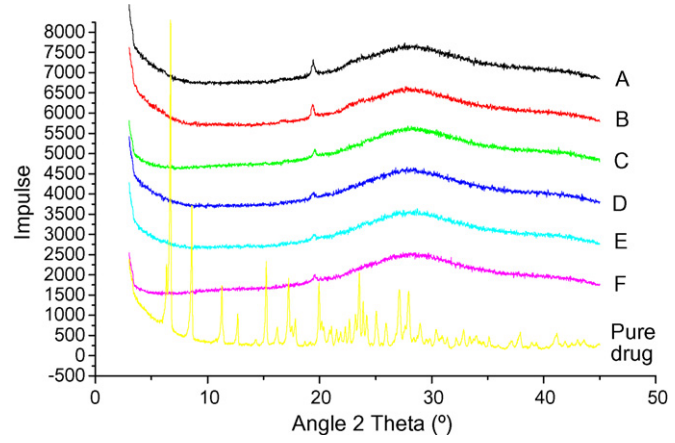

Fig. 3. WAXD patterns of the SLN: A = GF-SLN, B = GF-SLN 1%, C = GF-P90G-SLN, D = GF-P90G-SLN 0.5%, E = GF-P90G-SLN 0.75% and F = GF-P90G-SLN 1%.

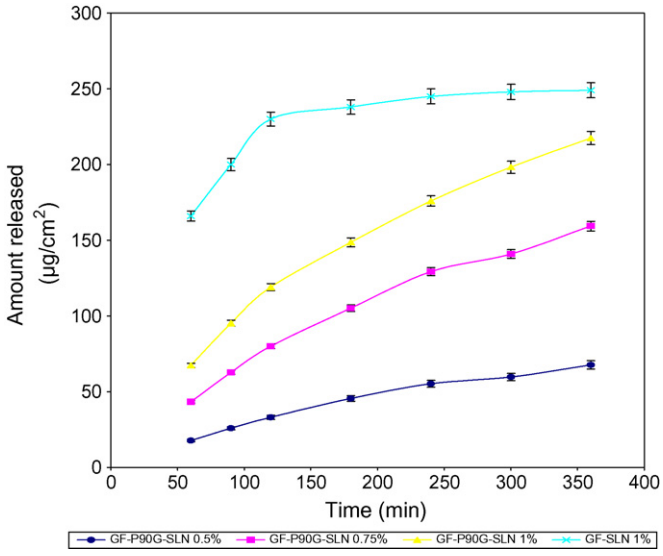

Fig. 4. Release profile of the diclofenac sodium-containing SLN (mean ± S.D., $n = 3$).

3.5. Drug release from the SLN

Fig. 4 shows the release profile of DNa in phosphate buffer (pH 7.4) using dialysis membrane (MCO 5000–8000) as the release barrier. There was burst release of DNa from SLN formulated without phospholipid within the first 125 min. This was due to the more crystalline nature of this SLN, which resulted in more drugs in the periphery and bulk aqueous medium than in the core of the lipid nanoparticles. The profiles of the phospholipid-containing SLN revealed sustained release of the active with regards to ocular delivery. Higher quantity of DNa was released in the batch loaded with higher amount of DNa. This showed phospholipid not only modified the encapsulation efficiency of the SLN but also modified the release properties. This was because of the complex structure of the phospholipid formed at the particle surface. Surface modification improves both the drug pay-load capacity and sustained release potential, which was not because of high crystallinity but due to structural effects. SLN without phospholipid was more crystalline.

3.6. Permeation studies

The permeated amount per unit area through HCC was higher at higher drug loading (Fig. 5). The resulting permeation fluxes J, i.e. the amount permeating per unit area per unit time were calculated and used for comparison. For convenience and for reason of stability, permeation studies were carried out using the SLN-containing phospholipid. The fluxes and permeation coefficients obtained for the different batches of SLN are presented in Table 2. Considering the high loading efficiency, the nanoparticle preparations permeated the cornea construct and could be used in the delivery of DNa to the eye to offer a sustained pharmacological effect in the treatment of post-operative inflammation in cataract surgery, control of ocular pain and discomfort associated with corneal epithelial defects, etc. The p-values obtained here are high, and were very close. They are

Table 2
Encapsulation efficiencies and permeation parameters obtained for the permeation of the diclofenac sodium-loaded SLN

Batch of SLN	Encapsulation efficiency (% ± S.D., $n = 3$)	Permeation parameter	
		Steady-state flux, J (μg/(cm^2 s))	Permeation coefficient, P (cm/s)
GF-P90G-SLN 0.5%	94.2 ± 1.5	0.4514	9.03×10^{-5}
GF-P90G-SLN 0.75%	94.0 ± 0.4	0.7440	9.92×10^{-5}
GF-P90G-SLN 1.0%	93.8 ± 1.2	0.9443	9.44×10^{-5}
GF-SLN 1.0%	61.7 ± 2.6		

comparable to p-values obtained for 0.5% DNa solution using human donor cornea and HCC (Reichl et al., 2005). Conventionally, permeability studies usually use isolated corneal tissue mounted in modified side-by-side diffusion chambers or Ussing chambers. Excised corneas from slaughtered animals or laboratory animals are frequently used (Praunitz and Noonan, 1998), in particular from rabbits, although anatomical and physiological differences between the rabbit and human eye suggest that *in vitro* permeation data could also differ. This necessitates the use of alternatives to this method of evaluation calling for cornea constructs which would eliminate species variability especially in terms of physiology, expression of metabolic enzymes and active transporters, efflux proteins, surface proteins and mucins. This study has further proved that it was possible to use human cornea constructs in ocular drug delivery studies.

Poor bioavailability of drugs from ocular dosage form is mainly due to the tear production, non-productive absorption, transient residence time, and impermeability of corneal epithelium. After topical ocular application, drugs may be absorbed into the eye through the corneal or conjunctival and scleral route. In fact, conjunctiva is a conduit for drug clearance into the systemic circulation (Hosoya et al., 2005). The route through conjunctiva and sclera is important mostly for very hydrophilic and large molecules that are not able to penetrate through the corneal barrier (Ahmed and Patton, 1985). Most clinically used ocular drugs have adequate lipophilicity for corneal absorption, and such properties are sought in the development of new ocular drug. This SLN formulation of DNa, which showed high *in vitro* ocular transport would offer two advantages in terms of ocular drug delivery. First, encapsulation of the DNa into the lipophilic particle would facilitate transport through the corneal route. Lipid formulations are also known to enhance the absorption of certain drugs and together with polysorbate 80, may also have inhibitory effects on some drug efflux transporters (P-glycoproteins) (Kreuter, 2001; Wasan, 2001). Second, the particulate nature of the formulation would ensure adherence to the surrounding membranes preventing tear wash out and providing sustained release of DNa, as SLN are highly adhesive (Müller-Goymann, 2004).

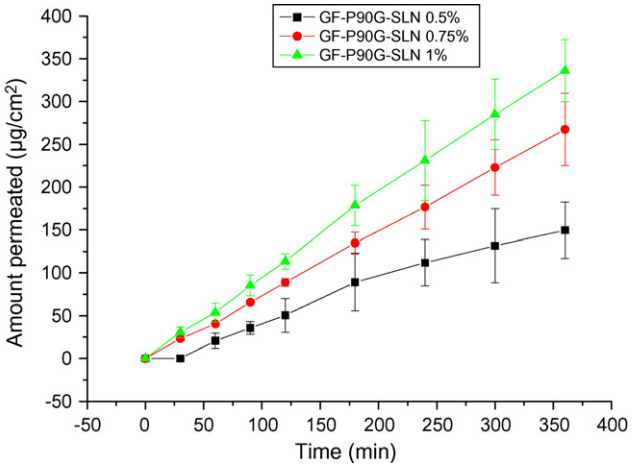

Fig. 5. Permeation profile of the drug-loaded SLN prepared with phospholipid (mean ± S.D., $n = 3$).

In order to overcome the problems of conventional ocular therapy, such as short residence time, drug drainage and frequent instillation, newer delivery systems such as SLN are being explored to improve ocular bioavailability of drugs. From the foregoing, SLN drug delivery systems exemplified by this study, seem to be ready to provide a solution to the challenge posed by non-successful ocular drug delivery. Particulate drug delivery systems like nanoparticles and microparticles are better tolerated by patients than larger particles and hence, microspheres and nanospheres represent very comfortable prolonged action ophthalmic drug delivery systems (Kaur et al., 2004). The ingredients used SLN formulation are generally regarded as safe (GRAS). The GRAS status (Wissing et al., 2004) of the ingredients used in the formulation of SLN makes it highly biocompatible unlike some polymeric systems, which have been shown to damage the corneal epithelium by disrupting the cell membrane (Calvo et al., 1994; Marchal-Heussler et al., 1993; Zimmer and Kreuter, 1991), and may produce toxic products on degradation.

4. Conclusion

The high encapsulation efficiency and high permeation achieved with this novel SLN formulation indicate this SLN could be an effective drug delivery system for ocular active drugs. HCC can be used as *in vitro* tool for screening ocular drugs or formulations for favourable pharmacokinetic properties, and can also be adapted for investigating the mechanism of ocular drug absorption. DNa-containing SLN evaluated in this work showed that the performance of this analgesic drug for ocular application could be improved by formulation as SLN.

Acknowledgement

Dr. A.A. Attama wishes to acknowledge the support of Alexander von Humboldt Stiftung, Bonn-Bad Godesberg, Germany through a fellowship award. We also thank Phospholipid

GmbH, for providing sample of Phospholipon 90G®, Nestec (Nestec Ltd., Nestlé Research Centre, Lausanne, Switzerland) for the supply of epithelial cells CEPI 17 CL 4 and Cornea Bank of MHH, Hannover for the supply of human donor corneas, and Ursula Jahn, Anjte Bieder and Dagma Hahne of Institut für Pharmazeutische Technologie, TU Braunschweig for X-ray studies, particle size analysis and human cornea construct experiments, respectively.

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